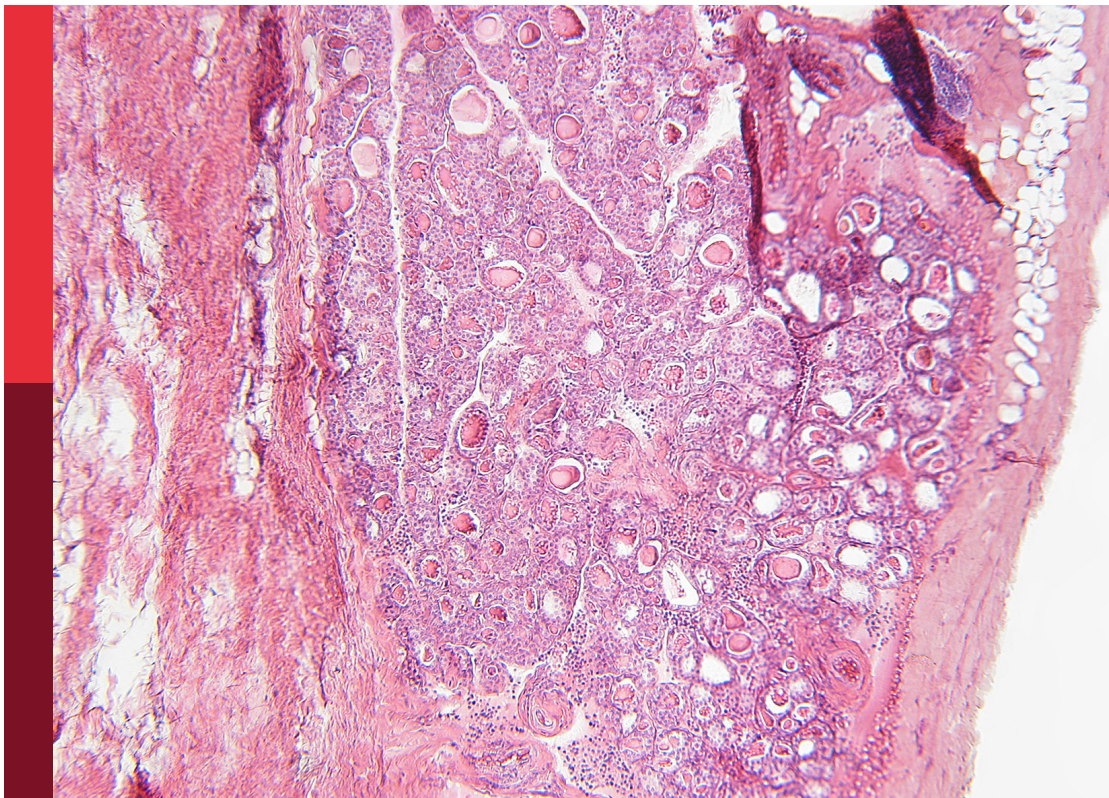
frontiers | Research Topics

# Safety and child health of assisted reproduction technology (ART)

**Edited by**
Yimin Zhu and Yiping Shen

**Published in**
Frontiers in Endocrinology
Frontiers in Physiology



ISSN 1664-8714
ISBN 978-2-8325-2572-2
DOI 10.3389/978-2-8325-2572-2

## About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

## Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

## Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

## What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

# Safety and child health of assisted reproduction technology (ART)

**Topic editors**

Yimin Zhu — Zhejiang University, China

Yiping Shen — Harvard Medical School, United States

**Citation**

Zhu, Y., Shen, Y., eds. (2023). *Safety and child health of assisted reproduction technology (ART)*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-2572-2

# Table of contents

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 07 February 2022
doi: 10.3389/fendo.2022.827978

# Evaluation of Bone Mineral Density in Children Conceived *via* Assisted Reproductive Technology

*Xinru Xia[1], Lingling Chen[1], Jing Wang[1], Xiang Yu[2], Li Gao[1], Yuan Zhang[1], Feiyang Diao[1], Yugui Cui[1], Jiayin Liu[1]* and Yan Meng[1]**

[1] State Key Laboratory of Reproductive Medicine, Center for Clinical Reproductive Medicine, First Affiliated Hospital, Nanjing Medical University, Nanjing, China, [2] Department of Pediatrics, First Affiliated Hospital, Nanjing Medical University, Nanjing, China

**Objectives:** To investigate bone mineral density (BMD) differences between assisted reproductive technology (ART)-conceived children and naturally conceived (NC) children.

**Study Design:** This retrospective cohort study included ART-conceived children and controls aged 1 to 12 years assessed with a follow-up protocol. Maternal and paternal background, birth condition, and growth and development indicators were analyzed.

**Results:** The ART and NC groups exhibited differences in maternal and paternal childbearing age; maternal weight; maternal body mass index (BMI); maternal alcohol consumption; paternal smoking; delivery method; and serum zinc, iron, and lead levels. Multifactor analysis adjusted for relevant factors showed that paternal childbearing age and group significantly affected the BMD Z score. In the subgroup analysis, *in vitro* fertilization (IVF) (p=0.026) or intracytoplasmic sperm injection (ICSI) (p=0.008) had a positive impact on the BMD Z score. Male infertility only (p=0.010) or male infertility combined with polycystic ovary syndrome (PCOS) (p=0.026) may affect the BMD Z score. In the embryo transfer cycle subgroup analysis, compared with natural conception, both stimulation cycle fresh embryo transfer (p=0.019) and natural cycle frozen embryo transfer (p=0.006) had a positive effect on the BMD Z score.

**Conclusions:** The BMD levels of the ART and control groups were generally in the normal range. Paternal childbearing age and the use of ART independently affected the BMD Z score of the offspring.

**Keywords: bone mineral density, bone development, infertility, assisted reproductive technology, childbearing age**

OPEN ACCESS

***Edited by:***
*Yimin Zhu,*
*Zhejiang University, China*

***Reviewed by:***
*Hang Ying Lou,*
*Zhejiang University, China*
*Zhiqi Liao,*
*Huazhong University of Science and Technology, China*

****Correspondence:***
*Yan Meng*
*ctmengyan@njmu.edu.cn*
*Jiayin Liu*
*jyliu_nj@126.com*

***Specialty section:***
*This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology*

***Received:*** *02 December 2021*
***Accepted:*** *11 January 2022*
***Published:*** *07 February 2022*

***Citation:***
*Xia X, Chen L, Wang J, Yu X, Gao L, Zhang Y, Diao F, Cui Y, Liu J and Meng Y (2022) Evaluation of Bone Mineral Density in Children Conceived via Assisted Reproductive Technology. Front. Endocrinol. 13:827978. doi: 10.3389/fendo.2022.827978*

## INTRODUCTION

Infertility has become an increasingly common health problem, affecting approximately 48.5 million couples worldwide (1). Due to its high prevalence, it is regarded as a social disease by the World Health Organization. There are many reasons for infertility, and they vary from person to person. Assisted reproductive technology (ART) is one of the three main treatment strategies used

for infertility. In recent decades, there has been great progress in ART, especially in the fields of fertility preservation, pre-implantation aneuploidy screening, uterine transplantation and mitochondrial replacement technology to prevent serious diseases, and previously incurable cases have been successfully treated (2–5). However, the risks of multiple birth, premature birth, very premature birth, low birth weight, very low birth weight, small for gestational age, congenital malformations, and birth defects are significantly increased in ART offspring (6–10). Bone density is the most sensitive early warning factor for osteoporosis. Osteoporosis is a serious disease, and there is inconsistent evidence regarding whether there is a difference in bone mineral density (BMD) in the offspring conceived by ART.

A study in 2015 showed that the speed of sound (SOS) level measured within 96 hours in ART preterm infants was lower than that measured in the naturally conceived (NC) group (11). However, another population-based study reported that there was no statistically significant difference in BMD between offspring aged 4 and 10 years who were born *via in vitro* fertilization (IVF) and fresh embryo transfer and those born *via* natural conception (12).

BMD is defined as the mass of bone mineral per unit volume. BMD is an indicator not only of the status of bone salt deposition but also of the status of bone development in children and adolescents (13). Childhood and adolescence are critical periods for bone development. Osteoporosis that occurs later in life is thought to have originated in childhood or adolescence (14). Thus, prevention and treatment of bone mineral deficiency in children is a key step to reducing the occurrence of osteoporosis in adulthood. The study of BMD in childhood has important healthcare significance.

BMD is affected by many factors, including genetic factors, birth state (15–17), age (18), sex (18), height (18, 19), nutrition (vitamin D and calcium intake) (19–21), pubertal status (19), life behavior (sun exposure, duration of breastfeeding or dietary pattern) (19, 22, 23), physical activity (19, 21), body composition (overall body mass, lean body mass or body fat mass) (24–26) and diseases (27–34).

Additionally, the health of offspring born after ART has been a focus of public attention. Whether ART itself or parental infertility affects the BMD of the offspring has not yet been reported.

Therefore, the purpose of this study was to determine whether the BMD of ART offspring is affected by infertility or ART itself and to explore the possible mechanism by which parental fertility and ART influence BMD to promote the bone health of ART-conceived children.

# MATERIALS AND METHODS

## Subjects

The children who were recruited were conceived *via* ART in our center (ART group) or were NC (NC group) and came to our hospital for a physical examination in the Child Health Department from 2012 to 2015. The ART-conceived offspring included live-born infants from 2001 to 2014 in the Clinic Center of Reproductive Medicine of Jiangsu Province Hospital. The two groups were matched according to their age in months. Local institutional (First Affiliated Hospital of Nanjing Medical University) ethical approval (2012-SR-048) was obtained prior to data collection.

The inclusion criteria for the ART group were as follows: willingness to participate voluntarily and cooperatively, prepubertal status, singleton birth, full-term birth, *in vitro* fertilization and embryo transfer (IVF-ET) or ICSI as the ART method, infertility factors that included polycystic ovary syndrome (PCOS) and/or male factors (oligoasthenospermia, spermatogenic dysfunction or obstructive azoospermia), and embryo transfer cycle that was carried out *via* a fresh embryo being transferred in a stimulated cycle or a frozen embryo being transferred in a natural cycle.

The inclusion criteria for the NC group were as follows: willingness to participate voluntarily and cooperatively, prepubertal status, singleton birth, full-term birth, and natural conception.

The exclusion criteria for the ART group were as follows: illness or use of drugs, trauma or fracture, high-intensity sports training, family history of metabolic diseases, use of glucocorticoids by the mother during pregnancy, and donated sperm or eggs.

The exclusion criteria for the NC group were as follows: illness or use of drugs, trauma or fracture, high-intensity sports training, family history of metabolic diseases, and use of glucocorticoids by the mother during pregnancy.

In total, 84 individuals were included in the ART group, and 123 individuals were included in the NC group. Informed consent was obtained from a guardian of each participant included in this study.

## Follow-Up Process

Two weeks before follow-up, the subjects were notified by telephone of the time, place and contact person for the follow-up assessment.

The day before the follow-up assessment, the participants were called to confirm whether they were free to participate. The pregnancy information of the ART group was retrieved and printed from the Center of Clinical Reproductive Medicine system.

On the day of the follow-up, the specific follow-up procedure was explained, and an informed consent form was signed by the patient. The Pediatrics department conducted general physical examinations (height and weight), trace element analyses, and bone density tests. Parents filled out the questionnaire. After all the items were completed, the information was checked, and it was confirmed that there were no omissions; then, the data were entered into the computer.

## Ultrasound Scans

Using an Omnisense 7000P ultrasonic bone densitometer (Sunlight Medical Co., Ltd., Tel Aviv, Israel), after calibration by professional operators each day, the ultrasonic propagation velocity value (SOS) in the middle of the left tibia was measured by a standard method. The Z score of the SOS value of Asian children of the same age and sex was used as the standard.

## Measurement of Height and Weight

Height and weight were measured at the Department of Children's Health Care by skilled personnel. The accuracy was within 0.1 cm and 0.1 kg, respectively. Body mass index (BMI)=weight (kg)/height $(m)^2$.

## Determination of Trace Elements

Peripheral blood was taken from the child's ring finger. The levels of zinc (Zn), copper (Cu), iron (Fe), calcium (Ca), magnesium (Mg) and lead (Pb) in peripheral blood were analyzed by an AA7000M atomic absorption spectrometer (East & West Analytical Instruments Co., Ltd., Beijing, China). The following normal ranges for different elements were applied: Zn (mg/L): 5–11.94; Cu (mg/L): 0.76–2.5; Fe (mg/L): 418.48–660.8; Ca (mg/L): 84–62.86; Mg (mg/L): 28.3–50.4; and Pb (μg/L): 0–100.

## Statistical Analysis

Data were analyzed using IBM SPSS 17.0 for Windows (SPSS, Inc., Chicago, IL, USA). In the single factor analysis, normally distributed continuous data are presented as the mean ± standard deviation (SD), and nonnormally distributed continuous data are presented as the median (interquartile range). Categorical data are presented as the frequency (%). Normally distributed continuous data were compared using an independent t test, and nonnormally distributed continuous data were compared using the Mann-Whitney U test. Unordered categorical data were compared using the chi-squared test, and ordered categorical data were compared using the rank-sum test. In the multifactor analysis, multiple linear regression analysis was used. Statistical significance was defined as $p < 0.05$.

# RESULTS

## Single Factor Analysis of Bone Mineral Density-Related Factors

We evaluated whether there was a difference in indicators between the two groups (**Table 1**) and found that there was no significant difference in maternal height, smoking, paternal height, paternal weight, paternal BMI, alcohol consumption, full-term birth, amniotic fluid characteristics, birth length, birth weight, sex, age, height, weight, BMI, Cu, Ca, or Mg between the ART and NC groups. On the one hand, there was a significant reduction in Zn (p=0.009) and Fe (p=0.001) in the ART group compared with the NC group; on the other hand, maternal childbearing age (p=0.002), maternal weight (p=0.031), maternal BMI (p=0.021), paternal childbearing age (p=0.001), cesarean section rate (p=0.000) and Pb level (p=0.000) were significantly higher in the ART group than in the NC group. There was also a significant difference in maternal alcohol consumption (p=0.016) and paternal smoking (p=0.000) prevalence between the two groups.

The medians of the Z scores of the two groups were within the normal range, indicating that the total bone density level was not significantly abnormal in the ART or NC children.

**TABLE 1** | Single-factor analysis of bone mineral density-related factors.

| | ART group (84) | NC group (123) | P value |
|---|---|---|---|
| Maternal childbearing age (years) | 30 (27, 32) | 28 (26, 30) | 0.002** |
| Maternal height (cm) | 160.5 (158.0, 165.0) | 160.0 (158.0, 165.0) | 0.694 |
| Maternal weight (kg) | 59.0 (53.0, 65.8) | 55.0 (51.0, 61.7) | 0.031* |
| Maternal BMI ($kg/m^2$) | 22.5 (20.4, 25.1) | 21.3 (20.0, 23.4) | 0.021* |
| Maternal smoking: Never | 77 (91.7) | 108 (87.8) | 0.826 |
| Ever | 0 | 2 (1.6) | |
| Occasionally | 3 (3.6) | 2 (1.6) | |
| Still | 0 | 1 (0.8) | |
| Missing | 4 (4.8) | 10 (8.1) | |
| Maternal alcohol use: Never | 68 (81.0) | 78 (63.4) | 0.016* |
| Ever | 3 (3.6) | 0 | |
| Occasionally | 11 (13.1) | 35 (28.5) | |
| Still | 0 | 0 | |
| Missing | 2 (2.4) | 10 (8.1) | |
| Paternal childbearing age (years) | 32.5 (30, 35) | 30 (28, 33) | 0.001** |
| Paternal height (cm) | 173.0 (170.0, 176.0) | 173.0 (170.0, 176.0) | 0.702 |
| Paternal weight (kg) | 70.0 (65.0, 76.0) | 75.0 (66.5, 80.0) | 0.181 |
| Paternal BMI ($kg/m^2$) | 24.3 ± 2.99 | 24.8 ± 3.44 | 0.309 |
| Paternal smoking: Never | 20 (23.8) | 31 (25.2) | 0.000*** |
| Ever | 7 (8.3) | 7 (5.7) | |
| Occasionally | 10 (11.9) | 20 (16.3) | |
| Still | 41 (48.8) | 52 (42.3) | |
| Missing | 6 (7.1) | 13 (10.6) | |
| Paternal alcohol use: Never | 18 (21.4) | 26 (21.1) | 0.973 |
| Ever | 5 (6.0) | 5 (4.1) | |
| Occasionally | 47 (56.0) | 60 (48.8) | |
| Still | 9 (10.7) | 18 (14.6) | |
| Missing | 5 (6.0) | 14 (11.4) | |
| Gestational age: Premature birth | 8 (9.5) | 6 (4.9) | 0.421 |
| Full-term birth | 73 (86.9) | 86 (69.9) | |
| Missing | 3 (3.6) | 31 (25.2) | |
| Delivery mode: Spontaneous delivery | 17 (23.9) | 54 (76.1) | 0.000*** |
| Caesarean section | 63 (54.3) | 53 (45.7) | |
| Missing | 4 (20.0) | 16 (80.0) | |
| Amniotic fluid characteristic: Clean | 74 (88.1) | 92 (74.8) | 0.096 |
| I° | 0 | 5 (4.1) | |
| II° | 1 (1.2) | 3 (2.4) | |
| III° | 1 (1.2) | 1 (0.8) | |
| Missing | 8 (9.5) | 22 (17.9) | |
| Birth height (cm) | 50.0 (50.0, 51.0) | 50.0 (50.0, 51.0) | 0.543 |
| Birth weight (kg) | 3.5 (3.0, 3.8) | 3.5 (3.2, 3.8) | 0.738 |
| Sex: Male | 46 (43) | 61 (57) | 0.482 |
| Female | 38 (38) | 62 (62) | |
| Age (months) | 35 (21.25, 46) | 35 (20, 60.25) | 0.381 |
| Height (cm) | 96.3 ± 13.85 | 98.6 ± 18.13 | 0.298 |
| Weight (kg) | 14.8 (12.4, 17.9) | 14.9 (11.7, 19.0) | 0.618 |
| BMI ($kg/m^2$) | 15.8 (14.9, 17.0) | 15.9 (15.0, 17.0) | 0.662 |
| Zn (mg/L) | 5.54 (5.27, 5.74) | 5.67 (5.38, 6.23) | 0.009** |
| Cu (mg/L) | 1.27 ± 0.167 | 1.26 ± 0.156 | 0.825 |
| Fe (mg/L) | 428.82 (424.83, 435.80) | 434.16 (426.67, 445.92) | 0.001** |
| Ca (mg/L) | 66.72 (65.31, 68.16) | 66.58 (64.87, 68.55) | 0.625 |
| Mg (mg/L) | 36.38 ± 3.817 | 35.88 ± 3.478 | 0.361 |
| Pb (μg/L) | 47.00 (44.00, 49.00) | 40.60 (32.21, 48.90) | 0.000*** |
| Z score | 0.6 (-0.1, 1.1) | 0.5 (-0.3, 1.0) | 0.243 |

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ° degree.*

## Multiple Linear Regression Analysis of Bone Mineral Density-Related Factors

After adjustments were made for the above confounding factors, paternal childbearing age (p=0.012) was still found to negatively affect the BMD Z score independently, which means that the higher the paternal age was, the lower the Z score would be. Grouping (p=0.004) positively affected the Z score of BMD, and the effect was greater than that of the father's age. In other words, the Z score of the ART group was higher than that of the control group (**Table 2**).

## Subgroup Analysis of Bone Mineral Density-Related Factors

Next, we divided the ART group into different subgroups according to the ART method, infertility factors and embryo transfer cycles to find the source of the difference in BMD between the two groups.

In the ART method subgroup analysis (**Table 3**) IVF (p=0.026) and ICSI (p=0.008) had a positive impact on the Z score of BMD, and ICSI had a greater impact. This finding indicates that IVF or ICSI technology may affect the Z score of BMD.

In the infertility factor subgroup analysis (**Table 4**), PCOS alone was not shown to affect the Z score of BMD compared with natural conception; however, male infertility (p=0.010) or male infertility combined with PCOS (p=0.026) positively affected the Z score, and the impact of male infertility combined with PCOS was greater. This finding indicates that paternal infertility may affect the BMD of offspring.

In the embryo transfer cycle subgroup analysis (**Table 5**), compared with natural conception, both stimulation cycle fresh embryo transfer (p=0.019) and natural cycle frozen embryo transfer (p=0.006) had a positive effect on the bone density Z score, and natural cycle frozen embryo transfer had a greater impact. This finding indicates that abnormal maternal hormone levels during fresh embryo transfer and frozen embryo technology may affect BMD.

**TABLE 2 |** Multiple linear regression analysis of bone mineral density group category.

| | β-value | 95% CI | | P value |
|---|---|---|---|---|
| Maternal childbearing age | 0.027 | -0.030 | 0.083 | 0.359 |
| Maternal weight | -0.012 | -0.057 | 0.034 | 0.611 |
| Maternal BMI | -0.006 | -0.136 | 0.125 | 0.932 |
| Maternal alcohol consumption | -0.131 | -0.320 | 0.057 | 0.301 |
| Paternal childbearing age | -0.059 | -0.104 | -0.013 | 0.012* |
| Paternal smoking | -0.062 | -0.188 | 0.064 | 0.333 |
| Delivery mode: spontaneous delivery Cesarean section(reference) | -0.239 | -0.588 | 0.111 | 0.179 |
| Zn | -0.177 | -0.477 | 0.124 | 0.247 |
| Fe | 0.001 | -0.004 | 0.006 | 0.692 |
| Pb | -0.010 | -0.022 | 0.003 | 0.123 |
| Group category: ART group NC group (reference) | 0.559 | 0.186 | 0.931 | 0.004** |

*p<0.05, **p<0.01.

**TABLE 3 |** Multiple linear regression analysis of bone mineral density- ART methods.

| | β-value | 95% CI | | P value |
|---|---|---|---|---|
| Maternal childbearing age | 0.027 | -0.030 | 0.084 | 0.350 |
| Maternal weight | -0.014 | -0.060 | 0.032 | 0.547 |
| Maternal BMI | 0.003 | -0.132 | 0.137 | 0.966 |
| Maternal alcohol consumption | -0.126 | -0.316 | 0.065 | 0.194 |
| Paternal childbearing age | -0.059 | -0.105 | -0.013 | 0.012* |
| Paternal smoking | -0.061 | -0.188 | 0.065 | 0.339 |
| Delivery mode: Spontaneous delivery Cesarean section(reference) | -0.235 | -0.586 | 0.115 | 0.186 |
| Zn | -0.167 | -0.470 | 0.137 | 0.279 |
| Fe | 0.001 | -0.004 | 0.006 | 0.715 |
| Pb | -0.010 | -0.023 | 0.003 | 0.121 |
| ART methods: IVF (48) | 0.494 | 0.059 | 0.929 | 0.026* |
| ICSI (36) | 0.639 | 0.174 | 1.105 | 0.008** |
| NC (reference) | | | | |

*p < 0.05, **p < 0.01.

**TABLE 4 |** Multiple linear regression analysis of bone mineral density—Infertility factors.

| | β-value | 95% CI | | P value |
|---|---|---|---|---|
| Maternal childbearing age | 0.025 | -0.032 | 0.082 | 0.386 |
| Maternal weight | -0.010 | -0.056 | 0.037 | 0.677 |
| Maternal BMI | -0.013 | -0.149 | 0.122 | 0.846 |
| Maternal alcohol consumption | -0.136 | -0.327 | 0.055 | 0.160 |
| Paternal childbearing age | -0.057 | -0.103 | -0.011 | 0.016* |
| Paternal smoking | -0.061 | -0.188 | 0.067 | 0.348 |
| Delivery mode: spontaneous delivery Cesarean section (reference) | -0.222 | -0.575 | 0.130 | 0.214 |
| Zn | -0.178 | -0.480 | 0.124 | 0.246 |
| Fe | 0.001 | -0.004 | 0.006 | 0.678 |
| Pb | -0.010 | -0.023 | 0.003 | 0.117 |
| Infertility factors: PCOS (22) | 0.443 | -0.095 | 0.982 | 0.106 |
| male infertility (52) | 0.554 | 0.132 | 0.976 | 0.010* |
| male infertility combined with PCOS (10) | 0.861 | 0.106 | 1.615 | 0.026* |
| NC (reference) | | | | |

*p < 0.05.

**TABLE 5 |** Multiple linear regression analysis of bone mineral density- embryo transfer cycles.

| | β-value | 95% CI | | P value |
|---|---|---|---|---|
| Maternal childbearing age | 0.023 | -0.035 | 0.080 | 0.439 |
| Maternal weight | -0.014 | -0.060 | 0.032 | 0.545 |
| Maternal BMI | 0.002 | -0.130 | 0.133 | 0.981 |
| Maternal alcohol consumption | -0.129 | -0.318 | 0.059 | 0.177 |
| Paternal childbearing age | -0.057 | -0.103 | -0.011 | 0.015* |
| Paternal smoking | -0.063 | -0.189 | 0.063 | 0.321 |
| Delivery mode: Spontaneous delivery Cesarean section (reference) | -0.247 | -0.596 | 0.103 | 0.165 |
| Zn | -0.180 | -0.480 | 0.121 | 0.239 |
| Fe | 0.001 | -0.004 | 0.006 | 0.682 |
| Pb | -0.010 | -0.022 | 0.003 | 0.126 |
| Embryo transfer cycles: fresh embryo transferred stimulation cycle (62) | 0.481 | 0.082 | 0.881 | 0.019* |
| Frozen embryo transferred natural cycle (22) NC (reference) | 0.765 | 0.228 | 1.303 | 0.006** |

*p < 0.05, **p < 0.01.

## DISCUSSION

ART has been used to treat infertility for more than 40 years. It has been confirmed that ART is associated with adverse perinatal outcomes, including premature birth, low birth weight, and increased risk of birth defects. A poor ART intrauterine environment and ART gamete manipulation, among other factors, are considered to be possible causes of poor ART pregnancy outcomes (35, 36). Therefore, the following question remains: as the child ages, is the growth and development of the ART-conceived offspring the same as that of the NC offspring?

Bone density characterizes bone development. Childhood is a critical period for bone development. Prevention and treatment of childhood bone mineral deficiency is important for reducing childhood rickets and osteoporosis in adulthood. Therefore, it is particularly important to assess the bone density of offspring conceived *via* ART.

Previous studies focused mostly on the BMD of ART-conceived offspring at birth or within a short period of time after birth. There is no long-term follow-up study on the bone development of ART-conceived offspring at preschool age. Therefore, this study focuses on the BMD of preschool-aged ART-conceived offspring. Whether there is a difference between the ART-conceived children and the NC offspring at the same age and the possible influencing factors were examined.

We found that the BMD Z scores of the offspring of the ART group were generally in the normal range and showed no obvious abnormality. This result is consistent with that of a 2007 study: There was no statistically significant difference between the BMD of the offspring conceived *via* IVF fresh embryo transfer at 4-10 years old and the NC offspring (12). Another study in 2015 showed that the SOS level measured within 96 hours in ART-conceived preterm infants was lower than that in infants in the NC group (11). The author suggested that this difference may be due to epigenetic changes in imprinted genes or other genes that undergo epigenetic modifications and participate in growth and the bone state. However, the number of participants in the study was relatively small, including only 37 ART-conceived infants (IVF or ICSI, no distinction between fresh embryos or frozen embryos) and 51 NC infants.

Our results revealed that the offspring of the ART group and the NC group had differences in maternal and paternal age, maternal weight, maternal BMI, maternal alcohol consumption, paternal smoking, delivery method, and serum zinc, iron, and lead levels.

The maternal and paternal ages in the ART group were higher than those in the NC group. This finding is consistent with previous studies (12, 37). Age is an independent risk factor affecting fertility; the older the age is, the higher the incidence of infertility. Thus, the childbearing age in the ART group was obviously higher than that in the NC group. Interestingly, after adjustments were made for the relevant variables in this study, maternal age no longer showed an independent effect on the BMD Z score. Instead, paternal age negatively affected the BMD Z score. A paternal childbearing age > 40 years is associated with an increased risk of spontaneous abortion (38–41), and advanced paternal age is associated with autosomal dominant disorders, such as Alport syndrome, achondroplasia and neurofibromatosis (42–46). There are also cohort studies and population studies showing that advanced paternal age is related to autism spectrum disorders and schizophrenia. A large prospective study of autism in Denmark with 1 million children found that the relative risk associated with a father's age from 40 to 44 years was 1.6 (47), and a cohort study in Israel found that the odds ratio for a father's age from 40 to 49 years was 5.75 (48). A large American study found that for every 10-year increase in paternal age, the relative risk of autism was 1.3 (49). Another study in Israel showed that the relative risk of having offspring with schizophrenia was 2.0 for fathers 45 to 49 years and 3.0 for fathers >50 years (50). Similar results have been observed in studies of other ethnic populations, including populations in Denmark, Sweden, and Japan (51–53).

Multifactor analysis with adjustments for relevant factors showed that paternal childbearing age and group category still significantly affected the Z score of the BMD. The possible reasons are as follows.

First, ART progeny may have subtle changes in the DNA methylation patterns of imprinted genes related to bone development. It has been reported in the literature that there is an increased risk of Beckwith-Wiedemwann syndrome in offspring conceived *via* ART (54–56). Although there is no description of the bone density of children with Beckwith-Wiedemwann syndrome, relatives of children with Beckwith-Wiedemann syndrome have been found to have higher childhood heights (12, 57). The phenotypic characteristics of being tall and having normal body weight confirm that the increase in the bone density Z score of ART-conceived offspring may be a subtle change in the DNA methylation pattern of imprinted genes related to skeletal development.

Second, ART-conceived offspring are exposed to supraphysiological doses of oestrogen *in utero*. The process of ART ovulation induces a high estrogen environment in the mother's uterus, and estrogen promotes the development of the child's bones. Estrogen can reduce the strain set point of the mechanical regulation system on the bone surface of the endosteum and increase the accumulation of bone in the cortex, causing thickening of the cortex (58, 59). In this study, to identify the possible reasons for the higher BMD of the ART offspring, the ART group was divided into different subgroups according to the ART method, infertility factors or embryo transfer cycle. In the ART group (62 cases), the BMD Z score was higher than that in the control group, verifying our hypothesis.

Skeletal development is affected by a variety of confounding factors, including genetics (parental bone development), socioeconomic background, nutritional status, and puberty. In our previous research, we collected data on the height of parents and found that the difference in height between the two groups was not statistically significant. Therefore, when we recruited participants, we chose families located in Nanjing, Jiangsu Province, to reduce the gap between family socioeconomic

backgrounds and increase follow-up compliance. This study failed to accurately collect data on the eating habits of each participant. When we collected the study data, we collected information regarding the family's dietary preferences in the questionnaire: vegetarian, meat-eaters, balanced diet or special eating habits. The vast majority of families consumed a balanced diet, and the offspring's nutritional status was evaluated during the physical examination. It can be roughly considered that there was no significant difference in nutritional status between the two groups. Both groups of offspring were prepubertal children, thus eliminating the influence of pubertal sex hormones on bone development. We used inclusion and exclusion criteria to reduce selection bias, but there may still be potential uncontrolled confounding factors.

## CONCLUSION

Paternal childbearing age can independently affect the BMD Z score, and the higher the father's age is, the lower the bone mineral density Z score will be. After adjustments were made for related confounding factors, the BMD Z scores of the ART group and the control group were still significantly different, that is, the bone density Z score in the ART group was significantly higher than that in the control group. The method of conception, ART indications and embryo transfer cycle all had a positive effect on the Z score of BMD.

### Limitations

The sample size of the subgroups still needs to be expanded to verify the results. However, additional longer-term, prospective follow-up multicenter studies are required to understand the increased risks among children conceived *via* ART.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethic Committee in First Affiliated Hospital of Nanjing Medical University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## AUTHOR CONTRIBUTIONS

XX wrote the draft of the manuscript and performed the data analyses. LC, JW, and XY conducted the health tracking survey and acquired the data. LG and YZ recruited the participants. FD and YC reviewed and edited the manuscript. JL and YM designed the study. All authors contributed to the article and approved the submitted version.

## FUNDING

This work was funded by the National Key Research and Development Program of China (2016YFC1000204, 2017YFC1001300), the National Nature Science Foundation of China (81730041), and The National Key Research and Clinical Centre of Reproductive Medicine in Jiangsu Province of China (YXZXB2016001).

## REFERENCES

1. Chiware TM, Vermeulen N, Blondeel K, Farquharson R, Kiarie J, Lundin K, et al. IVF and Other ART in Low- and Middle-Income Countries: A Systematic Landscape Analysis. *Hum Reprod Update* (2021) 27:213–28. doi: 10.1093/humupd/dmaa047
2. Szamatowicz M. Assisted Reproductive Technology in Reproductive Medicine - Possibilities and Limitations. *Ginekol Pol* (2016) 87:820–3. doi: 10.5603/GP.2016.0095
3. Inhorn MC, Patrizio P. Infertility Around the Globe: New Thinking on Gender, Reproductive Technologies and Global Movements in the 21st Century. *Hum Reprod Update* (2015) 21:411–26. doi: 10.1093/humupd/dmv016
4. Finnstrom O, Kallen B, Lindam A, Nilsson E, Nygren KG, Olausson PO. Maternal and Child Outcome After *In Vitro* Fertilization–A Review of 25 Years of Population-Based Data From Sweden. *Acta Obstet Gynecol Scand* (2011) 90:494–500. doi: 10.1111/j.1600-0412.2011.01088.x
5. Hyrapetian M, Loucaides EM, Sutcliffe AG. Health and Disease in Children Born After Assistive Reproductive Therapies (ART). *J Reprod Immunol* (2014) 106:21–6. doi: 10.1016/j.jri.2014.08.001
6. Lacamara C, Ortega C, Villa S, Pommer R, Schwarze JE. Are Children Born From Singleton Pregnancies Conceived by ICSI at Increased Risk for Congenital Malformations When Compared to Children Conceived Naturally? A Systematic Review and Meta-Analysis. *JBRA Assist Reprod* (2017) 21:251–9. doi: 10.5935/1518-0557.20170047
7. Giorgione V, Parazzini F, Fesslova V, Cipriani S, Candiani M, Inversetti A, et al. Congenital Heart Defects in IVF/ICSI Pregnancy: Systematic Review and Meta-Analysis. *Ultrasound Obstet Gynecol* (2018) 51:33–42. doi: 10.1002/uog.18932
8. Zheng D, Nguyen QN, Li R, Dang VQ. Is Intracytoplasmic Sperm Injection the Solution for All in Unexplained Infertility? *Semin Reprod Med* (2020) 38:36–47. doi: 10.1055/s-0040-1719085
9. Luke B, Brown MB, Wantman E, Forestieri NE, Browne ML, Fisher SC, et al. The Risk of Birth Defects With Conception by ART. *Hum Reprod* (2021) 36:116–29. doi: 10.1093/humrep/deaa272
10. Qin J, Liu X, Sheng X, Wang H, Gao S. Assisted Reproductive Technology and the Risk of Pregnancy-Related Complications and Adverse Pregnancy Outcomes in Singleton Pregnancies: A Meta-Analysis of Cohort Studies. *Fertil Steril* (2016) 105:73–85.e1-6. doi: 10.1016/j.fertnstert.2015.09.007
11. Armangil D, Gursoy T, Korkmaz A, Ozyuncu O. Decreased Bone Ultrasound Velocity in Premature Infants Conceived With Assisted Reproduction. *Turk J Pediatr* (2015) 57:17–25.
12. Miles HL, Hofman PL, Peek J, Harris M, Wilson D, Robinson EM, et al. *In Vitro* Fertilization Improves Childhood Growth and Metabolism. *J Clin Endocrinol Metab* (2007) 92:3441–5. doi: 10.1210/jc.2006-2465
13. Yu F, Xia W. The Epidemiology of Osteoporosis, Associated Fragility Fractures, and Management Gap in China. *Arch Osteoporos* (2019) 14:32. doi: 10.1007/s11657-018-0549-y
14. Henwood MJ, Binkovitz L. Update on Pediatric Bone Health. *J Am Osteopath Assoc* (2009) 109:5–12.

15. Bas EK, Bulbul A, Sirzai H, Arslan S, Uslu S, Bas V, et al. The Long-Term Impacts of Preterm Birth and Associated Morbidities on Bone Health in Preschool Children: A Prospective Cross-Sectional Study From Turkey. *J Matern Fetal Neonatal Med* (2020) 35(4):677–84. doi: 10.1080/14767058.2020.1730801
16. Nordman H, Voutilainen R, Laitinen T, Antikainen L, Jaaskelainen J. Birth Size, Body Composition, and Adrenal Androgens as Determinants of Bone Mineral Density in Mid-Childhood. *Pediatr Res* (2018) 83:993–8. doi: 10.1038/pr.2018.12
17. Baradaran Mahdavi S, Daniali SS, Farajzadegan Z, Bahreynian M, Riahi R, Kelishadi R. Association Between Maternal Smoking and Child Bone Mineral Density: A Systematic Review and Meta-Analysis. *Environ Sci Pollut Res Int* (2020) 27:23538–49. doi: 10.1007/s11356-020-08740-1
18. Sakka SD, Cheung MS. Management of Primary and Secondary Osteoporosis in Children. *Ther Adv Musculoskelet Dis* (2020) 12:1759720X20969262. doi: 10.1177/1759720X20969262
19. Al-Agha AE, Kabli YO, AlBeiruty MG, Milyani AA. Determinants of Bone Mineral Density Through Quantitative Ultrasound Screening of Healthy Children Visiting Ambulatory Paediatric Clinics. *Saudi Med J* (2019) 40:560–7. doi: 10.15537/smj.2019.6.24234
20. Closa-Monasterolo R, Zaragoza-Jordana M, Ferre N, Luque V, Grote V, Koletzko B, et al. Adequate Calcium Intake During Long Periods Improves Bone Mineral Density in Healthy Children. Data from the Childhood Obesity Project. *Clin Nutr* (2018) 37:890–6. doi: 10.1016/j.clnu.2017.03.011
21. Yang X, Zhai Y, Zhang J, Chen JY, Liu D, Zhao WH. Combined Effects of Physical Activity and Calcium on Bone Health in Children and Adolescents: A Systematic Review of Randomized Controlled Trials. *World J Pediatr* (2020) 16:356–65. doi: 10.1007/s12519-019-00330-7
22. Ren Y, Xi X, Hu D, Shang W, Peng S, Fan L, et al. Determinants for Low Bone Mineral Density in Pre-School Children: A Matched Case-Control Study in Wuhan, China. *J Pediatr Endocrinol Metab* (2019) 32:739–48. doi: 10.1515/jpem-2018-0554
23. Denova-Gutierrez E, Mendez-Sanchez L, Munoz-Aguirre P, Tucker KL, Clark P. Dietary Patterns, Bone Mineral Density, and Risk of Fractures: A Systematic Review and Meta-Analysis. *Nutrients* (2018) 10:1922. doi: 10.3390/nu10121922
24. Rokoff LB, Rifas-Shiman SL, Switkowski KM, Young JG, Rosen CJ, Oken E, et al. Body Composition and Bone Mineral Density in Childhood. *Bone* (2019) 121:9–15. doi: 10.1016/j.bone.2018.12.009
25. Soininen S, Sidoroff V, Lindi V, Mahonen A, Kroger L, Kroger H, et al. Body Fat Mass, Lean Body Mass and Associated Biomarkers as Determinants of Bone Mineral Density in Children 6-8years of Age - The Physical Activity and Nutrition in Children (PANIC) Study. *Bone* (2018) 108:106–14. doi: 10.1016/j.bone.2018.01.003
26. Fintini D, Cianfarani S, Cofini M, Andreoletti A, Ubertini GM, Cappa M, et al. The Bones of Children With Obesity. *Front Endocrinol (Lausanne)* (2020) 11:200. doi: 10.3389/fendo.2020.00200
27. Kindler JM, Kelly A, Khoury PR, Levitt Katz LE, Urbina EM, Zemel BS. Bone Mass and Density in Youth With Type 2 Diabetes, Obesity, and Healthy Weight. *Diabetes Care* (2020) 43:2544–52. doi: 10.2337/dc19-2164
28. Lee HS, Rho JG, Kum CD, Lim JS, Hwang JS. Low Bone Mineral Density at Initial Diagnosis in Children and Adolescents With Graves' Disease. *J Clin Densitom* (2021) 24:275–80. doi: 10.1016/j.jocd.2020.05.006
29. Mantovani A, Gatti D, Zoppini G, Lippi G, Bonora E, Byrne CD, et al. Association Between Nonalcoholic Fatty Liver Disease and Reduced Bone Mineral Density in Children: A Meta-Analysis. *Hepatology* (2019) 70:812–23. doi: 10.1002/hep.30538
30. Zhao Y, Qin R, Ma X, Qin Z, Yang Z, Hong H, et al. Adiposity Is Not Beneficial to Bone Mineral Density in 0-5 Year Old Chinese Children: The Jiangsu Bone Health Study. *Obes Res Clin Pract* (2020) 14:39–46. doi: 10.1016/j.orcp.2019.10.011
31. Ashritha A, Delhi Kumar CG, Sahoo J, Nalini P. Evaluation of Bone Mineral Density in Children With Hemophilia: An Observational Case-Control Study. *J Pediatr Hematol Oncol* (2019) 41:511–4. doi: 10.1097/MPH.0000000000001554
32. Fedewa MV, Bentley JL, Higgins S, Kindler JM, Esco MR, MacDonald HV. Celiac Disease and Bone Health in Children and Adolescents: A Systematic Review and Meta-Analysis. *J Clin Densitom* (2020) 23:200–11. doi: 10.1016/j.jocd.2019.02.003
33. Alvarez Zaragoza C, Vasquez Garibay EM, Garcia Contreras AA, Larrosa Haro A, Romero Velarde E, Rea Rosas A, et al. Bone Mineral Density and Nutritional Status in Children With Quadriplegic Cerebral Palsy. *Arch Osteoporos* (2018) 13:17. doi: 10.1007/s11657-018-0434-8
34. Inaba H, Cao X, Han AQ, Panetta JC, Ness KK, Metzger ML, et al. Bone Mineral Density in Children With Acute Lymphoblastic Leukemia. *Cancer* (2018) 124:1025–35. doi: 10.1002/cncr.31184
35. Aitken RJ. Age, the Environment and Our Reproductive Future: Bonking Baby Boomers and the Future of Sex. *Reproduction* (2014) 147:S1–S11. doi: 10.1530/REP-13-0399
36. Agarwal A, Durairajanayagam D, du Plessis SS. Utility of Antioxidants During Assisted Reproductive Techniques: An Evidence Based Review. *Reprod Biol Endocrinol* (2014) 12:112. doi: 10.1186/1477-7827-12-112
37. Bonduelle M, Wennerholm UB, Loft A, Tarlatzis BC, Peters C, Henriet S, et al. A Multi-Centre Cohort Study of the Physical Health of 5-Year-Old Children Conceived After Intracytoplasmic Sperm Injection, *In Vitro* Fertilization and Natural Conception. *Hum Reprod* (2005) 20:413–9. doi: 10.1093/humrep/deh592
38. Kleinhaus K, Perrin M, Friedlander Y, Paltiel O, Malaspina D, Harlap S. Paternal Age and Spontaneous Abortion. *Obstet Gynecol* (2006) 108:369–77. doi: 10.1097/01.AOG.0000224606.26514.3a
39. Nguyen BT, Chang EJ, Bendikson KA. Advanced Paternal Age and the Risk of Spontaneous Abortion: An Analysis of the Combined 2011-2013 and 2013-2015 National Survey of Family Growth. *Am J Obstet Gynecol* (2019) 221:476 e1–7. doi: 10.1016/j.ajog.2019.05.028
40. Slama R, Bouyer J, Windham G, Fenster L, Werwatz A, Swan SH. Influence of Paternal Age on the Risk of Spontaneous Abortion. *Am J Epidemiol* (2005) 161:816–23. doi: 10.1093/aje/kwi097
41. Maconochie N, Doyle P, Prior S, Simmons R. Risk Factors for First Trimester Miscarriage–Results From a UK-Population-Based Case-Control Study. *BJOG* (2007) 114:170–86. doi: 10.1111/j.1471-0528.2006.01193.x
42. Riccardi VM, Dobson CE2nd, Chakraborty R, Bontke C. The Pathophysiology of Neurofibromatosis: IX. Paternal Age as a Factor in the Origin of New Mutations. *Am J Med Genet* (1984) 18:169–76. doi: 10.1002/ajmg.1320180121
43. Orioli IM, Castilla EE, Scarano G, Mastroiacovo P. Effect of Paternal Age in Achondroplasia, Thanatophoric Dysplasia, and Osteogenesis Imperfecta. *Am J Med Genet* (1995) 59:209–17. doi: 10.1002/ajmg.1320590218
44. Carothers AD, McAllion SJ, Paterson CR. Risk of Dominant Mutation in Older Fathers: Evidence From Osteogenesis Imperfecta. *J Med Genet* (1986) 23:227–30. doi: 10.1136/jmg.23.3.227
45. Jadayel D, Fain P, Upadhyaya M, Ponder MA, Huson SM, Carey J, et al. Paternal Origin of New Mutations in Von Recklinghausen Neurofibromatosis. *Nature* (1990) 343:558–9. doi: 10.1038/343558a0
46. North K. Neurofibromatosis Type 1: Review of the First 200 Patients in an Australian Clinic. *J Child Neurol* (1993) 8:395–402. doi: 10.1177/088307389300800421
47. Lauritsen MB, Pedersen CB, Mortensen PB. Effects of Familial Risk Factors and Place of Birth on the Risk of Autism: A Nationwide Register-Based Study. *J Child Psychol Psychiatry* (2005) 46:963–71. doi: 10.1111/j.1469-7610.2004.00391.x
48. Reichenberg A, Gross R, Weiser M, Bresnahan M, Silverman J, Harlap S, et al. Advancing Paternal Age and Autism. *Arch Gen Psychiatry* (2006) 63:1026–32. doi: 10.1001/archpsyc.63.9.1026
49. Croen LA, Najjar DV, Fireman B, Grether JK. Maternal and Paternal Age and Risk of Autism Spectrum Disorders. *Arch Pediatr Adolesc Med* (2007) 161:334–40. doi: 10.1001/archpedi.161.4.334
50. Malaspina D, Harlap S, Fennig S, Heiman D, Nahon D, Feldman D, et al. Advancing Paternal Age and the Risk of Schizophrenia. *Arch Gen Psychiatry* (2001) 58:361–7. doi: 10.1001/archpsyc.58.4.361
51. Byrne M, Agerbo E, Ewald H, Eaton WW, Mortensen PB. Parental Age and Risk of Schizophrenia: A Case-Control Study. *Arch Gen Psychiatry* (2003) 60:673–8. doi: 10.1001/archpsyc.60.7.673
52. Tsuchiya KJ, Takagai S, Kawai M, Matsumoto H, Nakamura K, Minabe Y, et al. Advanced Paternal Age Associated With an Elevated Risk for Schizophrenia in Offspring in a Japanese Population. *Schizophr Res* (2005) 76:337–42. doi: 10.1016/j.schres.2005.03.004

53. Zammit S, Allebeck P, Dalman C, Lundberg I, Hemmingson T, Owen MJ, et al. Paternal Age and Risk for Schizophrenia. *Br J Psychiatry* (2003) 183:405–8. doi: 10.1192/bjp.183.5.405
54. Gicquel C, Gaston V, Mandelbaum J, Siffroi JP, Flahault A, Le Bouc Y. *In Vitro* Fertilization May Increase the Risk of Beckwith-Wiedemann Syndrome Related to the Abnormal Imprinting of the KCN1OT Gene. *Am J Hum Genet* (2003) 72:1338–41. doi: 10.1086/374824
55. DeBaun MR, Niemitz EL, Feinberg AP. Association of *In Vitro* Fertilization With Beckwith-Wiedemann Syndrome and Epigenetic Alterations of LIT1 and H19. *Am J Hum Genet* (2003) 72:156–60. doi: 10.1086/346031
56. Hattori H, Hiura H, Kitamura A, Miyauchi N, Kobayashi N, Takahashi S, et al. Association of Four Imprinting Disorders and ART. *Clin Epigenet* (2019) 11:21. doi: 10.1186/s13148-019-0623-3
57. Elliott M, Bayly R, Cole T, Temple IK, Maher ER. Clinical Features and Natural History of Beckwith-Wiedemann Syndrome: Presentation of 74 New Cases. *Clin Genet* (1994) 46:168–74. doi: 10.1111/j.1399-0004.1994.tb04219.x
58. Lanyon LE. Using Functional Loading to Influence Bone Mass and Architecture: Objectives, Mechanisms, and Relationship With Estrogen of the Mechanically Adaptive Process in Bone. *Bone* (1996) 18:37S–43S. doi: 10.1016/8756-3282(95)00378-9
59. Rodan GA. Mechanical Loading, Estrogen Deficiency, and the Coupling of Bone Formation to Bone Resorption. *J Bone Miner Res* (1991) 6:527–30. doi: 10.1002/jbmr.5650060602

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Physiology

ORIGINAL RESEARCH
published: 08 February 2022
doi: 10.3389/fphys.2022.827941

# Evaluation of Laser Confocal Raman Spectroscopy as a Non-Invasive Method for Detecting Sperm DNA Contents

*Mengge Li[1,2†], Yaxing Ji[3†], Dongmei Wang[4], Yanliang Zhang[4], Huan Zhang[3,5], Yi Tang[5], Ge Lin[1,3,5] and Liang Hu[1,3,5,6*]*

*[1]National Engineering and Research Center of Human Stem Cells, Changsha, China, [2]Hunan Guangxiu Hospital, Changsha, China, [3]NHC Key Laboratory of Human Stem Cell and Reproductive Engineering, Institute of Reproductive and Stem Cell Engineering, School of Basic Medical Science, Central South University, Changsha, China, [4]Thermo Fisher Scientific, Shanghai, China, [5]Clinical Research Center for Reproduction and Genetics in Hunan Province, Reproductive and Genetic Hospital of CITIC-XIANGYA, Changsha, China, [6]Hunan International Scientific and Technological Cooperation Base of Development and Carcinogenesis, Changsha, China*

OPEN ACCESS

**Edited by:**
*Yimin Zhu,*
*Zhejiang University, China*

**Reviewed by:**
*Alex C. Varghese, Astra Fertility Group, Canada*
*Sijia Lu, Yikon Genomics, China*

***Correspondence:**
*Liang Hu*
*lianghu7@gmail.com*

*[†]These authors have contributed equally to this work*

**Specialty section:**
*This article was submitted to Reproduction, a section of the journal Frontiers in Physiology*

**Received:** *02 December 2021*
**Accepted:** *13 January 2022*
**Published:** *08 February 2022*

**Citation:**
*Li M, Ji Y, Wang D, Zhang Y, Zhang H, Tang Y, Lin G and Hu L (2022) Evaluation of Laser Confocal Raman Spectroscopy as a Non-Invasive Method for Detecting Sperm DNA Contents. Front. Physiol. 13:827941. doi: 10.3389/fphys.2022.827941*

**Research Question:** Is Raman spectroscopy an efficient and accurate method to detect sperm chromosome balance state by DNA content differences?

**Design:** Semen samples were provided by diploid healthy men, and the analysis parameters met the current World Health Organization standards. The DNA content was assessed by analysis of the corresponding spectra obtained from a laser confocal Raman spectroscope. The sperm sex chromosome information was obtained by fluorescence *in situ* hybridization (FISH). Comparative analysis was performed between FISH results and Raman spectral analysis results.

**Results:** Different parts of the sperm head showed different spectral signal intensities, which indicated that there were different chemical components. Standard principal component analysis (PCA) can preliminarily classify sperm with different DNA contents into two groups. Further analysis showed that there were significant differences in the 785 DNA backbone peaks and 714–1,162 $cm^{-1}$ DNA skeleton regions among sperm with different DNA contents. The peak and regional peak of the DNA skeleton of X sperm were significantly higher than those of Y sperm (X vs. Y, $p < 0.05$). The above sperm types were confirmed by FISH. ROC curve analysis shows that there is a correlation between the Raman spectrum data and FISH results.

**Conclusion:** Raman spectroscopy can identify X and Y sperms by analyzing the DNA content difference. However, the accuracy of the detection still needs to be improved. Nevertheless, Raman spectroscopy has a potential application value in the field of sperm aneuploidy detection and may even be used as a non-invasive predictor of sperm aneuploid state in preimplantation genetic testing (PGT-A).

**Keywords: laser confocal Raman spectroscopy, human sperm, fluorescence *in situ* hybridization, DNA content, preimplantation genetic testing**

## INTRODUCTION

Spermatogenesis is a highly complex biological process in which spermatogonia undergo two meioses to produce sperm cells and transform into mature sperm through a series of nuclear and organelle changes (De Jonge and Barratt, 2006). In the process of spermatogenesis, if once meiosis is wrong, the normal chromosome balance changes, which leads to sperm aneuploidy (Rieger, 1968; McFeely, 1993). With the advancement of molecular diagnostic detection technology, it has been found that the normal chromosomes of males are also important factors for obtaining normal embryos, and sperm aneuploidy is directly related to embryo quality, which can lead to the failure of embryo implantation (Jan et al., 2004; Speyer et al., 2010) or repeated abortion during embryo development (Rodrigo et al., 2010; Scott et al., 2014; Ramasamy et al., 2015). Above all, aneuploid sperm fertilization not only affects embryo qualities but also leads to oocytes being wasted.

Preimplantation genetic testing of aneuploids (PGT-A) has become an indispensable technology in the field of assisted reproductive technology (ART; Franasiak et al., 2014). Currently, PGT-A for aneuploidy can detect defects in embryo chromosomes and select euploid embryos for transplantation (Eccles et al., 2017; Wilch and Morton, 2018). PGT-A uses different methods to identify the aneuploidy of all chromosomes, including the fluorescence *in situ* hybridization (FISH), array comparative genomic hybridization (CGH), and single nucleotide polymorphism (SNP) haplotypes combined with next-generation sequencing (NGS; Chen et al., 2018; Hu et al., 2018; Zhou et al., 2018). Despite the high accuracy of PGT-A, the embryo safety is still under doubt because the embryos must be biopsied. To date, little is known about the safety of the test samples obtained, although the initial report claimed that the method had no negative effects (Jiang et al., 2020). Furthermore, PGT-A can only be used to test normal embryos but cannot increase the number of transplantable embryos. Therefore, the non-invasive aneuploidy detection of gametes, especially sperm, may be an important way to improve the efficiency of assisted reproduction (Ranjith et al., 2014). Although the existing FISH and NGS technologies can detect the aneuploidy of single sperm, the sperm which is detected can no longer be used in ART. FISH detection only estimates the frequency of chromosomal abnormalities, which cannot guarantee the normal chromosomes of sperm used in ART, and there is no non-invasive technology to detect sperm aneuploidy (Patassini et al., 2013). Therefore, there is an urgent need for a non-invasive, efficient, and sensitive technique to directly assess sperm aneuploidy.

Raman spectroscopy is a non-invasive technique that allows the biochemical analysis of cellular components. As a vibration spectroscopy technique, Raman spectroscopy can identify chemical moieties through specific spectral patterns and can observe molecular changes with high specificity (Fragouli et al., 2017; Munné, 2018). It provides a rapid, simple, repeatable, non-destructive qualitative, and quantitative analysis of multicomponent materials by using the inelastic scattering of light, which has received increasing attention in research and clinical laboratories (Eberhardt et al., 2015). As early as 2014, there was study on non-invasive sex assessment in bovine semen by Raman spectroscopy combined with PCA analysis (De Luca et al., 2014). It has been reported that Raman spectroscopy can identify aneuploidy and aneuploidy embryos through embryo culture medium (Liang et al., 2019). In addition, Raman spectroscopy has detected oxidative DNA damage and mitochondrial damage caused by ultraviolet radiation (Konrad Meister et al., 2010; Mallidis et al., 2011). Therefore, the Raman technique may have great potential in the non-invasive detection of aneuploidy in germ cells, especially sperm.

In this study, we detected the human sperm chromosome DNA content by Raman spectroscopy and compared the Raman spectra data of X and Y sperms confirmed by FISH. Data analysis indicated that there were significant differences between the X and Y sperms Raman spectra data. Our results suggest Raman spectroscopy has a broad clinical application prospect in non-invasive sperm detection. However, more accurate and non-invasive improvement for detecting sperm aneuploidy is still needed.

## MATERIALS AND METHODS

### Semen Sample Collection

Semen samples were provided by three diploid healthy men after G-banded karyotype, and the analysis parameters met the current World Health Organization (WHO, 2010) standards. All the ejaculation sperm samples were obtained by masturbation after 2–7 days of sexual abstinence, followed by liquefaction (37°C, 30 min).

This study was approved by the Ethics Committee of Reproductive Medicine of Xiangya Reproductive Heredity Hospital of CITIC (LL-SC-2018-038). The written informed consent was acquired from all donors who voluntarily participated in the research. The present study was conducted in accordance with the principles of the Helsinki Declaration and medical ethics.

### Separation of Spermatozoa

The liquefied semen samples were placed in a centrifugal tube and centrifuged (Eppendorf 5810R, Germany) at 300 rcf for 15 min. A pipette was used to aspirate the supernatant and left the sediment at the centrifugal tube, and then, 1 ml phosphate-buffered saline (1 × PBS) was added to the centrifugal tube. After slowly whisking to mix and centrifuging at 300 rcf for 5 min, the semen samples were washed with PBS and recentrifuged at 300 rcf for 5 min. Aliquots of 1 μl of the sperm suspensions were coated on a glass slide (Fisher, Thermo Scientific), fixed in methanol/acetic acid (3:1) for 5 min, then cleaned by PBS, and air-dried.

### Optimization of the Spectral Acquisition Method

The stepwise optimization of the spectral acquisition system is displayed in the supplementary graph. The laser power was constant at 10 mw. Firstly, we found that scanning a point once in 0.5 s generated stronger signals than scanning three

times in 0.2 s did, which provided a better signal-to-noise ratio and clearer spectrum (**Supplementary Figure S1A**). Furthermore, we found that 15 scans in a point obtained better spectra than scanning only once (**Supplementary Figure S1B**).

## Laser Confocal Raman Microscope

We carried out analysis using a laser confocal Raman system (DXR Laser Microscopic Raman, Thermo Science). The system was equipped with an Olympus BX41 microscope, 532 nm laser (10 mW), adjustable confocal pinhole, automatic platform for microscopic sampling, standard resolution grating, XYZ drawing stage, and CCD inspection tester. The aperture of the pinhole collected by spectroscopy was 25 microns. The Olympus X 100 objective lens was used. We used a 532 nm laser and 10.0 mW laser power, and its image pixel size was 0.6 μm. The Raman signals of the thole sperm heads were acquired in the standard mode with an exposure time of 0.5 s, and each single sperm sample was scanned 15 times according to the above results. The spectra were obtained in a spectral range from 60 to 3,400 $cm^{-1}$, and the background control spectrum was reduced from each sample spectrum. The process of Raman analysis took approximately 15–30 min per sample. We collected the Raman spectra of more than 200 sperm heads.

## Fluorescence *in situ* Hybridization

We dropped 20 μl dithiothreitol (DTT) onto a glass slide and covered the coverslip, removed the coverslip quickly after 7 min, immersed the slides in 1X PBS for 2 min, and then dehydrated the slides in an ethanol series (75, 90, and 100%) for 2 min each. We put the glass slides an incubator at 37°C overnight. Because there was a significant difference in Y and X chromosome content, the sex chromosome of the sperm was determined and distinguished by FISH with the following commercial probes: the X chromosome (DXZ1, Xcen alphasatellite, SpectrumGreen) and the long arm of the Y chromosome (DYZ1, Yq12 satellite III, SpectrumRed), which were purchased from Abbott-Vysis (Downers Grove, IL, United States). After hybridization and washing, the glass slides were stained using distamycin A/4(,6-diamidino-2-phenylindole; DAPI, Millipore, Temecula, CA, United States), observed under the Olympus BX51 fluorescence microscope (Olympus, Tokyo, Japan), and utilized to capture fluorescence signals and photograph the images with VideoTesT FISH 2.0 (VideoTesT, St Petersburg, Russia; Carmen et al., 2019). The sperm cells analyzed by Raman spectroscopy matched with FISH analysis one by one. Firstly, we marked the reverse side of the slide in the scanning area of Raman microscope with a diamond pen and took photographs to record the shape of sperm distribution under the Raman microscope. After Raman scanning and FISH procedure, we found the labeled area and obtained the FISH results under the fluorescence microscope. The Raman microscope figures were then compared with FISH figures to find the corresponding sperms. Due to the sperm loss during FISH procedure, only about one-third of the sperms analyzed by Raman spectrum were retained.

## Analysis of Raman Spectra

Before spectrum analysis, all the Raman spectral data were processed using the TQ analyst EZ version. First, the Savitzky–Golay smoothing data points 11 and polynomial (5 degrees) options were used to correct the baseline and subtract the spectral background (**Supplementary Figure S2**), and then, the spectrum was filtered by using a denoising algorithm (denoising option) to improve the resolution without losing spectral information. The signals of 714.30–1161.86 $cm^{-1}$ (DNA-PO4 skeleton region) and 785 DNA skeleton peaks were used to present the sperm DNA content according to previous report (De Luca et al., 2014).

## Statistical Analysis

The measurements of all single sperm spectra data (over the 650–1,800 $cm^{-1}$ spectral range) were grouped together and analyzed by standard principal component analysis (PCA) and were computed to acquire the corresponding PC vectors. Next, the scores concerning PC vectors were plotted singly in triangle and circle shapes for each group. Clustering according to the triangle and circle groups was observed in the score space diagram, suggesting that the sperm samples could be separated based on the spectra PCA analysis calculated using the MATLAB (R2010a) software system.

Data analysis was performed using SPSS 23.0, and values of $p \leq 5\%$ were considered to be statistically significant. The data used in PCA were used for frequency distribution statistics. According to the results of FISH analysis, the corresponding sperm Raman spectrum data were divided into group x (X sperm) and group y (Y sperm). Mann–Whitney U-test was performed to calculate significant differences between the two groups. Then, the X and Y groups were categorized as dummy variables, and receiver operating characteristic (ROC) curve analysis was performed to evaluate the prediction value using the area under the curve (AUC) and to calculate the sex chromosome cutoff value.

# RESULTS

## Spectra and Mapping

To show the distribution of different chemical moieties clearly in the sperm head, we used the optimized final scheme to scan a single sperm. Four different colors of sperm head (blue, green, yellow, or red) were finally displayed, indicating the rich variety of chemical moieties in the sperm heads (**Figures 1A,B**). Then, we compared the Raman spectra of different parts of the human sperm head. The DNA skeleton, nucleotides, and phosphate skeleton were mainly located in the 725–1,162 $cm^{-1}$ region. As shown in **Figure 1C**, the changes and intensities of Raman peaks of the glass (black), acrosome (blue), and nucleus (red) of sperm head were very different in this region. Next, to verify the specificity of the sperm nucleus Raman spectrum, we tested the other single sperm heads, which were very close to the previous sperm nucleus spectrum (**Figures 2A–C**). When the spectra of sperm were combined into the same profile, the spectral trends of the sperm were completely consistent, and only the peak values were slightly different, especially at peaks at 785, 1,095, and 1,250 $cm^{-1}$ (**Figure 2D**).

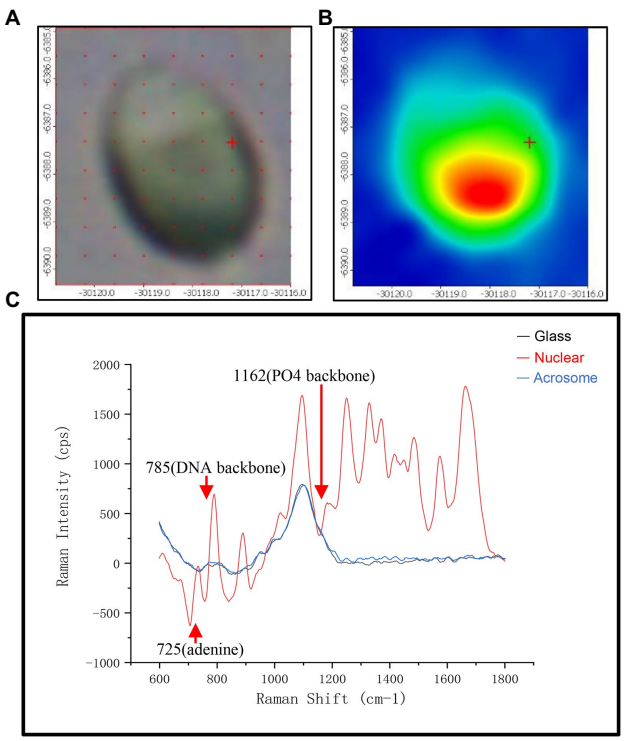



**FIGURE 1 |** The spectra of different regions in the sperm head. **(A)** Microscopic confocal picture of a human sperm cell head fixed on the glass slide. **(B)** The phase diagram of Raman microspectroscopy of the sperm head corresponding to **(A)**. **(C)** Raman spectra of three different positions with glass (black), acrosome (red) and DNA at the sperm head. The x-axis units are displacement wavenumber ($cm^{-1}$), nanometer (nm), and absolute wavenumber ($cm^{-1}$). The y-axis unit is generally Raman strength.

## Raman Microspectroscopy and Sperm FISH

To test the chromosomal difference of Raman scanning sperm, we subsequently performed FISH analysis with chromosome X and Y probes on the same slides. The distribution of X and Y sperm was approximately 2:1, as shown in **Figures 3A,B**. As shown in **Figure 3C**, the corresponding spectra of X sperm and Y sperm showed the same general characteristics and peak changing trend, but there were some differences in the corresponding DNA peaks. The peak intensity of X sperm at the DNA skeleton at 785 $cm^{-1}$ and the PO4 skeleton at 1095 $cm^{-1}$ was higher than that of Y sperm.

## PCA of the Spectra and Frequency Statistics

Due to the time-consuming nature of the Raman spectroscopy scan, we were able to scan 251 sperm with high quality and excellent signal. Most sperms were eluted during FISH processing, only 59 sperm had a one-to-one correspondence, including 39 X sperm and 20 Y sperm. The Raman spectra of each sample head were accumulated to obtain the average central spectrum. Among them, only 53 sperm were finally included in PCA (six sperms were excluded which could not be analyzed by PCA due to data deviation). These sperm were divided into two groups according to the calculation and calibration results of PCA: 22 sperm in group A and 31 sperm in group B (**Figure 4**), which was not exactly corresponded to the result of FISH. This result indicated that PCA could not distinguish the sperm sex chromosome completely and accurately.

Then, we conducted frequency distribution histogram statistics on the 59 sperm data, and the results showed that there were obvious frequency difference trends in the frequency distribution at I785 = 23,750 and Area714–1,162 = 3,250,000. For 20 Y sperm, the value of I785 mainly existed in the range of 17,000–23,000, and the mean value and median value were 21749.8 and 22,321, respectively. The main value range of Area (714–1,162) was 3,400,000–3,500,000, and the mean value and median values were 3,091,373 and 2,981,509, respectively. For 39 X sperm, the value of I785 mainly existed in the range of 29,000–35,000, and the mean value and median value were 26260.92 and 27,242, respectively. The main value range of Area (714–1,162) was 3,400,000–3,500,000, and the mean value and median value were 3,828,693 and 4,143,128, respectively. The analysis results indicated that these values could be the critical values of the difference between X and Y sperm (**Supplementary Figures S2A–D**).

## Discrimination of the Sperm With Different DNA Contents by Raman Spectroscopy

The total Raman spectra showed that there were significant differences between X sperm ($n = 39$) and Y sperm ($n = 20$) at 714–1162 $cm^{-1}$ and I785 (**Figure 5A**). Because the distribution of X and Y sperm data frequency does not accord with the normal distribution (**Supplementary Figures S3A–D**), we further analyzed the sperm of 59 cases by Mann–Whitney U-test rather than Student's t-test. The values of X and Y sperms in I785 ($p = 0.044$) and Area (714–1,162; $p = 0.0136$) were statistically different. Compared with the Y sperm group, the peak values at 785 and 714–1,162 $cm^{-1}$ in the X sperm group were higher than those in the Y sperm group, and the difference was more obvious in the regional peak (**Figure 5B**). ROC curve analysis was used to evaluate the sensitivity of the correlation between sperm DNA content and Raman spectra. The results showed that the corresponding thresholds of I785 = 24986.5 and Area714–1,162 $cm^{-1}$ = 3,748,990 were the best for distinguishing the two kinds of sperm. When the peak value of 785 or 714–1,162 $cm^{-1}$ exceeds this value, the possibility of X sperm is greatly increased. The AUCs of the ROC curves in both cases were 0.662 and 0.696, respectively (**Figures 5C**). Our results indicated that sperm DNA content has potential applicable value in the detection of sperm aneuploidy.

## DISCUSSION

Human sperm cells are usually divided into the head and the tail, including connecting pieces (neck) and flagella. The head is usually oval, with a length of 4–5.5 µm, a width of 2.5–3.5 mm, and an aspect ratio of approximately 1:1.5. The top of the

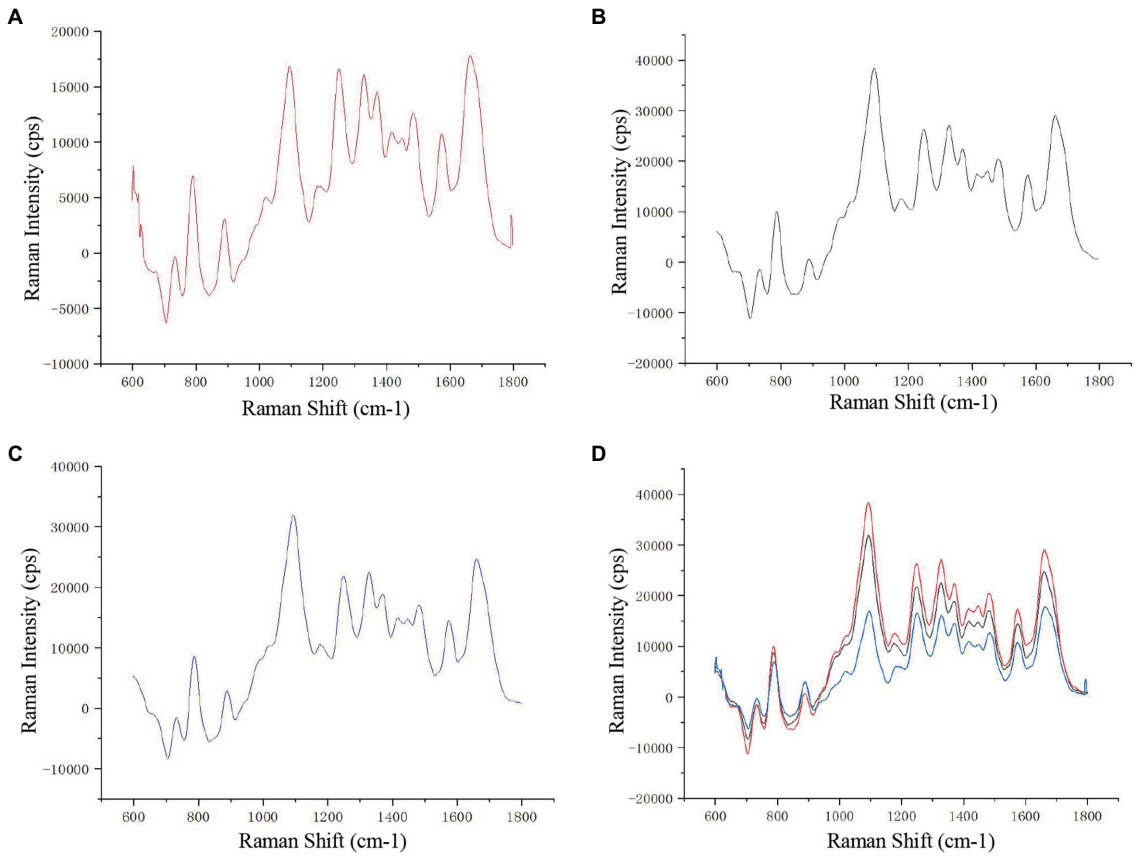



**FIGURE 2 |** Raman spectra of different sperm heads. **(A–C)** Three single sperm head Raman spectra. **(D)** The combined Raman spectra of the three sperms.

head is its acrosome area, covering approximately 40–70% of the head. The nucleus is located in the back of sperm head. It has been suggested that the aneuploidy of embryos may be caused by the fertilization of aneuploid sperm and oocytes, which may cause aneuploid blastocysts, resulting in implantation failure or loss of early pregnancy and waste of oocytes (Rodrigo et al., 2010; Scott et al., 2014; Arumugam et al., 2019). Although sperm FISH technology has become an important technology for male sperm aneuploidy detection (Ranjith et al., 2014), some shortcomings of the technology hinder its clinical application (Ioannou et al., 2018). FISH only analyzes the percentage of aneuploidic spermatozoa, and sperm treated with FISH cannot be used for normal fertilization again. Currently, the only criteria of better sperms in IVF lab remain to the sperm intensity, motility rate, and morphology, which cannot represent the euploidy of the sperms. Therefore, non-invasive sperm aneuploidy detection is very important to reduce the oocyte waste rate and improve the success rate of ART (Jiang et al., 2020).

Raman spectroscopy is a promising method for non-invasive sperm aneuploidy detection. It uses the inherent characteristics of light, and its intensity can be adjusted without labeling. It can identify the biological components of biological samples without affecting the integrity of cell structure and function (Agarwal and Said, 2003; Practice Committee of American Society for Reproductive Medicine in Collaboration with Society for Reproductive Endocrinology and Infertility, 2008; Con et al., 2014). In addition, because it is coupled with confocal microscopy, it is possible to analyze single cells. These characteristics make this technique very suitable for harmlessly assessing the biochemical properties of chemical moieties (Practice Committee of American Society for Reproductive Medicine in Collaboration with Society for Reproductive Endocrinology and Infertility, 2008; Con et al., 2014). Laser confocal Raman spectroscopy has been widely used in various fields and has become a new hot spot in the ART field. Previous research has used this technology to detect embryo culture medium to evaluate the aneuploidy of embryos (Liang et al., 2019). In addition, it was used to analyze sperm morphology and sperm head composition and evaluate the state of nuclear DNA to identify DNA damage (Kubasek et al., 1986; Mallidis et al., 2011; Davidson et al., 2013). PCA was used to distinguish X- and Y-bovine sperm cells based on single-cell Raman spectra, which could have a highly significant impact on animal production management systems as well as genetic improvement programs in farm animals (De Luca et al., 2014). Above all, numerous studies have shown that Raman spectroscopy may offer an alternative to the existing

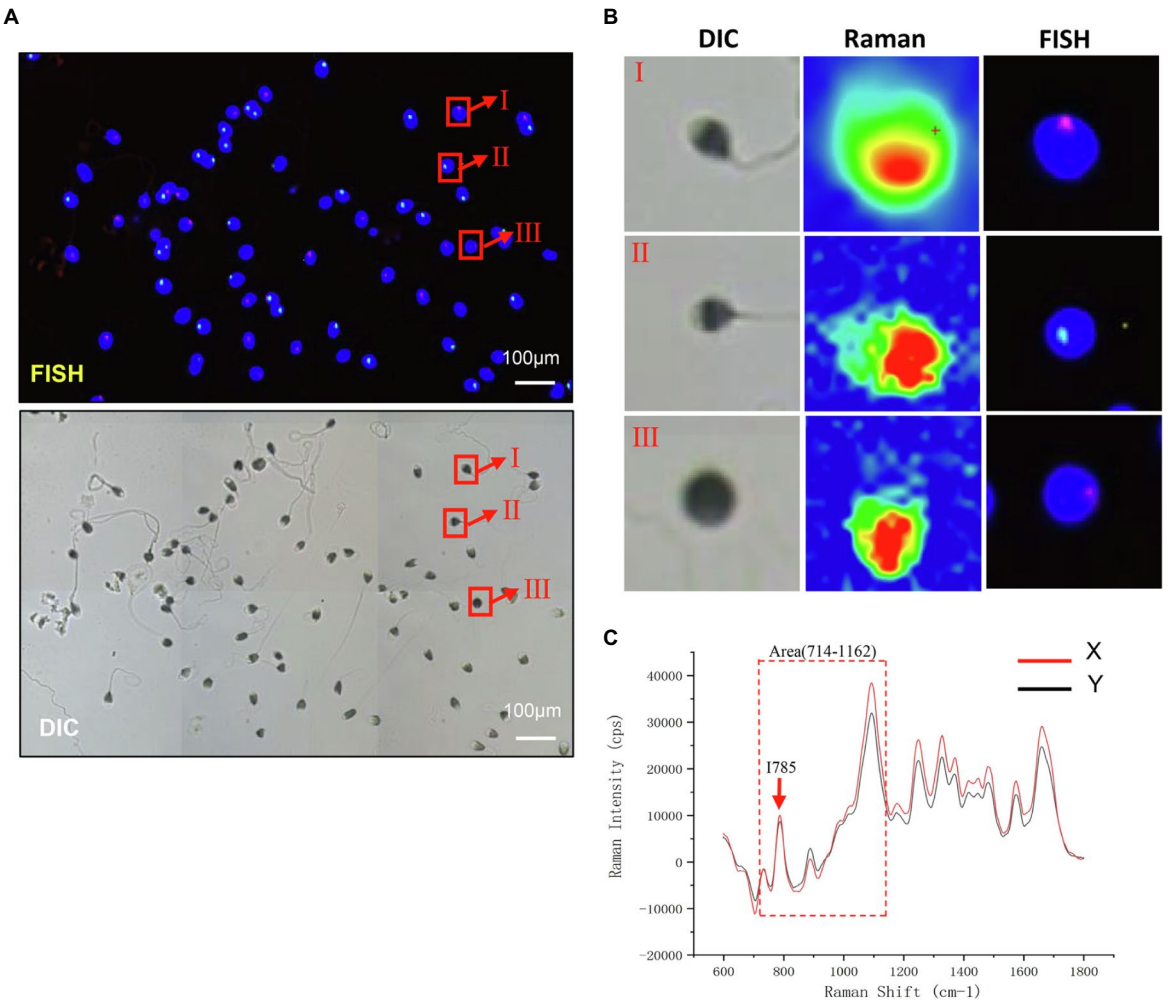



**FIGURE 3 |** Raman light micrograph and corresponding FISH result map. **(A)** Analysis of sperm sex chromosome status in FISH under a fluorescence microscope. The nucleus of sperm head is stained by DAPI. The red hybridization signal on the blue sperm head represents X sperm, and green represents Y sperm (scale bar, 100 μm). **(B)** Planar Map of Raman Spectrum Corresponding to FISH (scale bar, 100 μm). **(C)** The Raman spectra of X and Y sperm. Red arrows, I785; Dashed box, Area (714–1,162).

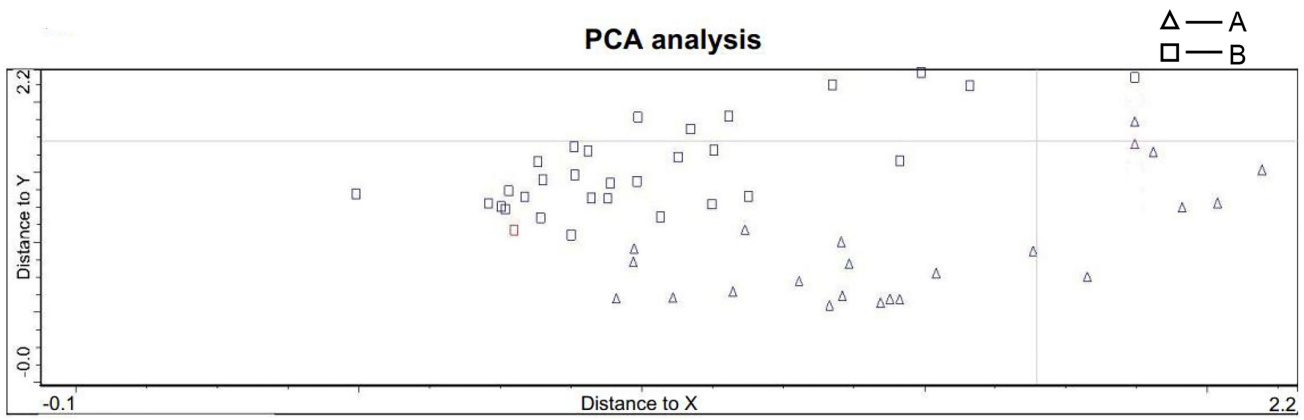



**FIGURE 4 |** PCA statistics of sperm spectra. The sperm were divided into two groups: group A included 22 sperm, and group B included 31 sperm (A: triangle B: circle).

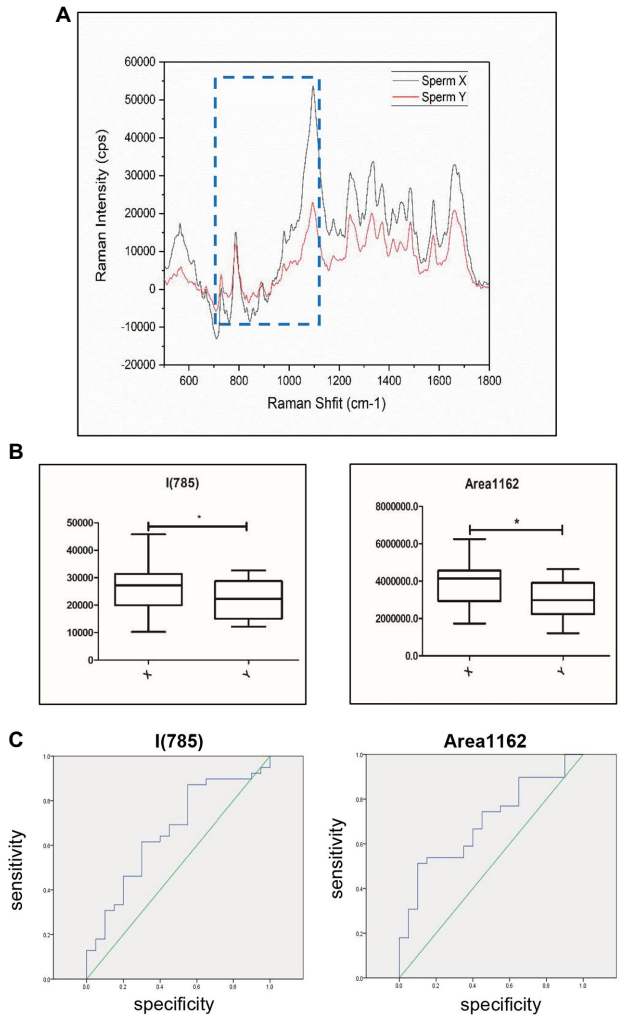



**FIGURE 5 |** Discrimination of sperm with different DNA contents by Raman spectroscopy. **(A)** Raman spectra of the 500–1800 $cm^{-1}$ area corresponding to X sperm and Y sperm. Black shows Y sperm and red shows X sperm. Raman peaks with the most significant difference for this study are highlighted by blue dotted lines. The x-axis units are displacement wavenumber ($cm^{-1}$), nanometer (nm). and absolute wavenumber ($cm^{-1}$). The y-axis unit is generally Raman strength. **(B)** The peak values of X and Y sperm in Area714-1,162 and I785. X, X sperm; Y, Y sperm. [$n$ (X) = 39, $n$ (Y) = 20, *, $p < 0.05$]. **(C)** Raman analysis data characteristics curve for sperm sex chromosomes. The area under the ROC curve indicates the prediction capacity of the model. Raman analysis data in I785 (AUC¼0.662). Raman analysis data in Area714-1,162 (AUC¼0.696).

methods, which avoids the clinical limitations of the existing methods.

In this study, we explored whether laser confocal Raman spectroscopy could be used as a potential non-invasive sperm chromosome aneuploidy detection technique in assisted reproduction. The total length of the X chromosome was 156 megabases (Mb), including 1973 genes, while that of the Y chromosome was 57 Mb, including 496 genes (Quintana-Murci and Fellous, 2001). Since X chromosome is much larger than Y chromosome, the DNA content of a normal haploid X sperm is slightly higher than that of a normal haploid Y sperm. In consideration of the difference in genetic material between the two kinds of sperm, we used laser confocal Raman spectroscopy to evaluate the DNA content of these two kinds of sperms. We used Raman spectroscopy to show the optical spectrum of the sperm head region. To compare the accuracy of different analysis strategy to evaluate the sperm head DNA, we calculated both the 714.30–1161.86 $cm^{-1}$ (DNA-PO4 skeleton region) and the 785 DNA skeleton peaks. Statistical analysis showed that there were significant differences between X sperm and Y sperm in the 714.30–1161.86 $cm^{-1}$ (DNA-PO4 skeleton region) and 785 DNA skeleton peaks. Moreover, the 714.30–1161.86 $cm^{-1}$ region spectral intensity was more accurate than 785 DNA skeleton peak intensity in distinguishing X and Y sperms. Our results showed that the Raman spectroscopy could distinguish the two kinds of sperms with different DNA contents. The difference of the chromosomal DNA content between aneuploid sperms and normal sperms is larger than that between X and Y sperms. According to this study, Raman spectroscopy combined with data analysis can screen out aneuploid sperms to avoid aneuploid embryos in ART.

However, due to the limitation of the current Raman spectral microscope, we can only collect the spectra of multiple fixed points of the sperm head instead of the whole sperm head spectra. This operation may miss the Raman spectra information of other sperm head regions, which may affect the accuracy of Raman analysis results. These may be the reason that the predicted X-Y sperm DNA difference is about 3%, while the X sperm spectral intensity of I785 and area (714–1,162) was 17 and 19% higher than that of Y sperm, respectively.

In our experiment, FISH process needed DNA denaturation, which resulted in dramatic sperm loss and reduced the number of corresponding sperms that could be analyzed. On the other hand, FISH with only X and Y numeric probes cannot detect the aneuploidy of other chromosomes, which may also affect the accuracy of the final results. Low-pass whole genomic sequencing may be a better approach to confirm the sperm aneuploidy status.

It remains challenging for quantitative analysis using Raman spectroscopy accurately to detect the unbalanced translocation of chromosomes, there is no consummate data analysis model to accurately analyze sperm Raman spectrum for accurate quantitative analysis, it is necessary to learn deeply to Establish a more perfect algorithm for data analysis. On the other hand, the current laser confocal Raman technology still uses the principle of infrared spectroscopy to detect the sperm, which must be washed and fixed on slides. These operations are still harmful to sperm and do not allow the sperm to be used for ART. Therefore, these methods cannot be used in clinical screening at present. In addition, the current Raman technology scanning is relatively time-consuming. The experimental steps and spectrum acquisition process still need to be further optimized to completely avoid sperm damage and improve efficiency. It is necessary to evaluate the safety of sperms under high-intensity laser before ART. In summary, the potential of Raman's clinical application still needs to be further explored.

In conclusion, our research shows that Raman microscopic spectroscopy can identify sperm with different DNA contents. To our knowledge, this was the first report which analyzed the sperm DNA content and confirmed the results with FISH technology. Current Raman spectroscopy is time-consuming, hazardous to sperm due to long-term laser exposure, and fixation requirement. Development of sensitive Raman flow cytometry and microfluidic technology may overcome the above drawbacks and may have a potential application value in the field of sperm aneuploidy detection.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of the Reproductive and Genetic Hospital of CITIC-Xiangya. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

LH designed the research. ML and YJ performed research. DW and YZ performed the standard principal component analysis. HZ and GL analyzed the data. ML analyzed the data and wrote the paper. All authors contributed to the article and approved the submitted version.

## FUNDING

This research was supported by the National Key R&D Program of China (grant 2018YFC1003100, to LH), the National Natural Science Foundation of China (grant 81873478, to LH), the Hunan Provincial Grant for Innovative Province Construction (2019SK4012), and the Natural Science Foundation of Hunan Province (2018JJ6088, to YT).

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.827941/full#supplementary-material

**Supplementary Figure S1 |** Optimization of spectral acquisition method. **(A)** Laser exposure time 0.5 s scanning once (red) and 0.2 s scanning three times spectrogram (black). **(B)** Laser exposure time 0.5 s scanning once and 15 times spectrogram. **(C)** Scanning map of a point in the sperm nucleus and its corresponding scanning position (scale bar, 100 μm). **(D)** The spectrogram of multiple sperm scans and corresponding Raman scans (scale bar, 100 μm).

**Supplementary Figure S2 |** Original and processed spectra. Comparison between original spectrum and spectrum after automatic correction and smoothing by software (purple line: origin, green line: correction; red line: correction and smoothing).

**Supplementary Figure S3 |** Frequency statistics of sperm spectra. **(A–D)** Frequency distribution histogram of 20 Y sperm and 39 X sperm data at I785 and Area (714–1,162); red dotted line: the potential critical value of the frequency distribution near the mean and median).

## REFERENCES

Agarwal, A., and Said, T. M. (2003). Role of sperm chromatin abnormalities and DNA damage in male infertility. *Hum. Reprod. Update* 9, 331–345. doi: 10.1093/humupd/dmg027

Arumugam, M., Shetty, D. P., Kadandale, J. S., and Nalilu, S. K. (2019). Association of sperm aneuploidy frequency and DNA fragmentation index in infertile men. *J. Reprod. Infertil.* 20, 121–126.

Carmen, R., Lorena, R., Carmen, G. P., Vanessa, P., Inmaculada, C. G., Sandra, G. H., et al. (2019). Clinical application of embryo aneuploidy testing by NGS. *Biol. Reprod.* 101, 1083–1090. doi: 10.1093/biolre/ioz019

Chen, L., Diao, Z., Xu, Z., Zhou, J., Yan, G., and Sun, H. (2018). The clinical application of single-sperm-based SNP haplotyping for PGD of osteogenesis imperfecta. *Syst Biol Reprod Med* 65, 75–80. doi: 10.1080/19396368.2018.1472315

Con, M., Victoria, S., Joachim, W., Frank, W., Martin, B., Carsten, F., et al. (2014). Raman microspectroscopy: shining a new light on reproductive medicine. *Hum. Reprod. Update*, 403–414. doi: 10.1093/humupd/dmt055

Davidson, B., Murray, A. A., Elfick, A., and Spears, N. (2013). Raman micro-spectroscopy can be used to investigate the developmental stage of the mouse oocyte. *PLoS One* 8:e67972. doi: 10.1371/journal.pone.0067972

De Jonge, C., and Barratt, C. (2006). *The Sperm Cell: Production, Maturation, Fertilization, Regeneration* Cambridge University Press.

De Luca, A. C., Managó, S., Ferrara, M. A., Rendina, I., Sirleto, L., Puglisi, R., et al. (2014). Non-invasive sex assessment in bovine semen by Raman spectroscopy. *Laser Phys. Lett.* 11:055604. doi: 10.1088/1612-2011/11/5/055604

Eberhardt, K., Stiebing, C., Matthäus, C., Schmitt, M., and Popp, J. (2015). Advantages and limitations of Raman spectroscopy for molecular diagnostics: an update. *Expert. Rev. Mol. Diagn.* 15, 773–787. doi: 10.1586/14737159.2015.1036744

Eccles, J., Morin, S. J., Zimmerman, R. S., and Iturriaga, A. (2017). Translocations, inversions and other chromosome rearrangements. *Fertil. Steril.* 107, 19–26. doi: 10.1016/j.fertnstert.2016.10.013

Fragouli, E., Alfarawati, S., Spath, K., Babariya, D., Tarozzi, N., Borini, A., et al. (2017). Analysis of implantation and ongoing pregnancy rates following the transfer of mosaic diploid–aneuploid blastocysts. *Hum. Genet.* 136, 805–819. doi: 10.1007/s00439-017-1797-4

Franasiak, J. M., Forman, E. J., Hong, K. H., Werner, M. D., Upham, K. M., Treff, N. R., et al. (2014). The nature of aneuploidy with increasing age of the female partner: a review of 15, 169 consecutive trophectoderm biopsies evaluated with comprehensive chromosomal screening. *Fertil. Steril.* 101, 656–663.e1. doi: 10.1016/j.fertnstert.2013.11.004

Hu, L., Wei, Y., Luo, K., Xie, P., Gong, F., Xiong, B., et al. (2018). Clinical outcomes in carriers of complex chromosomal rearrangements: a retrospective analysis of comprehensive chromosome screening results in seven cases. *Fertil. Steril.* 109, 486–492. doi: 10.1016/j.fertnstert.2017.11.021

Ioannou, D., Fortun, J., and Tempest, H. G. (2018). Meiotic nondisjunction and sperm aneuploidy in humans. *Reproduction* 157, R15–R31. doi: 10.1530/REP-18-0318

Jan, T., Ermanno, G., and Carmen, M. (2004). Late, but not early, paternal effect on human embryo development is related to sperm DNA fragmentation. *Hum. Reprod.* 19, 611–615. doi: 10.1093/humrep/deh127

Jiang, S., Peng, X., Gong, F., Huang, C., and Zhu, W. (2020). The role of total chromosomal disomy in human spermatozoa as a predictor of the outcome of pre-implantation genetic screening. *Fertil. Steril.* 113, 1196–1204.e1. doi: 10.1016/j.fertnstert.2020.02.004

Konrad Meister, D. A. S., Bründermann, E., and Havenith, M. (2010). Confocal Raman microspectroscopy as an analytical tool to assess the mitochondrial status in human spermatozoa. *Analyst* 135, 1370–1374. doi: 10.1039/b927012d

Kubasek, W. L., Wang, Y., Thomas, G. A., Patapoff, T. W., Schoenwaelder, K. H., Van, D. S., et al. (1986). Raman spectra of the model B-DNA oligomer d(CGCGAATTCGCG)2 and of the DNA in living salmon sperm show that both have very similar B-type conformations. *Biochemistry* 25, 7440–7445. doi: 10.1021/bi00371a028

Liang, B., Gao, Y., Xu, J., Song, Y., Xuan, L., Shi, T., et al. (2019). Raman profiling of embryo culture medium to identify aneuploid and euploid embryos. *Fertil. Steril.* 111, 753–762.e1. doi: 10.1016/j.fertnstert.2018.11.036

Mallidis, C., Wistuba, J., Bleisteiner, B., Damm, O. S., Gross, P., Wubbeling, F., et al. (2011). In situ visualization of damaged DNA in human sperm by Raman microspectroscopy. *Hum. Reprod.* 26, 1641–1649. doi: 10.1093/humrep/der122

McFeely, R. A. (1993). Chromosome abnormalities. *Vet. Clin. N. Am. Food Anim. Pract.* 9, 11–22. doi: 10.1016/S0749-0720(15)30667-8

Munné, S. (2018). Status of preimplantation genetic testing and embryo selection. *Reprod. BioMed. Online* 37, 393–396. doi: 10.1016/j.rbmo.2018.08.001

Patassini, C., Garolla, A., Bottacin, A., Menegazzo, M., Speltra, E., Foresta, C., et al. (2013). Molecular karyotyping of human single sperm by array- comparative genomic hybridization. *PLoS One* 8:e60922. doi: 10.1371/journal.pone.0060922

Practice Committee of American Society for Reproductive Medicine in Collaboration with Society for Reproductive Endocrinology and Infertility (2008). Optimizing natural fertility. *Fertil. Steril.* 90(5-supp-S), S1–S6. doi: 10.1016/j.fertnstert.2008.08.122

Quintana-Murci, L., and Fellous, M. (2001). The human Y chromosome: the biological role of a "functional wasteland". *J. Biomed. Biotechnol.* 1, 18–24. doi: 10.1155/S1110724301000080

Ramasamy, R., Scovell, J. M., Kovac, J. R., Cook, P. J., Lamb, D. J., and Lipshultz, L. I. (2015). Fluorescence in situ hybridization detects increased sperm aneuploidy in men with recurrent pregnancy loss. *Fertil. Steril.* 103, 906–909.e1. doi: 10.1016/j.fertnstert.2015.01.029

Ranjith, R., Stefan, B., and Dolores, J. L. (2014). Fluorescent in situ hybridization of human sperm: diagnostics, indications, and therapeutic implications. *Fertil. Steril.* 102, 1534–1539. doi: 10.1016/j.fertnstert.2014.09.013

Rieger, R. (1968). A glossary of genetics and cytogenetics, classical and molecular. *Springer Study* 44, 1669–1677.

Rodrigo, L., Peinado, V., Mateu, E., Remohí, J., Pellicer, A., Simón, C., et al. (2010). Impact of different patterns of sperm chromosomal abnormalities on the chromosomal constitution of preimplantation embryos. *Fertil. Steril.* 94, 1380–1386. doi: 10.1016/j.fertnstert.2009.05.061

Scott, S., Xiang, L., Jane, F., Khoury, C. D., and Potter, D. A. (2014). Determining parental origin of embryo aneuploidy: analysis of genetic error observed in 305 embryos derived from anonymous donor oocyte IVF cycles. *Mol. Cytogenet.* 7:68. doi: 10.1186/s13039-014-0068-5

Speyer, B. E., Pizzey, A. R., Ranieri, M., Joshi, R., Delhanty, J. D., and Serhal, P. (2010). Fall in implantation rates following ICSI with sperm with high DNA fragmentation. *Hum. Reprod.* 25, 1609–1618. doi: 10.1093/humrep/deq116

Wilch, E. S., and Morton, C. C. (2018). Historical and clinical perspectives on chromosomal translocations. *Adv. Exp. Med. Biol.* 1044, 1–14. doi: 10.1007/978-981-13-0593-1_1

Zhou, S., Cheng, D., Ouyang, Q., Xie, P., Lu, C., Gong, F., et al. (2018). Prevalence and authenticity of de-novo segmental aneuploidy (>16 Mb) in human blastocysts as detected by next-generation sequencing. *Reprod. BioMed. Online* 37, 511–520. doi: 10.1016/j.rbmo.2018.08.006

**Conflict of Interest:** DW and YZ were employed by company Thermo Fisher Scientific.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Endocrinology

SYSTEMATIC REVIEW
published: 11 February 2022
doi: 10.3389/fendo.2021.814648

# The Effect of Endometrial Thickness on Pregnancy, Maternal, and Perinatal Outcomes of Women in Fresh Cycles After IVF/ICSI: A Systematic Review and Meta-Analysis

*Zhiqi Liao[1], Chang Liu[2]*, Lei Cai[1], Lin Shen[1], Cong Sui[1], Hanwang Zhang[1]* and Kun Qian[1]*

[1] Reproductive Medicine Center, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [2] Reproductive Medicine Center, The Affiliated Drum Tower Hospital of Nanjing University Medical College, Nanjing, China

OPEN ACCESS

**Edited by:**
Claus Yding Andersen,
University of Copenhagen, Denmark

**Reviewed by:**
Yingpu Sun,
First Affiliated Hospital of Zhengzhou University, China
Meilan Mo,
Shenzhen Zhongshan Urological Hospital, China

***Correspondence:**
Chang Liu
lichi608@163.com
Hanwang Zhang
hwzhang605@126.com
Kun Qian
kunqian@tjh.tjmu.edu.cn

**Specialty section:**
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

**Received:** 14 November 2021
**Accepted:** 30 December 2021
**Published:** 11 February 2022

**Citation:**
Liao Z, Liu C, Cai L, Shen L, Sui C, Zhang H and Qian K (2022) The Effect of Endometrial Thickness on Pregnancy, Maternal, and Perinatal Outcomes of Women in Fresh Cycles After IVF/ICSI: A Systematic Review and Meta-Analysis. Front. Endocrinol. 12:814648. doi: 10.3389/fendo.2021.814648

**Background:** Thin endometrium on ovulation triggering day is associated with impaired pregnancy outcomes in women after *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI), but the role of thick endometrium on pregnancy outcomes remains controversial. Moreover, there has been insufficient evidence currently to analyze the influence of endometrial thickness (EMT) on obstetric complications and perinatal outcomes. Thus, we performed this meta-analysis to evaluate the effect of EMT on pregnancy, maternal, and perinatal outcomes in an enlarged sample size.

**Methods:** The databases Pubmed, Embase, Cochrane Libraries, and Web of Science were searched for English articles evaluating the correlation between EMT and pregnancy, maternal, or perinatal outcomes in women who underwent IVF/ICSI. We included studies that depicted a clear definition of outcomes and EMT grouping on ovulation triggering day. The EMT effect was analyzed in fresh cycle. Qualities of studies were assessed by the Newcastle-Ottawa Scale (NOS). Odds ratios (ORs) and weighted mean difference (WMD) with 95% confidence intervals (CIs) were calculated for analyzing dichotomous and continuous outcomes respectively, under a fixed or random effect model.

**Results:** A total of 22 pieces of literature were included for the final meta-analysis. A decreased trend towards pregnancy outcomes was observed, such as live birth rate (LBR), clinical pregnancy rate (CPR), and implantation rate (IR) in the thin endometrium groups (EMT <7 mm). In contrast, thick endometrium (EMT >14 mm) had no effect on pregnancy outcomes compared to medium EMT groups (EMT 7–14 mm). Moreover, thin endometrium (EMT <7.5 mm) enhanced the incidence of hypertensive disorders of pregnancy (HDP) and small-for-gestational-age (SGA) infants, and decreased the birthweight (BW) of babies.

**Conclusions:** Our studies indicated that thin endometrium not only had detrimental effect on pregnancy outcomes, but also increased the risk of HDP in women and SGA of babies, or decreased BW of babies. The thick endometrium does not have an adverse effect on IVF outcomes. Therefore, patients need to be informed on possible obstetric complications and perinatal outcomes caused by thin endometrium and are encouraged to actively cooperate with perinatal care.

**Systematic Review Registration:** (https://www.crd.york.ac.uk/PROSPERO/display_record.php?RecordID=242637), identifier CRD42021242637.

**Keywords: endometrium, *in vitro* fertilization, intracytoplasmic sperm injection, pregnancy rate, pregnancy complications**

## INTRODUCTION

Assisted reproductive technology (ART), namely, *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI), have been accepted as effective options for treating infertility (1). Multiple factors contribute to the success of IVF/ICSI, such as age, embryo quality and endometrial condition (2). Herein, endometrial thickness (EMT) measured by ultrasound has become a common indicator for monitoring endometrial condition, as the procedure of ultrasonographic examination is widely available and noninvasive (3). It has also been reported that EMT on ovulation triggering day was associated with the outcome of IVF/ICSI (4).

Many studies found that patients with thin endometrium had lower chances to be pregnant, both in fresh cycles and frozen-thawed embryo transfer (FET) cycles (5, 6). However, the relationship between increased EMT (>14 mm) and pregnancy outcomes remains controversial. Weissman et al. demonstrated that women with thick endometrium had lower implantation and pregnancy rate, and higher miscarriage rate (7). On the contrary, a study from Zhang et al. showed that increased EMT tended to improve IVF treatment outcomes, such as clinical pregnancy rate (CPR) (8). Therefore, there is lacking of consensus on the effect of thick endometrium on pregnancy outcomes of IVF/ICSI.

Furthermore, maternal perinatal complications and neonatal health are of great concern following ART as well (9, 10). Notably, recent evidence indicated that EMT has a strong correlation with maternal and perinatal outcomes (11–13). Guo et al. revealed that the incidence of small-for-gestational-age (SGA) infants was higher in thin endometrium group (13). Besides, Liu et al. also found that there was more risk of hypertensive disorders of pregnancy (HDP) in women with thin EMT (14). Nonetheless, the influence of thin EMT on obstetric complications and perinatal outcomes still lack evidence from a large sample size. Hence, this systematic review and meta-analysis aimed to assess the correlation between the EMT and pregnancy, maternal, and perinatal outcomes after IVF/ICSI.

## METHODS

We performed this review according to the Preferred Reporting Items for Systematic Review and Meta-analysis (PRISMA) statement and a registered protocol (PROSPERO registration number: CRD42021242637).

### Search Strategy and Data Collection

We searched four databases, namely, Pubmed, Embase, Cochrane Libraries, and Web of Science, for studies about the association between the EMT and outcomes of IVF/ICSI with no country or article type restrictions. Articles that published in English until April 2021 were recruited. The following terms were used: [(*in vitro* fertilization) OR (intracytoplasmic sperm injection) OR (artificial reproductive technology)] AND [(endometrial thickness) OR (endometrial sonographic parameters) OR (endometrial characters) OR (endometrial receptive)] AND [(live birth rate) OR (pregnancy outcomes) OR (neonatal outcomes) OR (maternal outcomes) OR (obstetric outcomes) OR (treatment outcomes)] (**Supplementary Table 1**).

After excluding duplicates, titles and abstracts were screened by two independent reviewers (ZL and LC). Studies relevant to our topic were assessed for eligibility. The flow chart of search strategy is shown in **Figure 1**. Full-text articles that met inclusion criteria were reviewed, data of which were extracted and recorded in pre-designed spreadsheets by two authors independently (ZL and LC). Any disagreement was resolved *via* discussion or consulting the third author (CL) if the consensus could not be reached. The following data were collected: authors, published year, type of study, time period, country, the number of live birth and clinical pregnancy, the number of implantation and miscarriage, the occurrence of obstetric complications [i.e., placenta previa (PP), placenta abruption (PA), and HDP], the incidence of perinatal outcomes [i.e., SGA, large-for-gestational-age (LGA), and preterm delivery (PTD)], the definition of outcomes, sample size of thin endometrial groups and thick endometrial groups, and other related information.

### Selection Criteria

Infertility women who underwent fresh cycles of IVF/ICSI treatment were included. Studies were included if these depicted the EMT of those women on ovulation triggering day and divided women into groups according to EMT. EMT, maximal distance from the endometrium–myometrium junction to the outer interfaces of the endometrium in the midsagittal plane of uterus, was measured by ultrasound

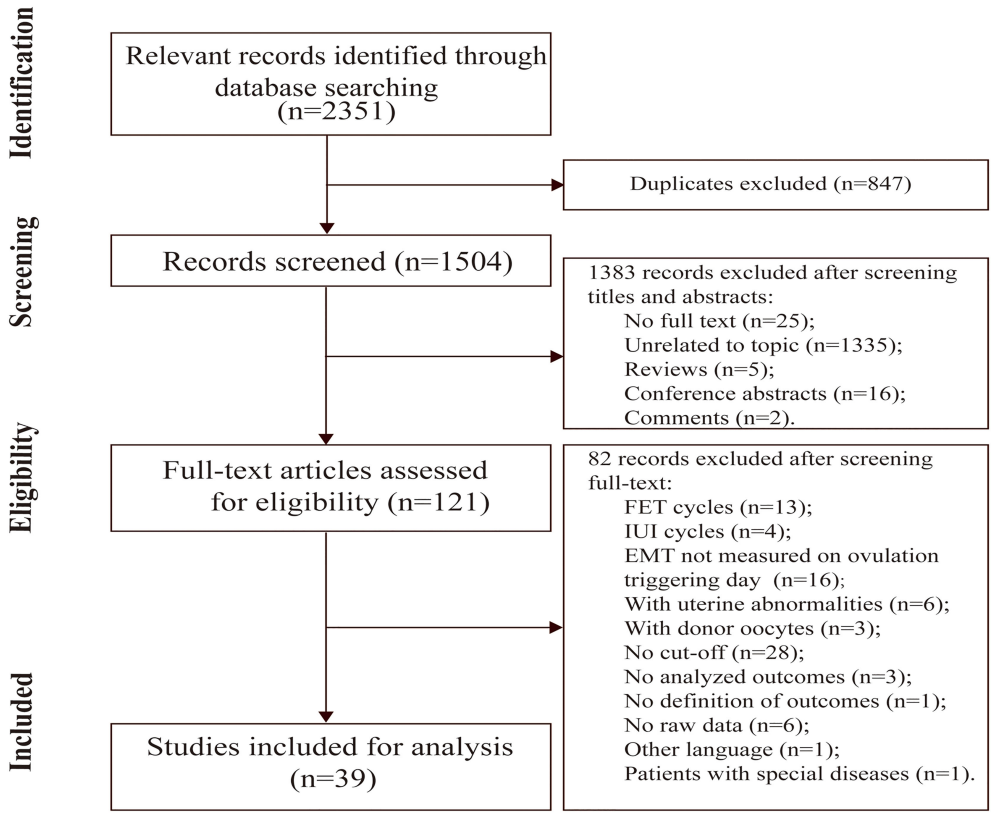



**FIGURE 1** | Flow chart of searching and screening strategy.

examination. Moreover, the outcomes in those studies should be related to pregnancy, maternal or neonatal outcomes, such as live birth rate (LBR), CPR, SGA, and so on. The definition of outcomes should be specific.

We excluded studies, in patients with uterine pathology, such as fibroids, polyps, adenomyosis, and so on. Besides, donor oocytes, as a confounding factor, may affect generalizing the result as well, so studies with donor oocytes treatments were excluded. Those studies with no definition of outcomes or no EMT groups were also excluded.

## Types of Outcomes

The primary outcome was LBR, which was defined as at least one live born baby was delivered per cycles, irrespective of the duration of pregnancy (15). In addition, data were provided concerning CPR (defined as the number of clinical pregnancies that diagnosed by ultrasonographic visualization of one or more gestational sacs after positive human chorionic gonadotrophin (hCG) tests per cycles), implantation rate (IR, defined as the ratio of the number of gestational sacs to number of embryos transferred), early miscarriage rate (EMR, defined as pregnancy loss before 12 weeks following clinical pregnancy), and miscarriage rate (MR, calculated as the ratio of any pregnancy loss after clinical pregnancy to the number of clinical pregnancy) in terms of pregnancy outcomes as well (15–17).

Maternal outcomes were PA (defined as premature separation of the normally implanted placenta from the uterus) and PP (defined as placenta implants in the lower segment of the uterus and may cover part or all of the opening of the cervix). Moreover, HDP were also analyzed. Herein, HDP included gestational hypertension (defined as blood pressure ≥140/90 mmHg after 20 gestational weeks), preeclampsia (coexistence of gestational hypertension and one or both of the following new disorders: proteinuria; dysfunction of other maternal organs) and eclampsia (onset of hyperreflexia, seizures, or coma in a previously diagnosed preeclamptic women) (13, 14).

The neonatal outcomes of dichotomous variables included SGA (defined as birthweight <10th percentile of the average body weight at the same gestational week), large-for-gestational-age (LGA, defined as birthweight >90th percentile of the average body weight at the same gestational week), preterm delivery (PTD, defined as delivery before 37 weeks of gestational age) (11, 13). The continuous variable was birthweight (BW).

## Quality Assessment of Studies

The Newcastle-Ottawa Scale (NOS) that comprises eight items was employed to assess the quality of studies. It was used for evaluating the bias from selection, comparability and the outcomes assessment. One or two stars were awarded to each item and studies that met all criteria of the NOS would receive a

maximum of nine stars. In the first item of NOS, representative cohorts were regarded as not restricted by diagnosis or by a type of ovarian stimulation protocol. Those cohort samples in fresh cycles that only received NC were also considered as unrepresentative. Moreover, studies adjusted for confounding factors (i.e., maternal age, body mass index, basal FSH, embryo score, chronic hypertension, previous pregnancy complications, and so on) by multivariable analysis or baseline data comparison were given a star or two stars in "comparability" item. Among them, age was the most important confounder that need to control. Besides, in "selection" and "outcome" items, information bias, such as the precision of measuring the EMT and evaluating the outcomes, were also taken into consideration. High-quality studies were considered as more than or equal 7 stars. Studies with medium quality had an total NOS score ≥5, but <7. Low quality studies had NOS score <5. Good quality and medium quality articles were included in the meta-analysis (18). Two investigators (ZL and LC) assessed risk of bias from each study *via* NOS independently. Disagreements between the two reviewers were settled by discussion and the third reviewer checked the accuracy of evaluation through view full-manuscript of those studies.

## Statistical Analysis

Given that most studies were retrospective cohort studies, we used odds ratios (OR) with 95%CI to measure dichotomous outcomes. Weighted mean difference (WMD) with 95%CI was used to analyze the association between EMT and BW. Results were combined for meta-analysis using Mantel–Haenszel fixed or random effects models which depended on heterogeneity. Q statistic and $I^2$ statistics were used to evaluate the heterogeneity of studies. P <0.10 indicated the presence of heterogeneity, and $I^2$ <50% indicated that the heterogeneity was acceptable, thus, a fixed-effects model was used; otherwise, a random-effect model was used. Results were expressed as forest plots. Sensitivity analysis was conducted to examine heterogeneity and the robustness of the results. For meta-analysis of more than 10 articles, we also analyzed publication bias, which was assessed by funnel plot asymmetry and Egger's test (P <0.05 considered as significant). When the publication bias existed, trim-fill adjustment method was used to assess the effect of this bias on outcomes. Statistics tests were calculated by the Review Manager software (version 5.3). Egger's test and trim-fill analysis were analyzed by R (version 4.0.3).

# RESULTS

## Literature Selection

There were 2,351 potential records by searching electronic database. After removing duplicates and screening titles and abstracts, 121 full-text articles related to our topic were retrieved for review. Of these, 82 records were excluded due to many reasons that are shown in **Figure 1**. Finally, 39 studies were eligible for further analysis. Considering that most studies select 7 and 14 mm as the threshold values for EMT grouping, we chose those thresholds as the cutoff values to explore the influence of thin (<7 mm) and thick (>14 mm) endometrium on pregnancy outcomes in the fresh cycles. Likewise, 7.5 mm was used as thin endometrial cut-off value for evaluating pregnancy complications and perinatal outcomes. Other studies (15 studies) that did not provide the above threshold information were not included for meta-analysis. Since most of studies were retrospective cohort studies, one prospective cross-sectional study was not suitable for meta-analysis, only for systematic review (19). In a study of fresh cycles, clomiphene (CC)-based minimal stimulation protocol was used, which was different from other controlled ovarian hyperstimulation (COH) protocols. Similarly, this study was only for systematic review as well (20). Therefore, 22 studies were included for final meta-analysis (5, 7, 11, 13, 16, 21–37).

## Description of Studies and Participants

Characteristics of included studies and patients are summarized in **Table 1**. The studies were published from 1991 to 2021. The articles used for meta-analysis were all observational studies, namely, retrospective and prospective cohort studies. Outcomes of most studies were LBR, CPR, IR, and MR. Herein, MR would be divided into two subgroups for meta-analysis, namely, EMR and MR. Only two articles described maternal (PP, PA, and HDP) and perinatal outcomes (SGA, BW, LGA, and PTD). Of these, BW was presented as mean with standard deviation (Mean ± SD).

All women underwent the fresh cycles of IVF/ICSI treatment. Women were divided into three groups depending on EMT when analyzed pregnancy outcomes (Thin endometrium/decreased EMT group: EMT <7mm; Medium endometrium group: EMT 7–14 mm; Thick endometrium/increased EMT group: EMT >14 mm). The effect of thin endometrium on obstetric complications and perinatal outcomes was evaluated in endometrial cut-off value of < 7.5cm versus >7.5cm. The total number of reported patients and cycles that were related to LBR was about 27,225 and 31,763 respectively. The number of patients enrolled for maternal and perinatal outcomes was 4,021. The mean of female age was approximately between 29 and 36 years. COH, such as GnRH-agonist long or short protocols and GnRH-antagonist protocols, were used in patients. On hCG triggering day, the mean of E2 level of these patients was about from 1,329.78 pg/ml to 3,489.62 pg/ml.

## Quality of Studies

The quality of studies based on NOS is shown in **Supplementary Table 2**. Qualities of 14 studies were good level, and 8 studies were medium level. Therefore, all 22 studies were included in analysis.

## Live Birth Rate

Women with thin endometrium (EMT <7 mm) had a significantly lower LBR compared to those women with EMT >7 mm in fresh cycles (OR 0.47, 95%CI: 0.37, 0.61, P <0.00001) (**Figure 2A**). However, significant heterogeneity was observed in

**TABLE 1 |** Characteristics of included references and participants.

| Author (year) | Type of studies | Time-period | No. of patients | No. of cycles | Stimulation protocol | ART treatment | Type of cycles | Female age (Mean ± SD) | E2 on ovulation triggering day | EMT group (mm) | EMT measured day | Outcomes |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Shakerian et al. (36) | Retrospective cohort study | 10/ 2016-08/ 2019 | NA | 273 | COH: GnRH-agonist/ antagonist protocol. | IVF | Fresh cycles | 36 (33–40)# | 1,353.12 ± 754.13 | <7, 7–14, >14. | hCG trigger | LBR, MR. |
| Simeonov et al. (37) | Retrospective cohort study | 01/ 2009-12/ 2017 | 2343 | 5133 | COH: GnRH-agonist/ antagonist protocol. | IVF/ICSI | Fresh cycles | NA | NA | <7, >7 | hCG trigger | LBR |
| Guo et al. (13) | Retrospective cohort study. | 01/ 2017-12/ 2018 | 3157 | NA | NC/Mild stimulation/COH: GnRH-agonist long/agonist short/antagonist protocol. | IVF/ICSI | Fresh cycles | 31.52 ± 4.17 | NA | <7.5, >7.5. | hCG trigger | PA/PP/ HDP/SGA/ LGA/PTD/ BW. |
| Lv et al. (21) | Retrospective cohort study. | 01/ 2013-12/ 2016 | 13909 | 15012 | COH: GnRH-agonist long/ agonist short/antagonist/ minimal-stimulation/ ultralong/other protocol. | IVF/ICSI | Fresh cycles | 31.23 ± 5.29 | 3,289.68 + 1,915.22 | <7, >7. | hCG trigger | LBR. |
| Tomicet al. (22) | Retrospective cohort study. | 2010-2017 | 552 | 552 | NC. | IVF | Fresh cycles | 33.93 ± 3.41 | 250.14 ± 70.87 | <7, 7–14, >14. | hCG trigger | CPR. |
| Nishihara et al. (20) | Retrospective cohort study | 11/ 2018-03/ 2019 | 746 | 746 | Clomiphene citrate-based minimal stimulation. | IVF/ICSI | Fresh cycles | 38.1 ± 0.1* | NA | <7, >7. | hCG trigger | CPR. |
| Eftekhar et al. (5) | Retrospective cohort study. | 05/ 2016-05/ 2018 | 1000 | 1000 | COH: GnRH-agonist/ antagonist protocol. | IVF/ICSI | Fresh cycles | NA | NA | <7, 7–14, >14. | hCG trigger | CPR |
| Ovayolu et al. (23) | Retrospective study. | 2005-2013 | 359 | 359 | COH: GnRH-agonist long/ antagonist protocol. | IVF/ICSI | Fresh cycles | 31.32 ± 4.01 | 2,299.56 ± 1,033.96 | <7, 7–14, >14. | hCG trigger | LBR. |
| Song et al. (24) | Retrospective cohort study. | 01/ 2013-12/ 2017 | 9511 | 4278 | COH: short GnRH-agonist long protocol/prolonged protocol. | IVF/ICSI | Fresh cycles | 28.93 ± 3.23 | NA | <7, 7–14, >14. | hCG trigger | CPR/IR |
| Chan et al. (25) | Retrospective cohort study. | 01/ 2012-12/ 2016 | 162 | 162 | COH: GnRH-agonist/ antagonist protocol. | IVF/ICSI | Fresh cycles | 33.81 ± 3.65 | 1,886.10 ± 1,399.90 | <7, 7–14, >14. | hCG trigger | LBR/CPR. |
| Holden et al. (26) | Retrospective cohort study. | 05/ 2004-12/ 2012 | 6331 | 6180 | COH: GnRH-agonist/ antagonist protocol. | IVF/ICSI | Fresh cycles | 35.6 (32.2–39.2) # | 1,711 (1,012–2,691) # | <7, >7. | hCG trigger. | LBR |
| Oron et al. (11) | Retrospective cohort study. | 01/ 2008-12/ 2014 | 864 | 5546 | NC; COH: GnRH-agonist long/agonist short/ antagonist protocol. | IVF/ICSI | Fresh cycles | 32.49 ± 5.12 | NA | <7.5, >7.5. | hCG trigger | PA/PP HDP/SGA/ LGA/BW. |
| Ribeiro et al. (16) | Retrospective cohort study. | 01/ 2010-12/ 2014 | 2827 | 3350 | COH: GnRH-antagonist protocol. | IVF/ICSI | Fresh cycles | NA | NA | <7, >7. | hCG trigger | LBR/CPR/ PTD/BW. |
| Wu et al. (27) | Retrospective cohort study. | 01/ 2011-12/ 2013 | 2106 | 2106 | COH: GnRH-antagonist protocol | IVF/ICSI | Fresh cycles | 31.94 ± 3.71 | 2,771.20 ± 1,649.66 | <7, 7–14, >14. | hCG trigger | CPR/IR |
| Zhao et al. (28) | Retrospective cohort study. | 01/ 2009-05/ 2011 | 1933 | 3319 | COH: HMG stimulation protocol. | IVF/ICSI | Fresh cycles | 31.20 ± 4.60 | 3,489.70 ± 2,112.20 | <7, 7–14, >14. | hCG trigger | CPR/IR |

*(Continued)*

**TABLE 1** | Continued

| Author (year) | Type of studies | Time-period | No. of patients | No. of cycles | Stimulation protocol | ART treatment | Type of cycles | Female age (Mean ± SD) | E2 on ovulation triggering day | EMT group (mm) | EMT measured day | Outcomes |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Aydin et al. (19) | Prospective cross-sectional study. | NA | 593 | 593 | COH: GnRH-agonist/ antagonist protocol. | IVF/ICSI | Fresh cycles | 26.86 ± 4.68 | NA | <7, 7–14, >14. | hCG trigger | CPR/IR |
| Zhao et al. (29) | Retrospective cohort study. | 01/ 2009-05/ 2011 | 1933 | 3319 | COH: HMG stimulation protocol. | IVF/ICSI | Fresh cycles | 31.18 ± 4.62 | 3,489.62 ± 2,112.21 | <7, 7–14, >14. | hCG trigger | CPR/IR |
| Chen et al. (30) | Retrospective cohort study. | 01/ 2003-12/ 2008 | 2896 | 2896 | COH: GnRH-agonist long protocol. | IVF/ICSI | Fresh cycles | 31.00 ± 3.90 | 2 107.30 ± 1,596.10 | <7, 7–14, >14. | hCG trigger | CPR |
| Okohue et al. (31) | Prospective study. | 05/ 2005-04/ 2006 | 251 | 251 | COH: GnRH-agonist long protocol. | IVF/ICSI | Fresh cycles | 30.58 ± 3.35 | NA | <7, 7–14, >14. | hCG trigger | CPR |
| Al-Ghamdi et al. (32) | Retrospective cohort study. | 01/ 2003-12/ 2005 | 2464 | 2464 | COH: GnRH-agonist long/ agonist short protocol. | IVF/ICSI | Fresh cycles | 30.83 ± 5.45 | NA | <7, 7–14, >14. | hCG trigger | CPR |
| Richter et al. (33) | Retrospective cohort study | 01/ 2002-12/ 2005 | 1294 | 1294 | COH | IVF/ICSI | Fresh cycles | 33.67 ± 3.47 | 2,553.67 ± 991.13 | <7, 7–14, >14. | hCG trigger | LBR/CPR |
| Yoeli et al. (34) | Prospective study. | 1998-2000 | 783 | 1218 | COH: GnRH-agonist long/ agonist short protocol. | IVF/ICSI | Fresh cycles | 32.86 ± 4.70 | 1,329.78 ± 1,053.67 | 7–14, >14. | hCG trigger | CPR/IR |
| Weissman et al. (7) | Retrospective cohort study. | 1994-1995 | NA | 717 | COH: GnRH-agonist long protocol. | IVF/ICSI | Fresh cycles | NA | NA | 7–14, >14. | hCG trigger | CPR/IR |
| Noyes et al. (35) | Prospective study. | 10/ 1991-06/ 1992 | 477 | 516 | COH: GnRH-agonist/Only Gn stimulation/CC+ Gn stimulation protocol. | IVF | Fresh cycles | 35.90 ± 4.20 | 1,465.00 ± 798.00 | <7, 7–14, >14. | hCG trigger | CPR/IR |

*ART, artificial reproductive technology; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; E2, estradiol; COH, controlled ovarian hyperstimulation; GnRH, gonadotropin releasing hormone; Gn, gonadotrophin; CC, Clomiphene citrate; NC, natural cycle; EMT, endometrial thickness; hCG, human chorionic gonadotrophin; PA, placenta abruption; PP, placenta previa; HDP, hypertensive disorders of pregnancy; SGA, small-for-gestational-age; LGA, large-for-gestational-age; PTD, preterm delivery; BW, birthweight; LBR, live birth rate; CPR, clinical pregnancy rate; MR, miscarriage rate; IR, implantation rate; NA, Not applicable. 2) E2 (pg/ml): data are presented as mean + SD. 3) *: mean± SEM; #: median (interquartile range).*

this result ($I^2$ = 62%). Hence, sensitivity analysis was conducted to detect the stability of result by removing each study and re-analyzing the remaining studies, which did not change the direction of the effect. When one study (21) was excluded, the substantial heterogeneity was decreased ($I^2$ declined from 62 to 0%). Women with thick endometrium (EMT >14 mm), had no significant higher LBR than those with medium EMT (7–14 mm) in fresh cycles (OR 1.08, 95%CI: 0.68, 1.72, P = 0.74, low heterogeneity: $I^2$ = 29%) (**Figure 2B**).

## Clinical Pregnancy Rate

Twelve studies that reported CPR of women with thin endometrium in fresh cycles are shown in **Figure 3**. Subgroup analysis was conducted according to whether women underwent COH protocols. In fresh cycles, lower CPR of decreased EMT group was observed both in COH stimulation group (OR 0.40; 95%CI: 0.31, 0.50, P <0.00001, low heterogeneity: $I^2$ = 40%) and in NC group. Since the analysis in COH stimulation group included more than 10 studies, funnel plot was presented (**Supplementary Figure 1**) and Egger' test (intercept = −0.4333, t = −2.99, P = 0.0135) was estimated. However, those results indicated the presence of publication bias, so the trim-fill adjustment method was analyzed. After adjustment, the ORs changed from 0.40 to 0.48, and the significant level did not change, which suggested that the existing results were not affected by publishing bias.

Thirteen studies that reported CPR of women with thick endometrium in fresh cycles are also shown in **Figure 3**. We performed subgroup analysis as well according to the study types. When only retrospective studies included for meta-analysis, result showed that women with thick endometrium had higher chances to conceive (OR 1.30; 95%CI: 1.09, 1.56, P = 0.004). When, notwithstanding, prospective studies were also included for analyzing, it seemed that there was no significant difference in CPR between thick endometrium and medium endometrium group in fresh cycles (OR 1.22; 95%CI: 1.00, 1.49, P = 0.05). It should be noted that substantial heterogeneity existed among all

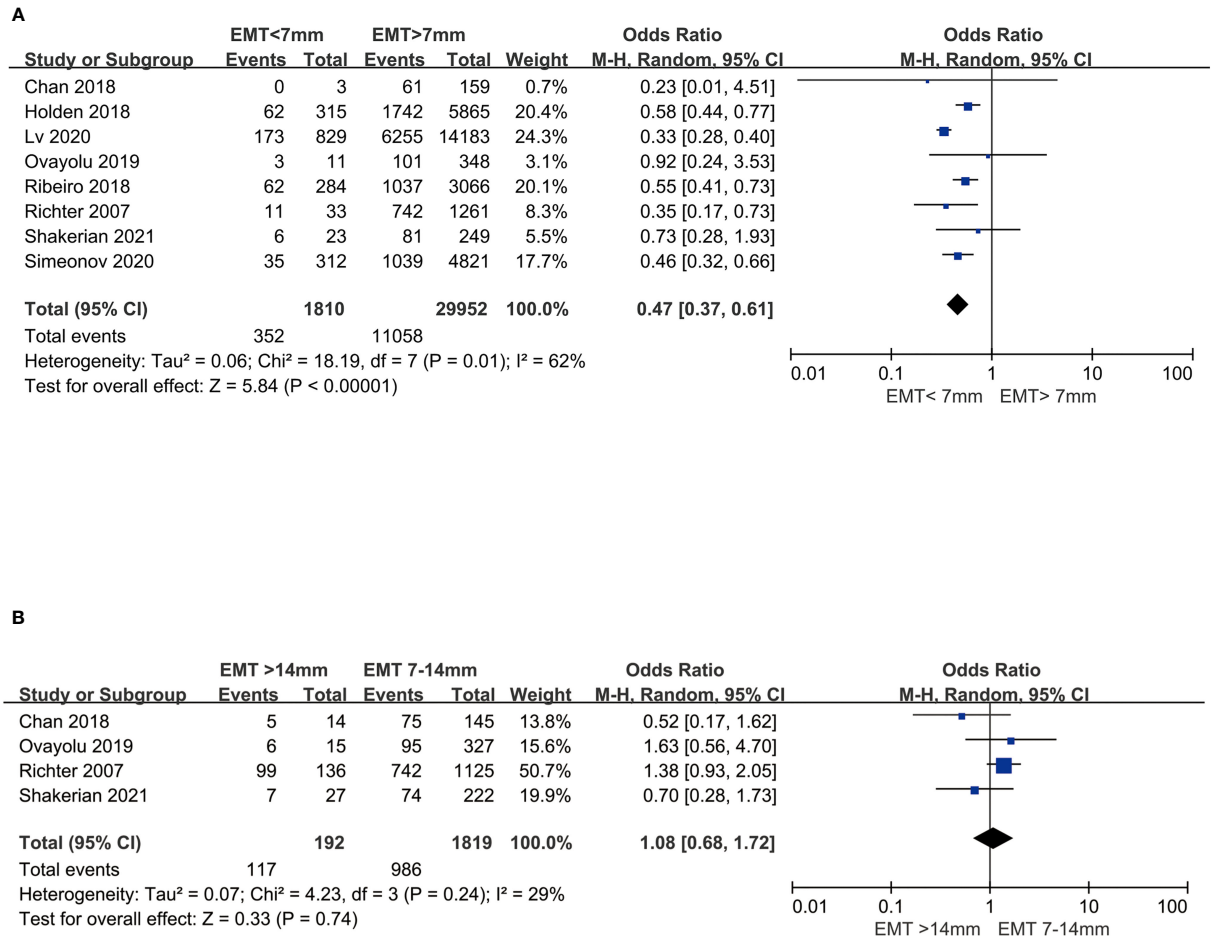



**FIGURE 2** | Comparison of LBR between EMT groups in fresh cycles. **(A)** Comparison between thin endometrium group and non-thin endometrium group. **(B)** Comparison between thick endometrium group and medium endometrium. LBR, Live birth rate; EMT, Endometrial thickness.

studies ($I^2$ = 64%), so we performed sensitivity analysis. When two (7, 31) of the studies was removed separately, the heterogeneity decreased ($I^2$ declined from 64 to 54%, or to 45% respectively) and the result changed (OR 1.29, 95%CI: 1.09, 1.54; OR 1.29, 95%CI: 1.11, 1.51 respectively). This analysis indicated that the result was not robust to some extent. Similarly, there were 10 studies in retrospective studies subgroup, so publication bias also estimated *via* funnel plot and Egger's test (**Supplementary Figure 2**). No publication bias was presented after assessed by Egger's test (intercept = 0.4283, t = −1.61, P = 0.1471).

## Implantation Rate

Similar to the results of LBR and CPR, thin endometrial patients had lower IR than those with EMT >7 mm as well (OR 0.27, 95% CI: 0.19, 0.39, P <0.00001, no heterogeneity: $I^2$ = 0) (**Figure 4A**). However, there was no significant difference among patients with thick endometrium compared to medium endometrium group (OR 1.14, 95%CI: 0.88, 1.47, P = 0.32), though the substantial heterogeneity ($I^2$ = 74%) existed (**Figure 4B**).

## Miscarriage Rate

In a subgroup analysis, no significant difference was observed in EMR (OR: 1.43, 95%CI: 0.32, 6.41, P = 0.64, no heterogeneity: $I^2$ = 0%) and MR (OR 1.42; 95%CI: 0.91, 2.22, P = 0.13, low heterogeneity: $I^2$ = 30%) in women with thin endometrium than those with EMT >7 mm in fresh cycles (**Figure 5A**). In an analysis about the effect of thick endometrium on EMR or MR, there was also no significant difference comparing thick endometrium groups to medium endometrium groups (MR: OR 1.04, 95%CI: 0.65, 1.68, P = 0.87, low heterogeneity $I^2$ = 30%; EMR: OR 0.75, 95%CI: 0.46, 1.20, P = 0.23, no heterogeneity $I^2$ = 0%) (**Figure 5B**).

## Systematic Review

A prospective cross-sectional study from Aydin et al. showed that there were significantly lower CPR and IR in thin endometrium group in fresh cycles (P <0.05, P <0.05, respectively), which also corroborated our results of meta-analysis. Furthermore, a study from Nishihara et al. also

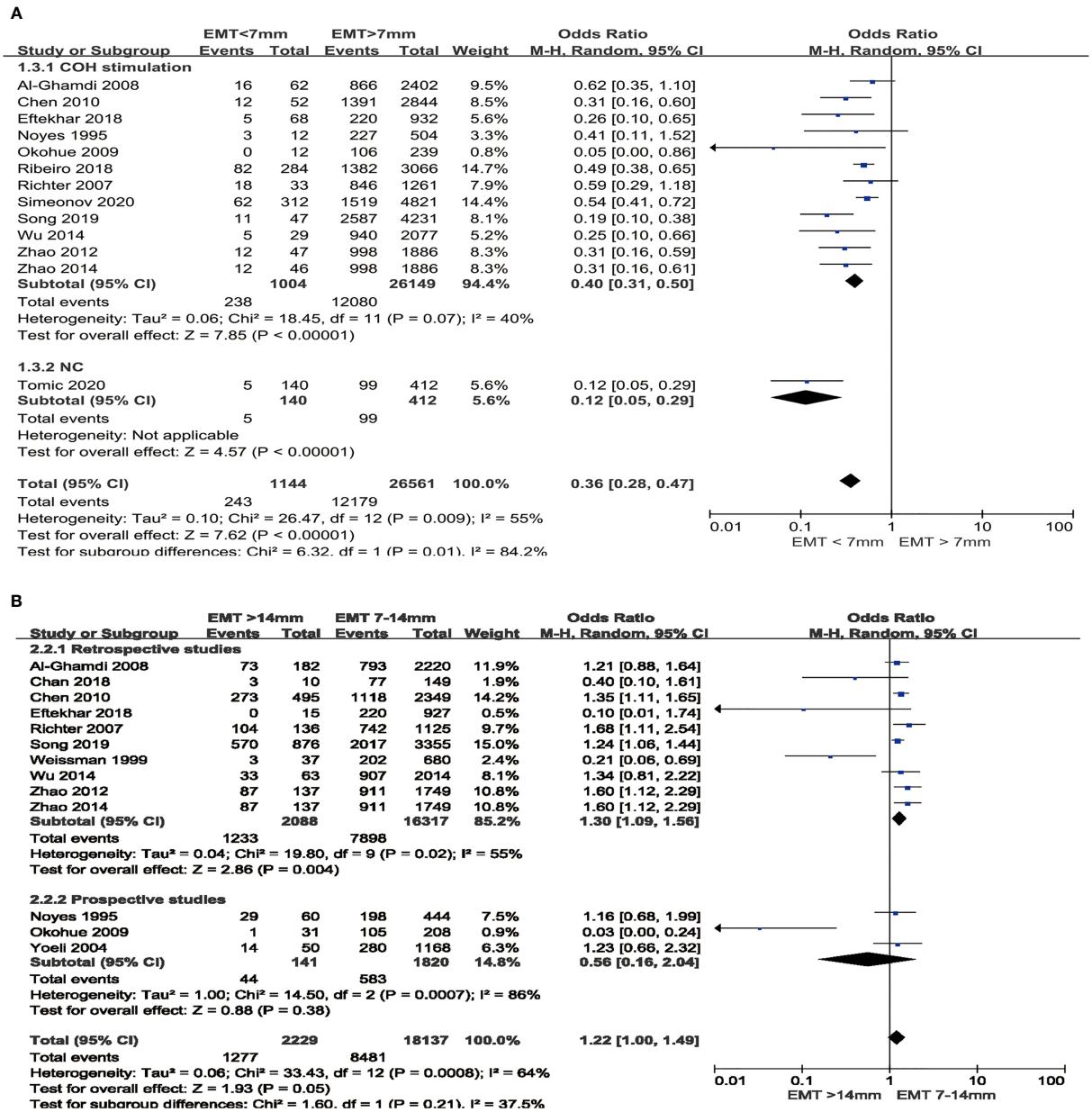



**FIGURE 3** | Comparison of CPR between EMT groups in fresh cycles. **(A)** Comparison between thin endometrium group and non-thin endometrium group. **(B)** Comparison between thick endometrium group and medium endometrium. CPR, Clinical pregnancy rate; COH, Controlled ovarian hyperstimulation; NC, Natural cycles.

showed that CPR was significantly decreased in women with thin endometrium in fresh cycles of CC-based stimulation (P <0.05).

## Maternal and Perinatal Outcomes

With respect to obstetric outcomes, as shown in **Figure 6**, thin endometrium (EMT <7.5 mm) had no effect on placenta previa (OR 0.49, 95%CI: 0.09, 2.55, P = 0.40, no heterogeneity $I^2$ = 0%) and placenta abruption (OR 0.47, 95%CI: 0.06, 3.46, P = 0.46, no heterogeneity $I^2$ = 0%). Incidence of hypertensive disorders of pregnancy was increased in women with thin endometrium, but there was no significant difference (OR 1.72, 95%CI: 1.01, 2.94, P = 0.05, no heterogeneity $I^2$ = 0%).

Besides, perinatal outcomes, such as small-for-gestational-age, large-for-gestational-age, and preterm delivery are shown in **Figure 7**. A higher incidence of SGA was observed in infants from decreased EMT group (OR 1.81; 95%CI: 1.16, 2.83; P = 0.009, no heterogeneity $I^2$ = 0%) and babies had significantly lower BW from women with thin endometrium (WMD: −0.12 kg, 95%CI: −0.19, −0.04, P = 0.004, no heterogeneity $I^2$ = 0%). No significant difference was observed in the incidence of LGA (OR

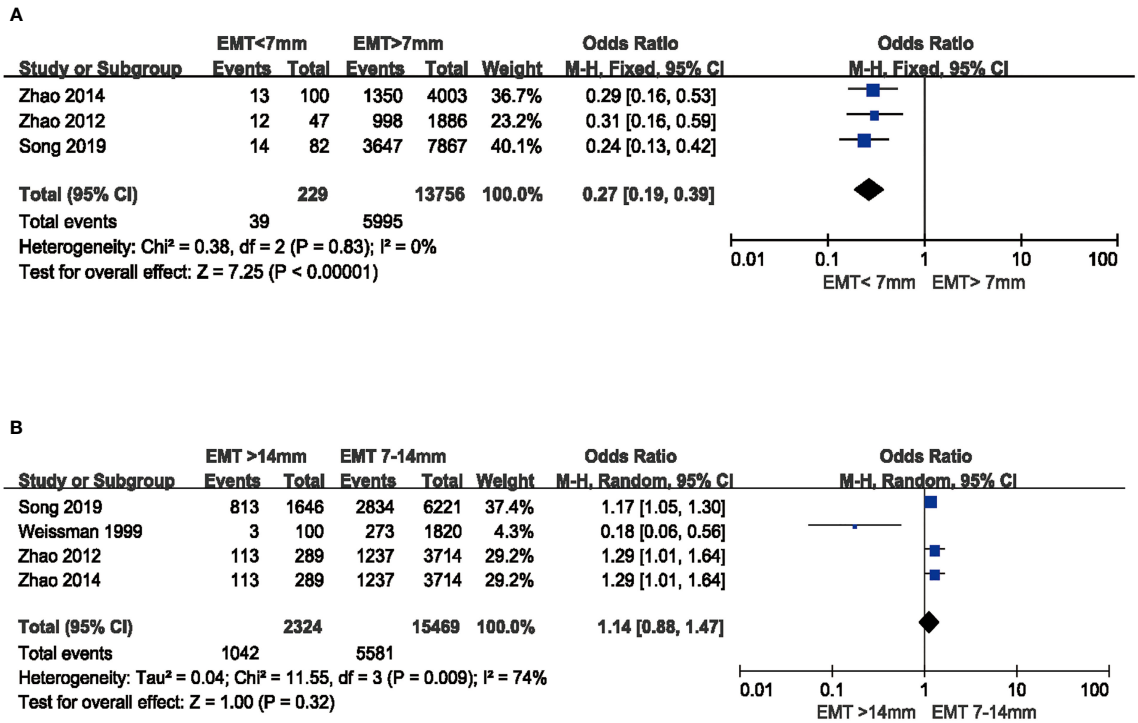



**FIGURE 4** | Comparison of IR between EMT groups in fresh cycles. **(A)** Comparison between thin endometrium group and non-thin endometrium group. **(B)** Comparison between thick endometrium group and medium endometrium. IR, Implantation rate.

0.96, 95%CI: 0.36, 2.56, P = 0.93, high heterogeneity: $I^2$ = 83%) and PTD (OR 1.34, 95%CI: 0.84, 2.13, P = 0.23, no heterogeneity $I^2$ = 0%) neonates in the thin endometrium group.

## DISCUSSION

In this review, we analyzed the effect of EMT on pregnancy, maternal, and perinatal outcomes in women after fresh cycles of IVF/ICSI. Because there was no consensus on the definition of thin or thick endometrium, we selected cutoffs of thin or thick endometrium reported in most studies for our meta-analysis, such as 7 and 14 mm in fresh cycles. Similarly, as the number of studies related to maternal and perinatal outcomes was not enough and the cutoffs of thin endometrium in these studies also have not reached an agreement, 7.5 mm that reported in most studies was selected for analyzing.

We found that LBR, CPR, and IR were lower in patients with thin endometrium, which were consistence with previous studies (4, 22, 38). The underlying reason might not only be related to high oxygen levels in basal layer of endometrium, but also relevant to abnormal transcriptional changes in thin endometrium (39–41). For instance, a recent study revealed that differentially expressed genes and microRNAs, which were enriched in angiogenesis, cell growth regulation, and Wnt signaling pathway, were detected in the mid-secretary phase of thin endometrium compared to adjacent normal endometrial cells (41). Moreover, our results showed that though a thin endometrium had no effect on MR, but had higher chance of early miscarriage. Although the reason behind this phenomenon is unclear, we speculated that decreased EMT had detrimental effect on decidualized endometrium, so this disrupt might contribute to some implanted embryos destined to miscarry before 12 weeks of gestation (42).

In terms of thick endometrium, there was no significant association between increased EMT and LBR, CPR, IR and MR. It should be noted that no significant difference was demonstrated between CPR in thick EMT and medium EMT group due to the substantial heterogeneity that existed among the studies. From the above results, it is clear that thick endometrium does not increase MR nor decrease CPR. Thus, thick endometrium does not have adverse effects on IVF outcomes, which is also supported by previous studies (4, 34, 43).

Apart from pregnancy outcomes, the obstetric complications (like HDP) and the perinatal outcomes (such as BW and SGA), were revealed to be influenced by EMT. Of these, the thickness of the endometrium has a negative relationship with the incidence of HDP or SGA and a positive correlation with BW, which were in accordance with previous studies (13, 14, 44). Notwithstanding, the number included in the studies is still insufficient, so it cannot make a firm conclusion and demands to be confirmed in a large sample prospective cohort study. Normal placental function and fetal development are both relied on the intrauterine environment (45). It is believed that the development of HDP and fetal growth

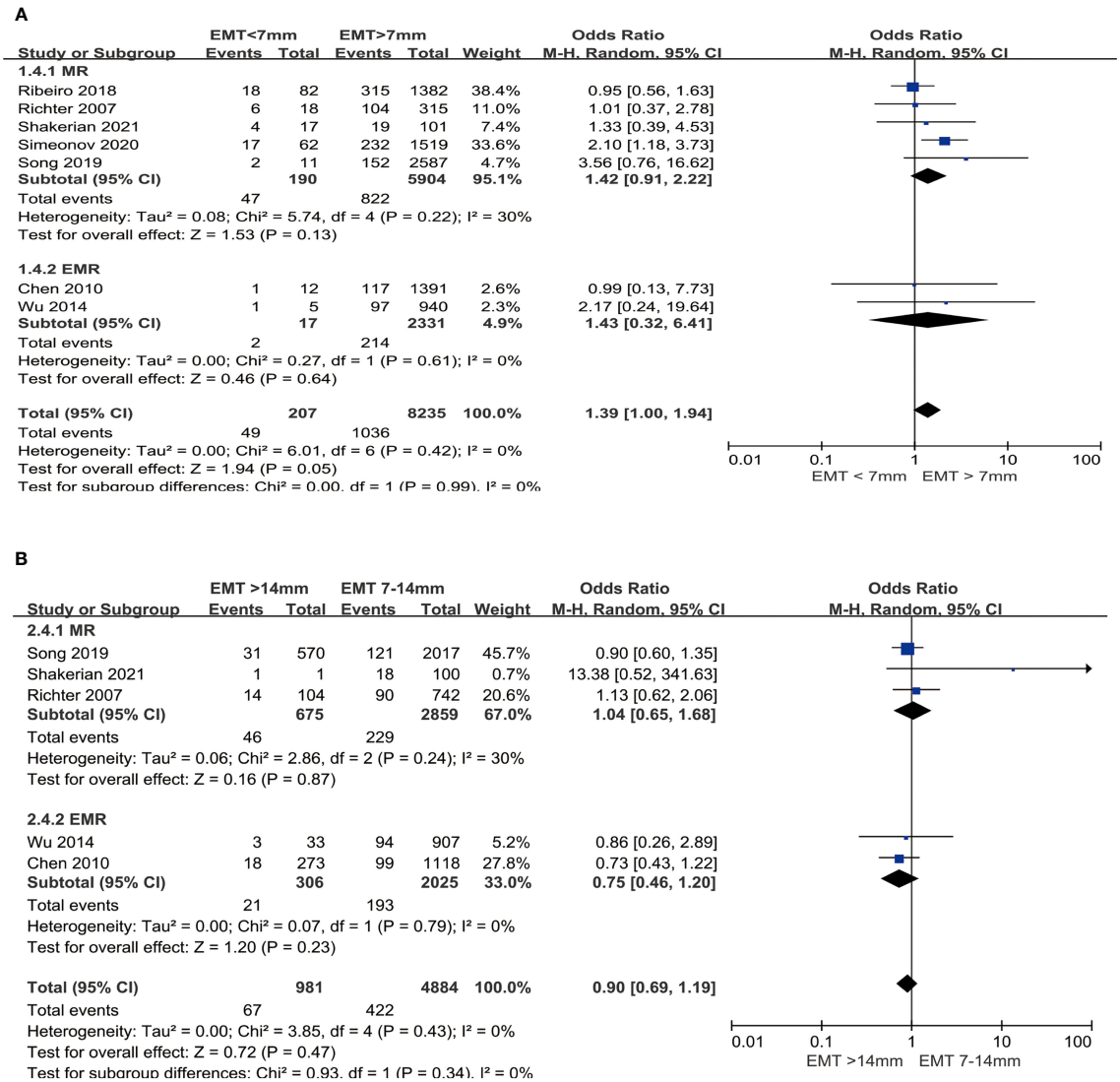



**FIGURE 5** | Comparison of MR between EMT groups in fresh cycles. **(A)** Comparison between thin endometrium group and non-thin endometrium group. **(B)** Comparison between thick endometrium group and medium endometrium. MR, Miscarriage rate; EMR, Early miscarriage rate.

restriction result from the failure of transformation of uterine spiral arteries into large vessels (45, 46). We speculated there was abnormal uterine artery blood flow in thin endometrium, as a consequence that intrauterine environment could not be maintained and the risk of HDP or SGA also increased. Moreover, a study revealed that thin endometrium appears to be associated with an aberrantly activated inflammatory environment (40). Thus, the increased immunological factors in thin endometrium may also impair placentation and contribute to the occurrence of SGA or preeclampsia (47, 48). However, the underlying mechanism for this phenomenon is still unclear and needs to be elucidated.

Our study provided evidence that thin endometrium not only dampened the pregnancy outcomes following in IVF/ICSI, but also suppressed the fetal development, namely, increased the risk of SGA and decreased the BW of the fetus. The incidence of HDP arose, suggesting thin endometrium might also contribute to abnormal placental functions. However, because of the small number of included studies, the conclusion needs to be drawn with caution. In general, clinicians need to inform patients of possible obstetric complications caused by thin endometrium after IVF/ICSI and encourage patients to actively cooperate with prenatal examinations and receive more perinatal care after conceiving.

Previous studies showed that thick endometrium had negative effect on IVF/ICSI pregnancy (7). Our results suggested that increased EMT did not adversely affect the pregnancy outcome. This phenomenon might be helpful for clinicians to make decisions about embryo transplantation when they encounter thicker endometrium.

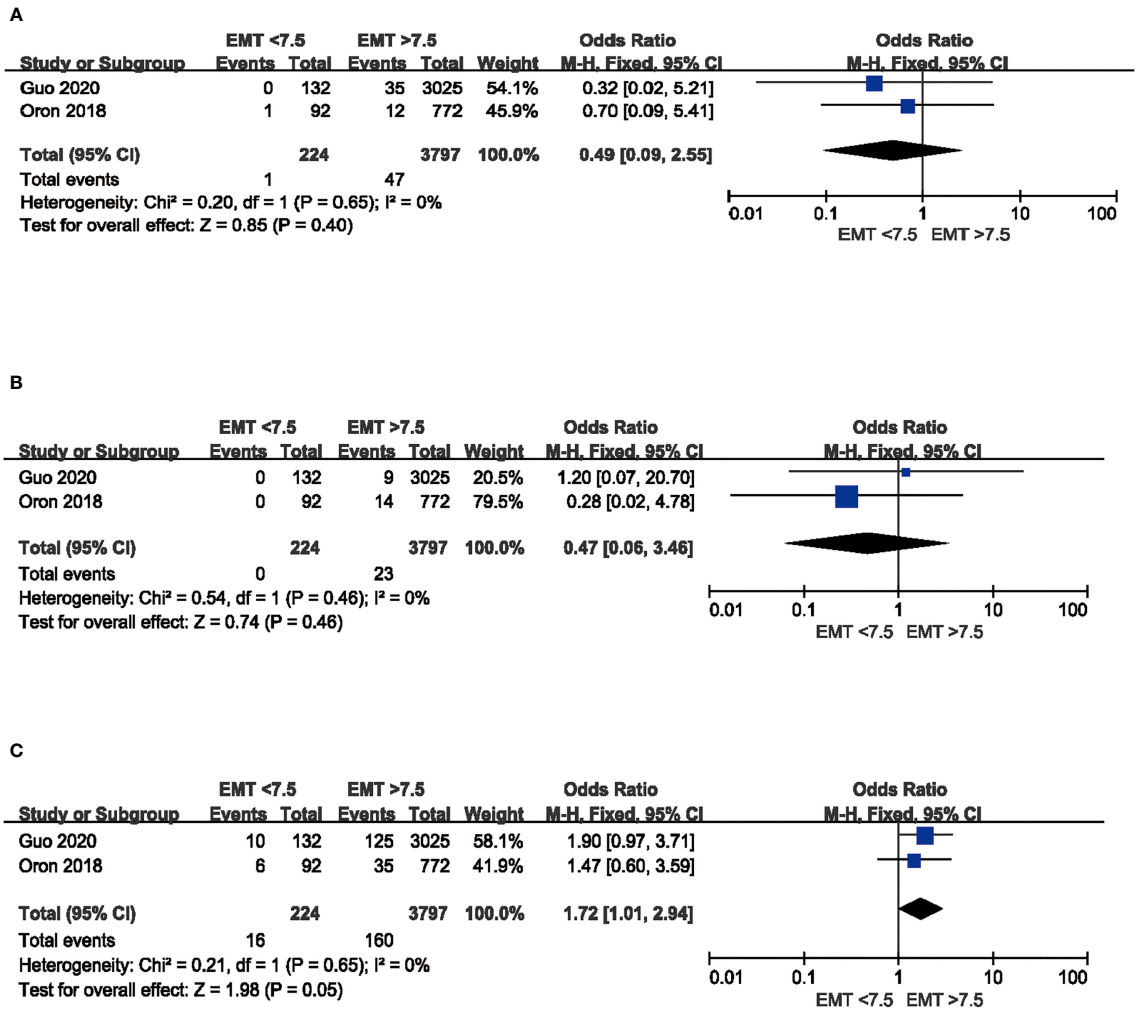



**FIGURE 6** | Comparison of maternal outcomes between EMT <7.5 mm and EMT >7.5 mm in fresh cycles. **(A)** Comparison of PP between thin endometrium group and non-thin endometrium group. **(B)** Comparison of PA between thin endometrium group and non-thin endometrium group. **(C)** Comparison of HDP between thin endometrium group and non-thin endometrium group. PP, Placenta previa; PA, Placenta abruption; HDP, hypertensive disorders of pregnancy.

This was, to the best of our knowledge, the first meta-analysis that not only explored the role of thick endometrium on pregnancy outcomes but also analyzed the effect of EMT on obstetric complications and perinatal outcomes after IVF/ICSI. Understanding these influences may enable evidence-based support to be provided.

There are also some limitations in this study. Firstly, substantial heterogeneity among studies existed in some analysis, such as when analyzing the effect of thin endometrium on LBR or CPR, and the influence of thick endometrium on CPR or IR. Secondly, many of the included studies were retrospective studies and this study type is relevant to an inevitable risk of bias. Thirdly, as the different sonographers and equipment cause, the measurements of EMT are inherent with inter- and intra-variability, which might also bring some bias. Additionally, the definition of thin endometrium has not reached an agreement (38). In our study, 7 mm was chosen as the cutoff value for thin EMT as most studies reported, and thus, this selection method might ignore the influence of other thresholds on outcomes. Fourthly, the cause of thin endometrium is unclear in studies and it is possible that scarred thin endometrium, such as following curettage, entails a poorer prognosis than "natively" thin endometrium, which might also affect the results (49). Lastly, because the number of studies related to maternal and perinatal outcomes is insufficient and the inclusion of any studies relating to impaired fetal growth did not refer to long term neuro development, more well-conducted prospective studies are required.

In conclusion, our study indicated that thin endometrium had an adverse effect on LBR, CPR, IR and BW of infants, and increased the incidence of HDP in women and SGA of babies. However, it had little effect on MR, PA, and PP of patients, or on

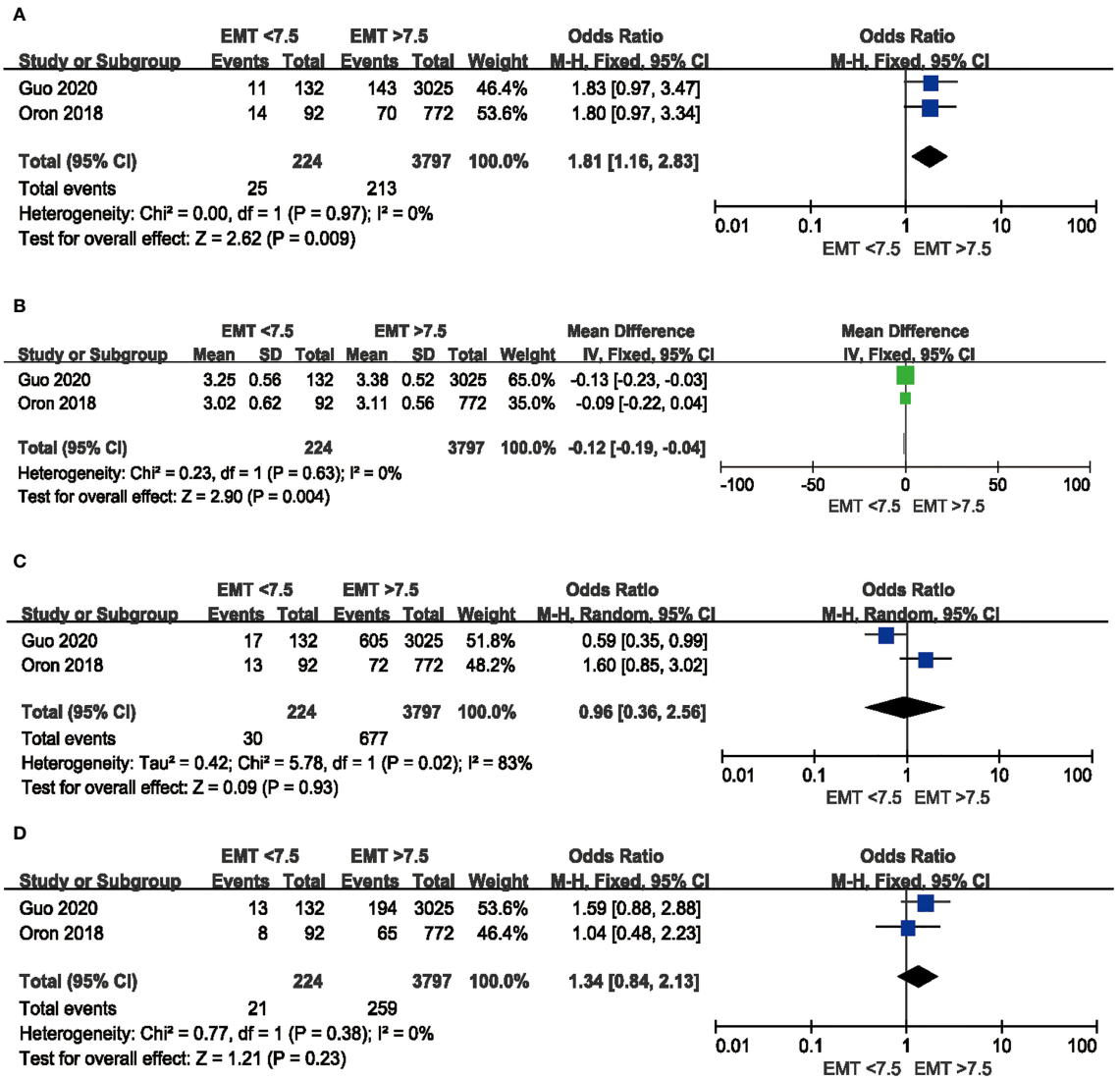



**FIGURE 7** | Comparison of perinatal outcomes between EMT <7.5 mm and EMT >7.5 mm in fresh cycles. **(A)** Comparison of SGA between thin endometrium group and non-thin endometrium group. **(B)** Comparison of BW between thin endometrium group and non-thin endometrium group. **(C)** Comparison of LGA between thin endometrium group and non-thin endometrium group. **(D)** Comparison of PTD between thin endometrium group and non-thin endometrium group. SGA, Small-for-gestational-age; BW, Birthweight; LGA, Large-for-gestational-age; PTD, Preterm delivery.

LGA and PTD among infants. More observational studies with large sample sizes and long-term follow-up or more randomized trials with preset protocols need to investigate the significance of the EMT on maternal or perinatal outcomes following in IVF/ICSI. The thick endometrium made no significant difference to pregnancy outcomes in fresh cycles.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

## AUTHOR CONTRIBUTIONS

ZL and CL contributed to the design of study. ZL, LC, and LS performed studies search and data collection. ZL and CL drafted the manuscript, which was revised by KQ, CS, and HZ. All authors contributed to the article and approved the submitted version.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.814648/full#supplementary-material

## REFERENCES


1. Farquhar C, Marjoribanks J. Assisted Reproductive Technology: An Overview of Cochrane Reviews. *Cochrane Database Syst Rev* (2018) 8. doi: 10.1002/14651858.CD010537.pub5
2. Vaegter KK, Lakic TG, Olovsson M, Berglund L, Brodin T, Holte J. Which Factors Are Most Predictive for Live Birth After *In Vitro* Fertilization and Intracytoplasmic Sperm Injection (IVF/ICSI) Treatments? Analysis of 100 Prospectively Recorded Variables in 8,400 IVF/ICSI Single-Embryo Transfers. *Fertil Steril* (2017) 107:641–8.e2. doi: 10.1016/j.fertnstert.2016.12.005
3. Basir GS, WS O, So WWK, Ng EHY, Ho PC. Evaluation of Cycle-to-Cycle Variation of Endometrial Responsiveness Using Transvaginal Sonography in Women Undergoing Assisted Reproduction. *Ultrasound Obstetr Gynecol* (2002) 19:484–9. doi: 10.1046/j.1469-0705.2002.00685.x
4. Fang R, Cai L, Xiong F, Chen J, Yang W, Zhao X. The Effect of Endometrial Thickness on the Day of Hcg Administration on Pregnancy Outcome in the First Fresh IVF/ICSI Cycle. *Gynecol Endocrinol* (2016) 32:473–6. doi: 10.3109/09513590.2015.1132304
5. Eftekhar M, Mehrjardi SZ, Molaei B, Taheri F, Mangoli E. The Correlation Between Endometrial Thickness and Pregnancy Outcomes in Fresh ART Cycles With Different Age Groups: A Retrospective Study. *Middle East Fertil Soc J* (2019) 24:16–20. doi: 10.1186/s43043-019-0013-y
6. Shalom-Paz E, Atia N, Atzmon Y, Hallak M, Shrim A. The Effect of Endometrial Thickness and Pattern on the Success of Frozen Embryo Transfer Cycles and Gestational Age Accuracy. *Gynecol Endocrinol* (2021) 37:428–32. doi: 10.1080/09513590.2020.1821359
7. Weissman A, Gotlieb L, Casper RF. The Detrimental Effect of Increased Endometrial Thickness on Implantation and Pregnancy Rates and Outcome in an *In Vitro* Fertilization Program. *Fertil Steril* (1999) 71:147–9. doi: 10.1016/s0015-0282(98)00413-0
8. Zhang X, Chen CH, Confino E, Barnes R, Milad M, Kazer RR. Increased Endometrial Thickness Is Associated With Improved Treatment Outcome for Selected Patients Undergoing *In Vitro* Fertilization-Embryo Transfer. *Fertil Steril* (2005) 83:336–40. doi: 10.1016/j.fertnstert.2004.09.020
9. Vermey BG, Buchanan A, Chambers GM, Kolibianakis EM, Bosdou J, Chapman MG, et al. Are Singleton Pregnancies After Assisted Reproduction Technology (ART) Associated With a Higher Risk of Placental Anomalies Compared With Non-ART Singleton Pregnancies? A Systematic Review and Meta-Analysis. *BJOG* (2019) 126:209–18. doi: 10.1111/1471-0528.15227
10. Henningsen A-KA, Pinborg A, Lidegaard Ø, Vestergaard C, Forman JL, Andersen AN. Perinatal Outcome of Singleton Siblings Born After Assisted Reproductive Technology and Spontaneous Conception: Danish National Sibling-Cohort Study. *Fertil Steril* (2011) 95:959–63. doi: 10.1016/j.fertnstert.2010.07.1075
11. Oron G, Hiersch L, Rona S, Prag-Rosenberg R, Sapir O, Tuttnauer-Hamburger M, et al. Endometrial Thickness of Less Than 7.5 Mm Is Associated With Obstetric Complications in Fresh IVF Cycles: A Retrospective Cohort Study. *Reprod BioMed Online* (2018) 37:341–8. doi: 10.1016/j.rbmo.2018.05.013
12. Rombauts L, Motteram C, Berkowitz E, Fernando S. Risk of Placenta Praevia Is Linked to Endometrial Thickness in a Retrospective Cohort Study of 4537 Singleton Assisted Reproduction Technology Births. *Hum Reprod* (2014) 29:2787–93. doi: 10.1093/humrep/deu240
13. Guo Z, Xu X, Zhang L, Zhang L, Yan L, Ma J. Endometrial Thickness Is Associated With Incidence of Small-for-Gestational-Age Infants in Fresh *In Vitro* Fertilization-Intracytoplasmic Sperm Injection and Embryo Transfer Cycles. *Fertil Steril* (2020) 113:745–52. doi: 10.1016/j.fertnstert.20619.12.014
14. Liu X, Wang J, Fu X, Li J, Zhang M, Yan J, et al. Thin Endometrium Is Associated With the Risk of Hypertensive Disorders of Pregnancy in Fresh IVF/ICSI Embryo Transfer Cycles: A Retrospective Cohort Study of 9,266 Singleton Births. *Reprod Biol Endocrinol* (2021) 19:55. doi: 10.1186/s12958-021-00738-9
15. Zegers-Hochschild F, Adamson GD, de Mouzon J, Ishihara O, Mansour R, Nygren K, et al. International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) Revised Glossary of ART Terminology, 2009. *Fertil Steril* (2009) 92:1520–4. doi: 10.1016/j.fertnstert.2009.09.009
16. Ribeiro VC, Santos-Ribeiro S, De Munck N, Drakopoulos P, Polyzos NP, Schutyser V, et al. Should We Continue to Measure Endometrial Thickness in Modern-Day Medicine? The Effect on Live Birth Rates and Birth Weight. *Reprod Biomed Online* (2018) 36:416–26. doi: 10.1016/j.rbmo.2017.12.016
17. Chaudhry K, Tafti D, Siccardi MA. Anembryonic Pregnancy. In: *Statpearls*. Treasure Island (FL: StatPearls Publishing Copyright © 2021, StatPearls Publishing LLC. (2021).
18. Marengoni A, Vetrano DL, Manes-Gravina E, Bernabei R, Onder G, Palmer K. The Relationship Between COPD and Frailty: A Systematic Review and Meta-Analysis of Observational Studies. *Chest* (2018) 154:21–40. doi: 10.1016/j.chest.2018.02.014
19. Aydin T, Kara M, Nurettin T. Relationship Between Endometrial Thickness and *In Vitro* Fertilization-Intracytoplasmic Sperm Injection Outcome. *Int J Fertil Steril* (2013) 7:29–34.
20. Nishihara S, Fukuda J, Ezoe K, Endo M, Nakagawa Y, Yamadera R, et al. Does the Endometrial Thickness on the Day of the Trigger Affect the Pregnancy Outcomes After Fresh Cleaved Embryo Transfer in the Clomiphene Citrate-Based Minimal Stimulation Cycle? *Reprod Med Biol* (2020) 19:151–7. doi: 10.1002/rmb2.12315
21. Lv H, Li X, Du J, Ling X, Diao F, Lu Q, et al. Effect of Endometrial Thickness and Embryo Quality on Live-Birth Rate of Fresh IVF/ICSI Cycles: A Retrospective Cohort Study. *Reprod Biol Endocrinol* (2020) 18:89. doi: 10.1186/s12958-020-00636-6
22. Tomic V, Kasum M, Vucic K. Impact of Embryo Quality and Endometrial Thickness on Implantation in Natural Cycle IVF. *Arch Gynecol Obstet* (2020) 301:1325–30. doi: 10.1007/s00404-020-05507-4
23. Ovayolu A, Gün I, Silfeler DB, Kutlu T. Live Birth and Endometrial Thickness in Unexplained Infertility. *Zeynep Kamil Tip Bulteni* (2019) 50:142–5. doi: 10.16948/zktipb.550114
24. Song J, Sun X, Qian K. Endometrial But Not Ovarian Response Is Associated With Clinical Outcomes and Can be Improved by Prolonged Pituitary Downregulation in Patients With Thin and Medium Endometrium. *Reprod Sci* (2019) 26:1409–16. doi: 10.1177/1933719118816835
25. Chan JM, Sukumar AI, Ramalingam M, Ranbir Singh SS, Abdullah MF. The Impact of Endometrial Thickness (EMT) on the Day of Human Chorionic Gonadotropin (Hcg) Administration on Pregnancy Outcomes: A 5-Year Retrospective Cohort Analysis in Malaysia. *Fertil Res Pract* (2018) 4:5. doi: 10.1186/s40738-018-0050-8
26. Holden EC, Dodge LE, Sneeringer R, Moragianni VA, Penzias AS, Hacker MR. Thicker Endometrial Linings Are Associated With Better IVF Outcomes: A Cohort of 6331 Women. *Hum Fertil (Camb)* (2018) 21:288–93. doi: 10.1080/14647273.2017.1334130
27. Wu Y, Gao X, Lu X, Xi J, Jiang S, Sun Y, et al. Endometrial Thickness Affects the Outcome of *In Vitro* Fertilization and Embryo Transfer in Normal Responders After Gnrh Antagonist Administration. *Reprod Biol Endocrinol* (2014) 12:96. doi: 10.1186/1477-7827-12-96
28. Zhao J, Zhang Q, Wang Y, Li Y. Endometrial Pattern, Thickness and Growth in Predicting Pregnancy Outcome Following 3319 IVF Cycle. *Reprod BioMed Online* (2014) 29:291–8. doi: 10.1016/j.rbmo.2014.05.011
29. Zhao J, Zhang Q, Li Y. The Effect of Endometrial Thickness and Pattern Measured by Ultrasonography on Pregnancy Outcomes During IVF-ET Cycles. *Reprod Biol Endocrinol* (2012) 10:100. doi: 10.1186/1477-7827-10-100
30. Chen S-L, Wu F-R, Luo C, Chen X, Shi X-Y, Zheng H-Y, et al. Combined Analysis of Endometrial Thickness and Pattern in Predicting Outcome of *In Vitro* Fertilization and Embryo Transfer: A Retrospective Cohort Study. *Reprod Biol Endocrinol* (2010) 8:30. doi: 10.1186/1477-7827-8-30
31. Okohue JE, Onuh SO, Ebeigbe P, Shaibu I, Wada I, Ikimalo JI, et al. The Effect of Endometrial Thickness on *In Vitro* Fertilization (IVF)-Embryo Transfer/Intracytoplasmic Sperm Injection (ICSI) Outcome. *Afr J Reprod Health* (2009) 13:113–21.
32. Al-Ghamdi A, Coskun S, Al-Hassan S, Al-Rejjal R, Awartani K. The Correlation Between Endometrial Thickness and Outcome of *In Vitro* Fertilization and Embryo Transfer (IVF-ET) Outcome. *Reprod Biol Endocrinol* (2008) 6:37. doi: 10.1186/1477-7827-6-37
33. Richter KS, Bugge KR, Bromer JG, Levy MJ. Relationship Between Endometrial Thickness and Embryo Implantation, Based on 1,294 Cycles of

*In Vitro* Fertilization With Transfer of Two Blastocyst-Stage Embryos. *Fertil Steril* (2007) 87:53–9. doi: 10.1016/j.fertnstert.2006.05.064
34. Yoeli R, Ashkenazi J, Orvieto R, Shelef M, Kaplan B, Bar-Hava I. Significance of Increased Endometrial Thickness in Assisted Reproduction Technology Treatments. *J Assist Reprod Genet* (2004) 21:285–9. doi: 10.1023/B:JARG.0000043701.22835.56
35. Noyes N, Liu HC, Sultan K, Schattman G, Rosenwaks Z. Endometrial Thickness Appears to be a Significant Factor in Embryo Implantation in *In-Vitro* Fertilization. *Hum Reprod* (1995) 10:919–22. doi: 10.1093/oxfordjournals.humrep.a136061
36. Shakerian B, Turkgeldi E, Yildiz S, Keles I, Ata B. Endometrial Thickness Is Not Predictive for Live Birth After Embryo Transfer, Even Without a Cutoff. *Fertil Steril* (2021) 116:130–7. doi: 10.1016/j.fertnstert.2021.02.041
37. Simeonov M, Sapir O, Lande Y, Ben-Haroush A, Oron G, Shlush E, et al. The Entire Range of Trigger-Day Endometrial Thickness in Fresh IVF Cycles Is Independently Correlated With Live Birth Rate. *Reprod Biomed Online* (2020) 41:239–47. doi: 10.1016/j.rbmo.2020.04.008
38. Kasius A, Smit JG, Torrance HL, Eijkemans MJ, Mol BW, Opmeer BC, et al. Endometrial Thickness and Pregnancy Rates After IVF: A Systematic Review and Meta-Analysis. *Hum Reprod Update* (2014) 20:530–41. doi: 10.1093/humupd/dmu011
39. Casper RF. It's Time to Pay Attention to the Endometrium. *Fertil Steril* (2011) 96:519–21. doi: 10.1016/j.fertnstert.2011.07.1096
40. Maekawa R, Taketani T, Mihara Y, Sato S, Okada M, Tamura I, et al. Thin Endometrium Transcriptome Analysis Reveals a Potential Mechanism of Implantation Failure. *Reprod Med Biol* (2017) 16:206–27. doi: 10.1002/rmb2.12030
41. Zong L, Zheng S, Meng Y, Tang W, Li D, Wang Z, et al. Integrated Transcriptomic Analysis of the miRNA-mRNA Interaction Network in Thin Endometrium. *Front Genet* (2021) 12:589408. doi: 10.3389/fgene.2021.589408
42. Larsen EC, Christiansen OB, Kolte AM, Macklon N. New Insights Into Mechanisms Behind Miscarriage. *BMC Med* (2013) 11:154. doi: 10.1186/1741-7015-11-154
43. Dietterich C, Check JH, Choe JK, Nazari A, Lurie D. Increased Endometrial Thickness on the Day of Human Chorionic Gonadotropin Injection Does Not Adversely Affect Pregnancy or Implantation Rates Following *In Vitro* Fertilization-Embryo Transfer. *Fertil Steril* (2002) 77:781–6. doi: 10.1016/s0015-0282(01)03276-9
44. Zhang J, Liu H, Mao X, Chen Q, Si J, Fan Y, et al. Effect of Endometrial Thickness on Birthweight in Frozen Embryo Transfer Cycles: An Analysis Including 6181 Singleton Newborns. *Hum Reprod* (2019) 34:1707–15. doi: 10.1093/humrep/dez103
45. Ridder A, Giorgione V, Khalil A, Thilaganathan B. Preeclampsia: The Relationship Between Uterine Artery Blood Flow and Trophoblast Function. *Int J Mol Sci* (2019) 20:3263. doi: 10.3390/ijms20133263
46. Turco MY, Moffett A. Development of the Human Placenta. *Dev (Cambridge England)* (2019) 146. doi: 10.1242/dev.163428
47. Vannuccini S, Clifton VL, Fraser IS, Taylor HS, Critchley H, Giudice LC, et al. Infertility and Reproductive Disorders: Impact of Hormonal and Inflammatory Mechanisms on Pregnancy Outcome. *Hum Reprod Update* (2016) 22:104–15. doi: 10.1093/humupd/dmv044
48. Sharps MC, Baker BC, Guevara T, Bischof H, Jones RL, Greenwood SL, et al. Increased Placental Macrophages and a Pro-Inflammatory Profile in Placentas and Maternal Serum in Infants With a Decreased Growth Rate in the Third Trimester of Pregnancy. *Am J Reprod Immunol (New York NY: 1989)* (2020) 84:e13267. doi: 10.1111/aji.13267
49. Shufaro Y, Simon A, Laufer N, Fatum M. Thin Unresponsive Endometrium–a Possible Complication of Surgical Curettage Compromising ART Outcome. *J Assist Reprod Genet* (2008) 25:421–5. doi: 10.1007/s10815-008-9245-y

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Physiology

ORIGINAL RESEARCH
published: 17 February 2022
doi: 10.3389/fphys.2022.808079

# Obstetric and Perinatal Outcomes After Assisted Reproductive Technology in Women With Cesarean Scar

*Yue Lin[1,2†], Qianqian Chen[2†], Xuefeng Huang[2], Ziliang Wang[3], Cuie Chen[4], Haiying Chen[5] and Fan Jin[1,6*]*

[1] Department of Reproductive Endocrinology, Women's Hospital, Zhejiang University School of Medicine, Hangzhou, China, [2] Reproductive Medicine Center, First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China, [3] NHC Key Laboratory of Reproduction Regulation (Shanghai Institute for Biomedical and Pharmaceutical Technologies), School of Public Health, Fudan University, Shanghai, China, [4] Department of Obstetrics and Gynecology, Affiliated Yueqing Hospital of Wenzhou Medical University, Wenzhou, China, [5] Department of Obstetrics and Gynecology, Wenzhou Central Hospital, Wenzhou Maternal and Child Health Care Hospital, Wenzhou, China, [6] Women's Reproductive Health Laboratory of Zhejiang Province, Key Laboratory of Reproductive Genetics, National Ministry of Education (Zhejiang University), Hangzhou, China

OPEN ACCESS

**Edited by:**
*Yimin Zhu,*
*Zhejiang University, China*

**Reviewed by:**
*Clarissa Lim Velayo,*
*University of the Philippines Manila, Philippines*
*Li Jin,*
*Fudan University, China*

***Correspondence:**
*Fan Jin*
*jinfan@zju.edu.cn*

*† These authors have contributed equally to this work and share first authorship*

**Specialty section:**
*This article was submitted to Reproduction, a section of the journal Frontiers in Physiology*

**Received:** *03 November 2021*
**Accepted:** *25 January 2022*
**Published:** *17 February 2022*

**Citation:**
*Lin Y, Chen Q, Huang X, Wang Z, Chen C, Chen H and Jin F (2022) Obstetric and Perinatal Outcomes After Assisted Reproductive Technology in Women With Cesarean Scar. Front. Physiol. 13:808079. doi: 10.3389/fphys.2022.808079*

**Introduction:** Assisted reproductive technology (ART) and previous Cesarean section (CS) are independently associated with the risk of adverse obstetric and perinatal outcomes in general. Few studies have focused on the association between adverse obstetric and perinatal outcomes and ART used in the high-risk population of women with previous CS.

**Materials and Methods:** A retrospective cohort study including 14,099 women with a previous delivery and a subsequent delivery between April 2014 and April 2020 was conducted at our hospital. We assessed the risk of adverse obstetric and perinatal outcomes in pregnancies conceived by ART in women with previous CS, using log-binomial regression models.

**Results:** In women with previous CS, ART singleton pregnancies were associated with an increased risk of maternal complications, such as pregnancy complications, placental anomalies of implantation, postpartum hemorrhage, and preterm birth (PTB), as compared to spontaneously conceived pregnancies. The implementation of ART and previous CS interacted in a synergistic manner to increase the likelihood of the placenta accreta spectrum in women with singleton pregnancies [adjusted relative risk (aRR) 5.30, 95% confidence interval (CI) 4.01–7.00; relative risk due to interaction: 1.41, 95%CI 0.07–2.75]. In women with previous CS who underwent ART, women with singletons conceived through intracytoplasmic sperm injection were at increased risk of velamentous placenta (aRR 2.46, 95%CI 1.35–4.48) compared with those with singletons conceived through *in vitro* fertilization (IVF), whereas women with singletons conceived through cleavage-stage embryo transfer (ET) were at increased risk of gestational diabetes mellitus (GDM) (aRR 1.74, 95%CI 1.16-2.60) than those with singletons conceived through blastocyst–stage ET.

**Conclusion:** Pregnancies conceived through ART were at increased risk for adverse obstetric and perinatal outcomes in women who had previously delivered by CS, particularly for placental anomalies of implantation. In women with previous CS undergoing ART, IVF and blastocyst–stage ET may be a relatively safe treatment.

**Keywords: assisted reproductive techniques, Cesarean section, complications, interaction, offspring health, safety**

# INTRODUCTION

It is well-documented that Cesarean section (CS) might increase the incidence of adverse obstetric and perinatal outcomes in subsequent conceptions, including persistent complete placenta previa, placental abruption, uterine Cesarean scar rupture, preterm birth (PTB), and low birth weight (LBW) (Ventura Laveriano and Redondo, 2013; Hu et al., 2018; Granfors et al., 2020). Thus, women with a Cesarean scar are a high–risk population for obstetric and perinatal complications in subsequent conceptions. Over the past decade, the number of infertile women with a Cesarean scar who seek assisted reproductive technology (ART) has been steadily increasing (Zhang et al., 2016). However, pregnancies conceived through ART have been suggested to have a higher risk of adverse obstetric and perinatal outcomes than spontaneously conceived (SC) pregnancies (Qin et al., 2016; Vannuccini et al., 2018; Yanaihara et al., 2018). Hence, the prevalence of adverse obstetric and perinatal outcomes in pregnancies conceived by ART in women with previous CS should be investigated. Nevertheless, few studies have focused on this topic. In addition, little is known about the effect of the type of ART procedure used in such women in relation to obstetric and perinatal outcomes.

The present retrospective cohort study aimed to assess the prevalence of adverse obstetric and perinatal outcomes associated with ART in women with previous CS precisely and to elucidate how to implement ART safely in infertile women with a Cesarean scar.

# MATERIALS AND METHODS

## Study Design and Participants

We conducted a retrospective cohort study, including all multipara women with a single previous full-term delivery and a subsequent delivery between April 2014 and April 2020 at the First Affiliated Hospital of Wenzhou Medical University. Obstetric and perinatal data of live newborns delivered after the 28th week of gestation were obtained from the delivery records. The exclusion criteria were as follows: (1) congenital uterine malformations, including uterus unicornis, uterus bicornis, septate uterus, and duplex uterus; (2) previous uterine myomectomy; and (3) stillbirths at current delivery. ART included *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI)–embryo transfer (ET), and frozen-thawed ET (FET). In women with a history of CS, pregnancies conceived through ART were assigned to the CS–ART group, whereas spontaneously conceived pregnancies were categorized into the CS–SC group. In women with a history of vaginal delivery (VD), pregnancies conceived through ART were assigned to the VD–ART group, whereas spontaneously conceived pregnancies were categorized into the VD–SC group.

Subsequently, the ART pregnancy group was divided into IVF and ICSI subgroups according to the fertilization mode, into the ET and FET subgroups according to different ET methods, and into blastocyst and cleavage-stage ET subgroups according to different embryo developmental stages. Using the unique personal identification number, all data were retrospectively collected from computer databases and stored in a deidentified database. Validation was performed on the data to check for errors and inconsistencies in documentation and coding.

## Outcomes

The study outcomes consisted of four parts: pregnancy complications, including gestational hypertension, preeclampsia, gestational diabetes mellitus (GDM); placental anomalies of implantation (Vahanian et al., 2015; Jauniaux et al., 2020), including placenta previa, low-lying placenta, velamentous placenta, placenta accreta spectrum (defined as abnormal adherence of the placenta to the implantation site) (Miller et al., 2021); other complications, including placental abruption, postpartum hemorrhage, uterine rupture, preterm prelabor rupture of the membranes (pPROM, defined as rupture of the fetal membranes prior to 37 weeks of completed gestation), and perinatal outcomes, including PTB (delivery at $< 37$ completed weeks of gestation), very PTB (gestational age $< 32$ weeks), LBW (weight $< 2{,}500$ g), macrosomia (weight $> 4{,}000$ g), and Apgar score $< 7$ at 1 min.

## Statistical Analysis

Categorical variables are presented as numbers with percentages. In women with previous CS, the risks of obstetric and perinatal outcomes in ART pregnancies (vs. non-ART) stratified by birth plurality were assessed using log-binomial regression models. The adjusted risk ratios (aRRs) with 95% confidence intervals (CIs) for each outcome and the interaction models were calculated after controlling for maternal age at the time of delivery ($\leq 35$, 36–39, and $\geq 40$ years), interpregnancy interval ($< 6$, 6–12, and $> 12$ months) (Kangatharan et al., 2017), other previous intrauterine operation, body mass index at the time of delivery ($< 24$ and $\geq 24$ kg/m$^2$), and education level ($\leq 9$ and $> 9$ years). Other previous intrauterine operations included curettage, surgical termination of pregnancy, and evacuation of retained conception products. In the ART pregnancy group of women with previous CS, we compared the incidence of obstetric and perinatal complications between different fertilization modes, between different ET methods, and between different embryo

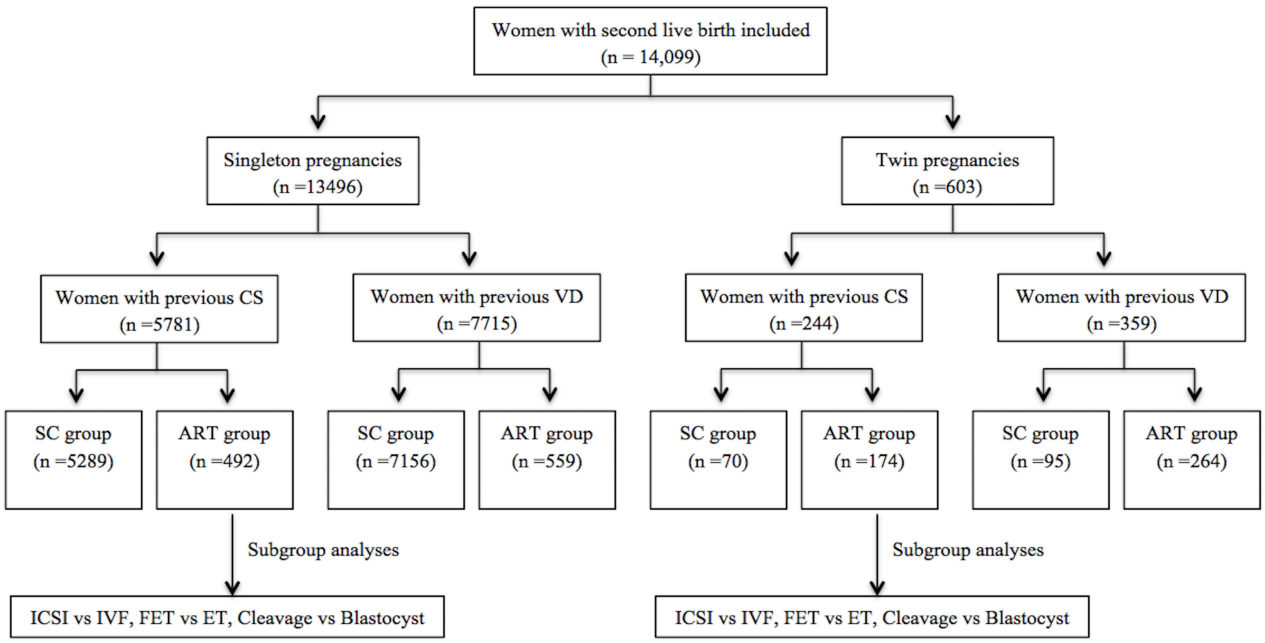



**FIGURE 1 |** Flow chart of the population in a study of obstetric and perinatal outcomes in women with a single previous full-term delivery. ART, assisted reproductive technology; SC, spontaneously conceived; CS, Cesarean section; VD, vaginal delivery; IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection; ET, embryo transfer; FET, frozen-thawed ET.

developmental stages, to elucidate how to implement ART safely in infertile women with a Cesarean scar. For comparison between different fertilization modes, we additionally adjusted for ET methods and embryo developmental stages. For comparison between different ET methods, we additionally adjusted for fertilization modes and embryo developmental stages. For comparison between different embryo developmental stages, we additionally adjusted for fertilization modes and ET methods. For maternal outcomes, such as pregnancy complications, placental anomalies of implantation and other obstetric complications, as the dependent variable, the unit of analysis was the delivery. For perinatal outcomes as the dependent variable, the unit of analysis was the offspring. In addition, for perinatal outcomes in twin pregnancies, generalized estimating equations were used to account for the correlation between the twins of the same mother (Grove et al., 1993).

In addition, we investigated the interaction between ART implementation and previous CS on the risk of obstetric and perinatal complications stratified by birth plurality, in which the VD–SC group were used as the reference group. The interaction measure between ART implementation and previous CS on the risk of obstetric and perinatal complications should only be used if the two exposure factors are risk factors for obstetric and perinatal outcomes. The relative excess risk due to interaction (RERI) along with 95%CI on the additive scale were calculated using the method described by Hosmer and Lemeshow (1992). RERI represents the extent to which risk increases due to the interaction of two exposures, rather than the sum of the individual risks (Knol and VanderWeele, 2012). Positive interactions on the additive scale were represented by a RERI greater than 0. A positive interaction on the additive scale indicates that the estimated joint effect of the two exposure factors exceeds the sum of their individual effects. SPSS statistical software (version 25; SPSS Inc., Armonk, NY, United States) was used for data analysis.

## Ethics Approval

All participants provided informed consent before undergoing routine treatment. Using the unique personal identification number, all data were retrospectively collected from computer databases and stored in a deidentified database. This study was approved by the ethics committee of the First Affiliated Hospital of Wenzhou Medical University (no. 2020–04) and performed according to the principles embodied in the Declaration of Helsinki.

# RESULTS

A total of 14,099 participants were included in the analysis (**Figure 1**). We identified 6,025 women with previous CS and 8,074 women with previous VD. Of the women with previous CS, 666 were in the CS-ART group and 5,359 were in the CS–SC group. Of the women with previous VD, 823 were in the VD-ART group and 7,251 were in the VD–SC group.

The maternal characteristics of singleton pregnancies and twin pregnancies are shown in **Tables 1**, **2**, respectively. Obstetric and perinatal outcomes of singleton pregnancies are summarized in **Table 3**, and those of twin pregnancies are shown in **Table 4**. Uterine rupture occurred in 18 women with previous CS, 14 of

**TABLE 1 |** Maternal characteristics in subsequent singleton pregnancies according to the mode of delivery at the first birth.

| | Previous CS | | | | | | | | Previous VD |
|---|---|---|---|---|---|---|---|---|---|
| | **Spontaneous conception (*n* = 5,289)** | **ART (*n* = 492)** | **IVF (*n* = 365)** | **ICSI (*n* = 127)** | **Fresh ET (*n* = 173)** | **FET (*n* = 319)** | **Cleavage (*n* = 360)** | **Blastocyst (*n* = 132)** | **Spontaneous conception (*n* = 7,156)** |
| | ***n* (%)** | ***n* (%)** | ***n* (%)** | ***n* (%)** | ***n* (%)** | ***n* (%)** | ***n* (%)** | ***n* (%)** | ***n* (%)** |
| **Maternal age** | | | | | | | | | |
| ≤ 35 years | 4,106 (77.6) | 276 (56.1) | 196 (53.7) | 80 (63.0) | 98 (56.6) | 178 (55.8) | 186 (51.7) | 90 (68.2) | 5,823 (81.4) |
| 36–39 years | 934 (17.7) | 150 (30.5) | 115 (31.5) | 35 (27.6) | 55 (31.8) | 95 (29.8) | 118 (32.8) | 32 (24.2) | 978 (13.7) |
| ≥ 40 years | 249 (4.7) | 66 (13.4) | 54 (14.8) | 12 (9.4) | 20 (11.6) | 46 (14.4) | 56 (15.6) | 10 (7.6) | 355 (5.0) |
| **Cause of infertility** | | | | | | | | | |
| PCOS | ND[a] | 54 (11.0) | 42 (11.5) | 12 (9.4) | 0 (0.0) | 54 (16.9) | 33 (9.2) | 21 (15.9) | ND[a] |
| Tubal factor | ND[a] | 343 (69.7) | 259 (71.0) | 84 (66.1) | 109 (63.0) | 234 (73.4) | 241 (66.9) | 102 (77.3) | ND[a] |
| Endometriosis | ND[a] | 44 (8.9) | 36 (9.9) | 8 (6.3) | 9 (5.2) | 35 (11.0) | 26 (7.2) | 18 (13.6) | ND[a] |
| Male factor | ND[a] | 188 (38.2) | 72 (19.7) | 116 (91.3) | 57 (32.9) | 131 (41.1) | 139 (38.6) | 49 (37.1) | ND[a] |
| Unexplained | ND[a] | 56 (11.4) | 45 (12.3) | 11 (8.7) | 19 (11.0) | 37 (11.6) | 37 (10.3) | 19 (14.4) | ND[a] |
| **Interpregnancy interval** | | | | | | | | | |
| < 6 months | 158 (3.0) | 24 (4.9) | 24 (6.6) | 0 (0.0) | 12 (6.9) | 12 (3.8) | 16 (4.4) | 8 (6.1) | 247 (3.5) |
| 6–12 months | 322 (6.1) | 92 (18.7) | 73 (20.0) | 19 (15.0) | 28 (16.2) | 64 (20.1) | 77 (21.4) | 15 (11.4) | 4,445 (62.1) |
| > 12 months | 4,809 (90.9) | 376 (76.4) | 268 (73.4) | 108 (85.0) | 133 (76.9) | 243 (76.2) | 267 (74.2) | 109 (82.6) | 2,464 (34.4) |
| **First birth conceived through ART** | | | | | | | | | |
| Yes | 0 (0.0) | 126 (25.6) | 84 (23.0) | 42 (33.1) | 46 (26.6) | 80 (25.1) | 82 (22.8) | 44 (33.3) | 0 (0.0) |
| No | 5,289 (100.0) | 366 (74.4) | 281 (77.0) | 85 (66.9) | 127 (73.4) | 239 (74.9) | 278 (77.2) | 88 (66.7) | 7,156 (100.0) |
| **Education level** | | | | | | | | | |
| ≤ 9 years | 3,188 (60.3) | 293 (59.6) | 219 (60.0) | 74 (58.3) | 95 (54.9) | 198 (62.1) | 206 (57.2) | 87 (65.9) | 4,634 (64.8) |
| > 9 years | 2,101 (39.7) | 199 (40.4) | 146 (40.0) | 53 (41.2) | 78 (45.1) | 121 (37.9) | 154 (42.8) | 45 (34.1) | 2,522 (35.2) |
| **Smoking** | | | | | | | | | |
| Yes | 0 (0.0) | 2 (0.4) | 2 (0.5) | 0 (0.0) | 0 (0.0) | 2 (0.6) | 1 (0.3) | 1 (0.8) | 0 (0.0) |
| No | 5,289 (100.0) | 490 (99.6) | 363 (99.5) | 127 (100.0) | 173 (100.0) | 317 (99.4) | 359 (99.7) | 131 (99.2) | 7,156 (100.0) |
| **Alcohol consumption** | | | | | | | | | |
| Yes | 1 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 3 (0.0) |
| No | 5,288 (100.0) | 492 (100.0) | 365 (100.0) | 127 (100.0) | 173 (100.0) | 319 (100.0) | 360 (100.0) | 132 (100.0) | 7,153 (100.0) |
| **Other previous intrauterine operation[b]** | | | | | | | | | |
| Yes | 4,637 (87.7) | 444 (90.2) | 322 (88.2) | 122 (96.1) | 160 (92.5) | 284 (89.0) | 327 (90.8) | 117 (88.6) | 6,381 (89.2) |
| No | 652 (12.3) | 48 (9.8) | 43 (11.8) | 5 (3.9) | 13 (7.5) | 35 (11.0) | 33 (9.2) | 15 (11.4) | 775 (10.8) |
| **Maternal body mass index** | | | | | | | | | |
| < 24 kg/m$^2$ | 777 (14.7) | 147 (29.9) | 106 (29.0) | 41 (32.3) | 42 (24.3) | 105 (32.9) | 97 (26.9) | 50 (37.9) | 1,516 (21.2) |
| ≥ 24 kg/m$^2$ | 4,512 (85.3) | 345 (70.1) | 259 (71.0) | 86 (67.7) | 131 (75.7) | 214 (67.1) | 263 (73.1) | 82 (62.1) | 5,640 (78.8) |

*ART, assisted reproductive technology; CS, Cesarean section; VD, vaginal delivery; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; ET, embryo transfer; FET, frozen-thawed ET; ND, not defined. Cause of infertility of someone may be more than 1 cause possible.*

[a] *Because of zero counts in one cell.*

[b] *Included curettage, surgical termination of pregnancy and evacuation of retained products of conception.*

**TABLE 2 |** Maternal characteristics in subsequent twin pregnancies according to mode of delivery at the first birth.

| | Previous CS | | | | | | | | Previous VD |
|---|---|---|---|---|---|---|---|---|---|
| | Spontaneous conception (*n* = 70) | ART (*n* = 174) | IVF (*n* = 126) | ICSI (*n* = 48) | Fresh ET (*n* = 69) | FET (*n* = 105) | Cleavage (*n* = 132) | Blastocyst (*n* = 42) | Spontaneous conception (*n* = 95) |
| | *n* (%) | *n* (%) | *n* (%) | *n* (%) | *n* (%) | *n* (%) | *n* (%) | *n* (%) | *n* (%) |
| **Maternal age** | | | | | | | | | |
| ≤ 35 years | 56 (80.0) | 116 (66.7) | 83 (65.9) | 33 (68.8) | 48 (69.6) | 68 (64.8) | 83 (62.9) | 33 (78.6) | 83 (87.4) |
| 36–39 years | 10 (14.3) | 51 (29.3) | 37 (29.4) | 14 (29.3) | 18 (26.1) | 33 (31.4) | 43 (32.6) | 8 (19.0) | 10 (10.5) |
| ≥ 40 years | 4 (5.7) | 7 (4.0) | 6 (4.8) | 1 (4.0) | 3 (4.3) | 4 (3.8) | 6 (4.5) | 1 (2.4) | 2 (2.1) |
| **Cause of infertility** | | | | | | | | | |
| PCOS | ND[a] | 22 (12.6) | 16 (12.7) | 6 (12.5) | 0 (0.0) | 22 (21.0) | 16 (12.1) | 6 (14.3) | ND[a] |
| Tubal factor | ND[a] | 115 (66.1) | 104 (82.5) | 11 (22.9) | 47 (68.1) | 68 (64.8) | 88 (66.7) | 27 (64.3) | ND[a] |
| Endometriosis | ND[a] | 27 (15.5) | 20 (15.9) | 7 (14.6) | 6 (8.7) | 21 (20.0) | 21 (15.9) | 6 (14.3) | ND[a] |
| Male factor | ND[a] | 82 (47.1) | 41 (32.5) | 41 (85.4) | 28 (40.6) | 54 (51.4) | 51 (38.6) | 31 (73.8) | ND[a] |
| Unexplained | ND[a] | 6 (3.4) | 6 (4.8) | 0 (0.0) | 3 (4.3) | 3 (2.9) | 6 (4.5) | 0 (0.0) | ND[a] |
| **Interpregnancy interval** | | | | | | | | | |
| < 6 months | 3 (4.3) | 4 (2.3) | 2 (1.6) | 2 (4.2) | 0 (0.0) | 4 (3.8) | 2 (1.5) | 2 (4.8) | 5 (5.3) |
| 6–12 months | 9 (12.9) | 40 (23.0) | 27 (21.4) | 13 (27.1) | 14 (20.3) | 26 (24.8) | 34 (25.8) | 6 (14.3) | 76 (80.0) |
| > 12 months | 58 (82.9) | 130 (74.7) | 97 (77.0) | 33 (68.8) | 55 (79.7) | 75 (71.4) | 96 (72.7) | 34 (81.0) | 14 (14.7) |
| **First birth conceived through ART** | | | | | | | | | |
| Yes | 0 (0.0) | 45 (25.9) | 28 (22.2) | 17 (35.4) | 12 (17.4) | 33 (31.4) | 28 (21.2) | 17 (40.5) | 0 (0.0) |
| No | 70 (100.0) | 129 (74.1) | 98 (77.8) | 31 (64.6) | 57 (82.6) | 72 (68.6) | 104 (78.8) | 25 (59.5) | 95 (100.0) |
| **Education level** | | | | | | | | | |
| ≤ 9 years | 52 (74.3) | 96 (55.2) | 70 (55.6) | 26 (54.2) | 48 (69.6) | 48 (45.7) | 71 (53.8) | 25 (59.5) | 81 (85.3) |
| > 9 years | 18 (25.7) | 78 (44.8) | 56 (44.4) | 22 (45.8) | 21 (30.4) | 57 (54.3) | 61 (46.2) | 17 (40.5) | 14 (14.7) |
| **Smoking** | | | | | | | | | |
| Yes | 0 (0.0) | 1 (0.6) | 1 (0.8) | 0 (0.0) | 0 (0.0) | 1 (1.0) | 1 (0.8) | 0 (0.0) | 0 (0.0) |
| No | 70 (100.0) | 173 (99.4) | 125 (99.2) | 48 (100.0) | 69 (100.0) | 104 (99.0) | 131 (99.2) | 42 (100.0) | 95 (100.0) |
| **Alcohol consumption** | | | | | | | | | |
| Yes | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| No | 70 (100.0) | 174 (100.0) | 126 (100.0) | 48 (100.0) | 69 (100.0) | 105 (100.0) | 132 (100.0) | 42 (100.0) | 95 (100.0) |
| **Other previous intrauterine operation**[b] | | | | | | | | | |
| Yes | 58 (82.9) | 162 (93.1) | 116 (92.1) | 46 (95.8) | 61 (88.4) | 101 (96.2) | 123 (93.2) | 39 (92.9) | 89 (90.5) |
| No | 12 (17.1) | 12 (6.9) | 10 (7.9) | 2 (4.2) | 8 (11.6) | 4 (3.8) | 9 (6.8) | 3 (7.1) | 9 (9.5) |
| **Maternal body mass index** | | | | | | | | | |
| < 24 kg/m$^2$ | 6 (82.9) | 57 (32.8) | 41 (32.5) | 16 (33.3) | 22 (31.9) | 35 (33.3) | 42 (31.8) | 15 (35.7) | 7 (7.4) |
| ≥ 24 kg/m$^2$ | 64 (91.4) | 117 (67.2) | 85 (67.5) | 32 (66.7) | 47 (68.7) | 70 (66.7) | 90 (68.2) | 27 (64.3) | 88 (92.6) |

*ART, assisted reproductive technology; CS, Cesarean section; VD, vaginal delivery; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; ET, embryo transfer; FET, frozen-thawed ET; ND, not defined. Cause of infertility of someone may be more than 1 cause possible.*

[a] *Because of zero counts in one cell.*

[b] *Included curettage, surgical termination of pregnancy and evacuation of retained products of conception.*

**TABLE 3 |** Obstetric and perinatal outcomes in subsequent singleton pregnancies according to mode of delivery at the first birth.

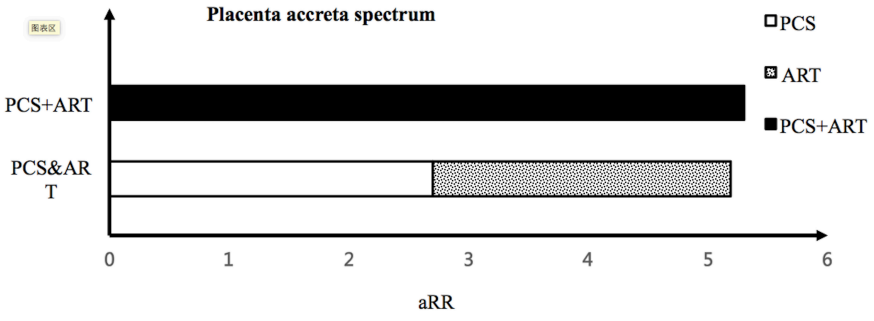
| | Previous CS | | | | | | | | Previous VD |
|---|---|---|---|---|---|---|---|---|---|
| | **Spontaneous conception (*n* = 5,289)** | **ART (*n* = 492)** | **IVF (*n* = 365)** | **ICSI (*n* = 127)** | **Fresh ET (*n* = 173)** | **FET (*n* = 319)** | **Cleavage (*n* = 360)** | **Blastocyst (*n* = 132)** | **Spontaneous conception (*n* = 7,156)** |
| | ***n* (%)** | ***n* (%)** | ***n* (%)** | ***n* (%)** | ***n* (%)** | ***n* (%)** | ***n* (%)** | ***n* (%)** | ***n* (%)** |
| **Pregnancy complications** | | | | | | | | | |
| Gestational hypertension | 169 (3.2) | 43 (8.7) | 30 (8.2) | 13 (10.2) | 9 (5.2) | 34 (10.7) | 25 (6.9) | 18 (13.6) | 195 (2.7) |
| Preeclampsia | 110 (2.1) | 16 (3.3) | 10 (2.7) | 6 (4.7) | 6 (3.5) | 10 (3.1) | 9 (2.5) | 7 (5.3) | 109 (1.5) |
| GDM | 907 (17.1) | 131 (26.6) | 91 (24.9) | 40 (31.5) | 47 (27.2) | 84 (26.3) | 109 (30.3) | 22 (16.7) | 937 (13.1) |
| **Placental anomalies of implantation** | | | | | | | | | |
| Placenta previa | 132 (2.5) | 19 (3.9) | 18 (4.9) | 1 (0.8) | 6 (3.5) | 13 (4.1) | 15 (4.2) | 4 (3.0) | 102 (1.4) |
| Low-lying placenta | 33 (0.6) | 7 (1.4) | 5 (1.4) | 2 (1.6) | 1 (0.6) | 6 (1.9) | 6 (1.7) | 1 (0.8) | 41 (0.6) |
| Velamentous placenta | 186 (3.5) | 42 (8.5) | 26 (7.1) | 16 (12.6) | 10 (5.8) | 32 (10.0) | 29 (8.1) | 13 (9.8) | 354 (4.9) |
| Placenta accreta spectrum | 409 (7.7) | 85 (17.3) | 68 (18.6) | 17 (13.4) | 21 (12.1) | 64 (20.1) | 60 (16.7) | 25 (18.9) | 203 (2.8) |
| **Other complications** | | | | | | | | | |
| Placental abruption | 38 (0.7) | 5 (1.0) | 3 (0.8) | 2 (1.6) | 0 (0.0) | 5 (1.6) | 4 (1.1) | 1 (0.8) | 77 (1.1) |
| Postpartum hemorrhage | 18 (0.3) | 10 (2.0) | 7 (1.9) | 3 (2.4) | 3 (1.7) | 7 (2.2) | 5 (1.4) | 5 (3.8) | 33 (0.5) |
| pPROM | 544 (10.3) | 55 (11.2) | 47 (12.9) | 8 (6.3) | 21 (12.1) | 34 (10.7) | 48 (13.3) | 7 (5.3) | 1,403 (19.6) |
| Uterine rupture | 14 (0.3) | 3 (0.6) | 3 (0.8) | 0 (0.0) | 1 (0.6) | 2 (0.6) | 3 (0.8) | 0 (0.0) | 0 (0.0) |
| Cesarean section | 4,767 (90.1) | 471 (95.7) | 349 (95.6) | 122 (96.1) | 169 (97.7) | 302 (94.7) | 350 (97.2) | 121 (91.7) | 920 (12.9) |
| **Infants** | | | | | | | | | |
| PTB | 394 (7.4) | 55 (11.2) | 46 (12.6) | 9 (7.1) | 24 (13.9) | 31 (9.7) | 48 (13.3) | 7 (5.3) | 482 (6.7) |
| Very PTB | 51 (1.0) | 9 (1.8) | 9 (2.5) | 0 (0.0) | 3 (1.7) | 6 (1.9) | 7 (1.9) | 2 (1.5) | 73 (1.0) |
| LBW | 181 (3.4) | 26 (5.3) | 23 (6.3) | 3 (2.4) | 11 (6.4) | 15 (4.7) | 22 (6.1) | 4 (3.0) | 212 (3.0) |
| Macrosomia | 310 (5.9) | 29 (5.9) | 19 (5.2) | 10 (7.9) | 2 (1.2) | 27 (8.5) | 14 (3.9) | 15 (11.4) | 441 (6.2) |
| Apgar score < 7 at 1 min | 67 (1.3) | 6 (1.2) | 6 (1.6) | 0 (0.0) | 4 (2.3) | 2 (0.6) | 3 (0.8) | 3 (2.3) | 73 (1.0) |

*ART, assisted reproductive technology; CS, Cesarean section; VD, vaginal delivery; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; ET, embryo transfer; FET, frozen-thawed ET; GDM, gestational diabetes mellitus; pPROM, preterm prelabor rupture of the membranes; PTB, preterm birth; LBW, low birthweight.*

**TABLE 4 |** Obstetric and perinatal outcomes in subsequent twin pregnancies according to mode of delivery at the first birth.

| | Previous CS | | | | | | | | Previous VD |
|---|---|---|---|---|---|---|---|---|---|
| | **Spontaneous conception (*n* = 70)** | **ART (*n* = 174)** | **IVF (*n* = 126)** | **ICSI (*n* = 48)** | **Fresh ET (*n* = 69)** | **FET (*n* = 105)** | **Cleavage (*n* = 132)** | **Blastocyst (*n* = 42)** | **Spontaneous conception (*n* = 95)** |
| | ***n* (%)** | ***n* (%)** | ***n* (%)** | ***n* (%)** | ***n* (%)** | ***n* (%)** | ***n* (%)** | ***n* (%)** | ***n* (%)** |
| **Pregnancy complications** | | | | | | | | | |
| Gestational hypertension | 10 (14.3) | 32 (18.4) | 26 (20.6) | 6 (12.5) | 7 (10.1) | 25 (23.8) | 20 (15.2) | 12 (28.6) | 8 (8.4) |
| Preeclampsia | 7 (10.0) | 14 (8.0) | 10 (7.9) | 4 (8.3) | 3 (4.3) | 11 (10.5) | 9 (6.8) | 5 (11.9) | 4 (4.2) |
| GDM | 14 (20.0) | 48 (27.6) | 34 (27.0) | 14 (29.2) | 19 (27.5) | 29 (27.6) | 32 (24.2) | 16 (38.1) | 17 (17.9) |
| **Placental anomalies of implantation** | | | | | | | | | |
| Placenta previa | 0 (0.0) | 16 (9.2) | 9 (7.1) | 7 (14.6) | 8 (11.6) | 8 (7.6) | 14 (10.6) | 2 (4.8) | 1 (1.1) |
| Low-lying placenta | 0 (0.0) | 5 (2.9) | 4 (3.2) | 1 (2.1) | 3 (4.3) | 2 (1.9) | 4 (3.0) | 1 (2.4) | 1 (1.1) |
| Velamentous placenta | 6 (8.6) | 23 (13.2) | 17 (13.5) | 6 (12.5) | 12 (17.4) | 11 (10.5) | 23 (17.4) | 0 (0.0) | 5 (5.3) |
| Placenta accreta spectrum | 9 (12.9) | 22 (12.6) | 15 (11.9) | 7 (14.6) | 2 (2.9) | 20 (19.0) | 19 (14.4) | 3 (7.1) | 12 (12.6) |
| **Other complications** | | | | | | | | | |
| Placental abruption | 1 (1.4) | 1 (0.6) | 0 (0.0) | 1 (2.1) | 0 (0.0) | 1 (1.0) | 1 (0.8) | 0 (0.0) | 0 (0.0) |
| Postpartum hemorrhage | 0 (0.0) | 10 (5.7) | 7 (5.6) | 3 (6.3) | 4 (5.8) | 6 (5.7) | 8 (6.1) | 2 (4.8) | 0 (0.0) |
| pPROM | 7 (10.0) | 36 (20.7) | 26 (20.6) | 10 (20.8) | 10 (14.5) | 26 (24.8) | 25 (18.9) | 11 (26.2) | 16 (16.8) |
| Uterine rupture | 1 (1.4) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Cesarean section | 69 (98.6) | 167 (96.0) | 119 (94.4) | 48 (100.0) | 64 (92.8) | 103 (98.1) | 127 (96.2) | 40 (95.2) | 82 (86.3) |
| **Infants** | | | | | | | | | |
| PTB | 82/140 (58.6) | 240/348 (69.0) | 168/252 (66.7) | 72/96 (75.0) | 90/138 (65.2) | 150/210 (71.4) | 178/264 (67.4) | 62/84 (73.8) | 98/190 (51.6) |
| Very PTB | 4/140 (2.9) | 24/348 (6.9) | 18/252 (7.1) | 6/96 (6.3) | 12/138 (8.7) | 12/210 (5.7) | 20/264 (7.6) | 4/84 (4.8) | 4/190 (2.1) |
| LBW | 27/140 (19.3) | 149/348 (42.8) | 100/252 (39.7) | 49/96 (51.0) | 62/138 (44.9) | 87/210 (41.4) | 115/264 (43.6) | 34/84 (40.5) | 28/190 (14.7) |
| Macrosomia | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Apgar score < 7 at 1 min | 3/140 (2.1) | 25/348 (7.2) | 7/252 (2.8) | 18/96 (18.8) | 10/138 (7.2) | 15/210 (7.1) | 14/264 (5.3) | 11/84 (13.1) | 8/190 (4.2) |

*ART, assisted reproductive technology; CS, Cesarean section; VD, vaginal delivery; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; ET, embryo transfer; FET, frozen-thawed ET; GDM, gestational diabetes mellitus; pPROM, preterm prelabor rupture of the membranes; PTB, preterm birth; LBW, low birthweight.*




| PCS | ART | aRR | 95% CI |
|---|---|---|---|
| + | − | 2.7 | 2.29-3.19 |
| − | + | 2.49 | 1.74-3.57 |
| + | + | 5.3 | 4.01-7.00 |

**FIGURE 2 |** The interaction between the implementation of ART and previous CS on the risk of placenta accreta spectrum. ART, assisted reproductive technology; CS, Cesarean section; aRR, adjusted risk ratio; CI, confidence interval.

whom were in CS–SC group, whereas none of the women with previous VD had uterine rupture. An overwhelming majority (> 90%) of women with previous CS had subsequent CS delivery, regardless of conception method and birth plurality.

## Comparison of Obstetric and Perinatal Outcomes in Women With Previous Cesarean Section Who Conceived by Assisted Reproductive Technology or Spontaneously, Stratified by Birth Plurality

In women with previous CS, ART singleton pregnancies were associated with an increased risk of gestational hypertension (aRR 2.06, 95%CI 1.27–3.35), GDM (aRR 1.39, 95%CI 1.15–1.67), velamentous placenta (aRR 2.46, 95%CI 1.70–3.56), placenta accreta spectrum (aRR 2.07, 95%CI 1.61–2.66), postpartum hemorrhage (aRR 8.65, 95%CI 3.83–19.57), and PTB (aRR 1.34, 95%CI 1.01–1.77), as compared to singletons in the CS–SC group (**Table 5**).

The implementation of ART and previous CS (**Supplementary Table 1**) are both risk factors for GDM and placenta accreta spectrum, when using VD–SC group as the reference group. We then investigated the interaction between the implementation of ART and previous CS on the risk of GDM and placenta accreta spectrum in singleton pregnancies (**Supplementary Table 2**). In singleton pregnancies, women with previous CS undergoing ART were found to have a significantly increased risk of placenta accreta spectrum (aRR 5.30, 95%CI 4.01-7.00; RERI 1.41, 95%CI 0.07–2.75), as compared to VD–SC group. This was due to a positive interaction on the additive scale between the implementation of ART and previous CS (**Figure 2**).

In women with previous CS, twins born following ART had an increased risk of LBW (aRR 2.34, 95% CI 1.37–3.98) compared to twins in CS–SC group (**Table 6**). Previous CS is not a risk factor for LBW in twin pregnancies (**Supplementary Table 3**); therefore, the interaction between ART implementation and previous CS on the risk of LBW in twin pregnancies was not assessed.

## Obstetric and Perinatal Outcomes Between Different Types of Assisted Reproductive Technology Procedure Used in Cesarean Section-Assisted Reproductive Technology Group

As shown in **Table 5**, women with singletons conceived through ICSI were at an increased risk of velamentous placenta (aRR 2.46, 95%CI 1.35–4.48) as compared to those with singletons conceived through IVF. Women with singletons conceived through cleavage-stage ET were 1.74 times more likely to develop GDM (95%CI 1.16–2.60) than those involving singletons conceived through blastocyst-stage ET (**Table 5**). As shown in **Table 6**, no significantly increased incidence of GDM (aRR 0.54, 95%CI 0.29–1.01) was observed between twins conceived through blastocyst-stage ET and through cleavage-stage ET.

# DISCUSSION

In this study, women with singletons in CS–ART group were at increased risk for adverse obstetric and perinatal outcomes when compared to those with singletons in CS–SC group. The risk was particularly increased for placental anomalies of implantation. In addition, the implementation of ART and previous CS interact synergistically to increase the likelihood of placenta accreta spectrum in women with singleton pregnancies. The obstetric and perinatal outcomes between different types of ART procedures used in women with previous CS were also examined: women with singletons conceived through ICSI were at increased risk of velamentous placenta compared with those with singletons conceived through IVF; whereas women with singletons conceived through cleavage-stage ET were at increased

**TABLE 5 |** The effect of ART procedures on obstetric and perinatal outcomes of singletons in women with previous CS.

| | ART vs. Spontaneous conception | | ICSI vs. IVF | | FET vs. ET | | Cleavage vs. Blastocyst | |
|---|---|---|---|---|---|---|---|---|
| | aRR[b] (95%CI) | *P*-value | aRR[c] (95%CI) | *P*-value | aRR[d] (95%CI) | *P*-value | aRR[e] (95%CI) | *P*-value |
| **Pregnancy complications** | | | | | | | | |
| Gestational hypertension | 2.06 (1.27–3.35) | 0.003 | 1.39 (0.77–2.52) | 0.280 | 1.38 (0.64–2.97) | 0.411 | 0.53 (0.28–0.99) | 0.047 |
| Preeclampsia | 1.51 (0.76–3.00) | 0.243 | 1.83 (0.68–4.91) | 0.230 | 0.50 (0.16–1.57) | 0.231 | 0.27 (0.09-0.84) | 0.024 |
| GDM | 1.39 (1.15–1.67) | <0.001 | 1.26 (0.93–1.73) | 0.142 | 0.97 (0.71–1.32) | 0.969 | 1.74 (1.16–2.60) | 0.007 |
| **Placental anomalies of implantation** | | | | | | | | |
| Placenta previa | 1.31 (0.75–2.28) | 0.338 | 0.16 (0.02–1.20) | 0.074 | 1.22 (0.44–3.35) | 0.703 | 1.51 (0.47–4.83) | 0.485 |
| Low-lying placenta | 1.69 (0.54–5.35) | 0.370 | 0.92 (0.18–4.64) | 0.914 | 5.08 (0.60–42.86) | 0.136 | 4.21 (0.49–36.10) | 0.190 |
| Velamentous placenta | 2.46 (1.70–3.56) | <0.001 | 2.46 (1.35–4.48) | 0.003 | 1.75 (0.86–3.56) | 0.122 | 0.85 (0.45–1.62) | 0.625 |
| Placenta accreta spectrum | 2.07 (1.61–2.66) | <0.001 | 0.77 (0.47–1.27) | 0.312 | 1.62 (1.00–2.63) | 0.053 | 1.19 (0.75–1.87) | 0.465 |
| **Other complications** | | | | | | | | |
| Placental abruption | 1.82 (0.68–4.82) | 0.231 | 1.39 (0.23–8.48) | 0.719 | ND[a] | | 2.52 (0.28–22.37) | 0.407 |
| Postpartum hemorrhage | 8.65 (3.83–19.57) | <0.001 | 1.13 (0.30–4.32) | 0.858 | 0.81 (0.17–3.80) | 0.792 | 0.34 (0.08–1.53) | 0.165 |
| pPROM | 0.93 (0.67–1.28) | 0.646 | 0.49 (0.24–1.00) | 0.049 | 1.10 (0.65–1.85) | 0.719 | 2.69 (1.21–5.95) | 0.015 |
| Uterine rupture | 2.66 (0.75–9.51) | 0.132 | ND[a] | | 1.60 (0.16–16.10) | 0.690 | ND[a] | |
| **Infants** | | | | | | | | |
| PTB | 1.34 (1.01–1.77) | 0.045 | 0.52 (0.26–1.03) | 0.059 | 0.87 (0.52–1.46) | 0.603 | 2.24 (1.00–5.01) | 0.050 |
| Very PTB | 1.21 (0.53–2.77) | 0.650 | ND[a] | | 0.89 (0.21–3.83) | 0.871 | 1.05 (0.19–5.65) | 0.959 |
| LBW | 1.42 (0.93–2.16) | 0.097 | 0.34 (0.10–1.10) | 0.070 | 0.87 (0.40–1.90) | 0.719 | 1.67 (0.56–4.98) | 0.362 |
| Macrosomia | 1.12 (0.76–1.65) | 0.561 | 1.58 (0.76–3.30) | 0.224 | 6.07 (1.39–26.59) | 0.017 | 0.59 (0.28–1.26) | 0.174 |
| Apgar score < 7 at 1 min | 0.98 (0.40–2.38) | 0.966 | ND[a] | | 0.11 (0.02–0.76) | 0.025 | 0.13 (0.02–0.77) | 0.025 |

*ART, assisted reproductive technology; CS, Cesarean section; VD, vaginal delivery; aRR, adjusted risk ratio; CI, confidence interval; ND, not defined; GDM, gestational diabetes mellitus; pPROM, preterm prelabor rupture of the membranes; PTB, preterm birth; LBW, low birthweight.*

[a]*Because of zero counts in one cell.*

[b]*RRs were adjusted for maternal age and body mass index at the time of delivery, interpregnancy interval, other previous intrauterine operation, and education level.*

[c]*RRs were adjusted for maternal age and body mass index at the time of delivery, interpregnancy interval, other previous intrauterine operation, education level, embryo transfer methods, and embryo developmental stage.*

[d]*RRs were adjusted for maternal age and body mass index at the time of delivery, interpregnancy interval, other previous intrauterine operation, education level, fertilization modes, and embryo developmental stage.*

[e]*RRs were adjusted for maternal age and body mass index at the time of delivery, interpregnancy interval, other previous intrauterine operation, education level, fertilization modes, and embryo transfer methods.*

**TABLE 6 |** The effect of ART procedures on obstetric and perinatal outcomes of twins in women with previous CS.

| | ART vs. Spontaneous conception | | ICSI vs. IVF | | FET vs. ET | | Cleavage vs. Blastocyst | |
|---|---|---|---|---|---|---|---|---|
| | aRR[b] (95%CI) | *P*-value | aRR[c] (95%CI) | *P*-value | aRR[d] (95%CI) | *P*-value | aRR[e] (95%CI) | *P*-value |
| **Pregnancy complications** | | | | | | | | |
| Gestational hypertension | 1.45 (0.70–3.01) | 0.318 | 0.54 (0.24–1.22) | 0.137 | 1.75 (0.71–4.35) | 0.226 | 0.54 (0.26–1.11) | 0.090 |
| Preeclampsia | 0.97 (0.38–2.51) | 0.952 | 0.84 (0.28–2.51) | 0.758 | 0.87 (0.22–3.51) | 0.848 | 0.44 (0.15-1.32) | 0.141 |
| GDM | 1.46 (0.75–2.86) | 0.268 | 1.07 (0.63–1.79) | 0.813 | 0.75 (0.41–1.40) | 0.367 | 0.54 (0.29–1.01) | 0.053 |
| **Placental anomalies of implantation** | | | | | | | | |
| Placenta previa | ND[a] | | 1.69 (0.67–4.31) | 0.268 | 0.61 (0.23–1.59) | 0.310 | 1.62 (0.34–7.87) | 0.547 |
| Low-lying placenta | ND[a] | | 0.62 (0.08–4.92) | 0.647 | 0.07 (0.04-1.22) | 0.068 | 0.46 (0.03–6.00) | 0.549 |
| Velamentous placenta | 2.44 (0.96–6.19) | 0.061 | 1.02 (0.44–2.39) | 0.963 | 1.05 (0.48–2.31) | 0.907 | ND[a] | |
| Placenta accreta spectrum | 1.44 (0.63–3.30) | 0.395 | 1.48 (0.66–3.27) | 0.340 | 7.28 (1.74–30.50) | 0.007 | 3.38 (1.05–10.83) | 0.041 |
| **Other complications** | | | | | | | | |
| Placental abruption | 0.40 (0.03–6.34) | 0.518 | ND[a] | | ND[a] | | ND[a] | |
| Postpartum hemorrhage | ND[a] | | 1.22 (0.33–4.58) | 0.766 | 1.05 (0.27–4.00) | 0.949 | 1.24 (0.23–6.60) | 0.799 |
| pPROM | 2.24 (0.97–5.18) | 0.059 | 0.88 (0.46–1.68) | 0.697 | 1.57 (0.75–3.30) | 0.235 | 0.75 (0.39–1.46) | 0.399 |
| Uterine rupture | ND[a] | | ND[a] | | ND[a] | | ND[a] | |
| **Infants** | | | | | | | | |
| PTB | 1.11 (0.87–1.41) | 0.419 | 1.11 (0.91–1.37) | 0.303 | 1.08 (0.87–1.34) | 0.509 | 0.97 (0.77–1.23) | 0.808 |
| Very PTB | 2.41 (0.55–10.51) | 0.240 | 0.88 (0.24–3.21) | 0.842 | 0.72 (0.22–2.31) | 0.579 | 1.34 (0.27–6.75) | 0.722 |
| LBW | 2.34 (1.37–3.98) | 0.002 | 1.10 (0.79–1.53) | 0.569 | 0.74 (0.54–1.02) | 0.062 | 0.88 (0.59–1.32) | 0.540 |
| Macrosomia | ND[a] | | ND[a] | | ND[a] | | ND[a] | |
| Apgar score < 7 at 1 min | 3.35 (0.73–15.51) | 0.122 | 6.45 (2.08–19.97) | 0.001 | 0.45 (0.18–1.08) | 0.074 | 0.27 (0.11–0.68) | 0.006 |

*ART, assisted reproductive technology; CS, Cesarean section; VD, vaginal delivery; aRR, adjusted risk ratio; CI, confidence interval; ND, not defined; GDM, gestational diabetes mellitus; pPROM, preterm prelabor rupture of the membranes; PTB, preterm birth; LBW, low birthweight.*

[a] *Because of zero counts in one cell.*

[b] *RRs were adjusted for maternal age and body mass index at the time of delivery, interpregnancy interval, other previous intrauterine operation, and education level.*

[c] *RRs were adjusted for maternal age and body mass index at the time of delivery, interpregnancy interval, other previous intrauterine operation, education level, embryo transfer methods, and embryo developmental stage.*

[d] *RRs were adjusted for maternal age and body mass index at the time of delivery, interpregnancy interval, other previous intrauterine operation, education level, fertilization modes, and embryo developmental stage.*

[e] *RRs were adjusted for maternal age and body mass index at the time of delivery, interpregnancy interval, other previous intrauterine operation, education level, fertilization modes, and embryo transfer methods.*

risk of GDM than those with singletons conceived through blastocyst-stage ET.

Women with singletons in CS–ART group were associated with an increased risk of maternal complications, such as gestational hypertension, GDM, velamentous placenta, placenta accreta spectrum, postpartum hemorrhage, as well as PTB, as compared with those with singletons in CS–SC group. This finding was in line with several recent cohort studies, and a 2016 meta–analysis including 50 cohort studies that showed high relative risks for adverse obstetric and perinatal outcomes in the ART group as compared with the spontaneous conception group (Qin et al., 2016; Zhu et al., 2016; Vannuccini et al., 2018; Yanaihara et al., 2018). Notably, the incidence of placenta accreta spectrum of singletons conceived through ART in our study (17.3%) was higher than that reported in a large-sample retrospective cohort study (6.9%) (Zhu et al., 2016). One of the possible explanations for this inconsistent result may be that our study was restricted to a high-risk population of women with previous CS, in contrast to previous studies. In the present study, women with singletons in the CS-ART group were 5.30 (95%CI 4.01–7.00) times more likely to develop placenta accreta spectrum than those with singletons in VD–SC group, which resulted from the positive interaction on the additive scale between the implementation of ART and previous CS. Although previous studies have identified previous CS as a risk factor for placenta accreta spectrum (Fitzpatrick et al., 2012), our study provided additional evidence suggesting that the implementation of ART and previous CS interacted synergistically to increase the likelihood of placenta accreta spectrum. This means that the joint effect of ART and previous CS exceeded the mere sum of their individual effects on placenta accreta spectrum.

The current hypothesis for the development of placenta accreta spectrum is that of a secondary defect of the endometrial–myometrial interface, leading to a failure of normal decidualization in the area of the uterine scar, allowing abnormally deep placentation (Jauniaux and Burton, 2018). Maternal pelvic factors, such as morphological, structural, and biological changes in the endometrium, are associated with infertility. Stimulation protocols or hormonal support in ART could also wholly or partly contribute to the incidence of placental disorders (Simon et al., 2003; Nakamura et al., 2015; Zhao et al., 2019; Jauniaux et al., 2020). The underlying mechanisms by which a Cesarean scar and ART interact in a synergistic manner to increase the risk of placenta accreta spectrum might require further investigation.

A recent cohort study confirmed the association between velamentous placenta and IVF and found that the odds ratio for velamentous placenta in women with IVF pregnancy was 1.72 (Yanaihara et al., 2018). However, the study involved only uncomplicated singletons conceived by IVF, and did not include ICSI. Our study established that ICSI had an enhanced effect on the incidence of velamentous placenta, as compared to IVF, in singleton pregnancies of women with previous CS. Possible mechanisms for this observation may relate to the genetic or epigenetic changes in trophectoderm cells due to ICSI, resulting in abnormal placentation (Tarín et al., 2014). Furthermore, our results indicated that cleavage-stage ET increased the risk of GDM in singleton pregnancies of women with previous CS, as compared with blastocyst-stage ET. Dysregulation of placental function may contribute to the pathogenesis of GDM (Souvannavong-Vilivong et al., 2019). Our findings raise the possibility that the improvement of uterine and embryonic synchronicity due to the prolonged *in vitro* culture of the trophectoderm cell may contribute to abnormal production or function of various placenta secrete molecules (Ming et al., 2012), which influences the pathogenesis of obstetric and perinatal outcomes. The exact mechanism by which different types of ART procedures might be related to placental abnormalities and subsequent obstetric and perinatal outcomes should be studied further.

Our study showed an increased risk for obstetric and perinatal outcomes in ART singletons as compared with spontaneously conceived neonates, but unexpectedly, we did not observe a similar trend in twin pregnancies. The reasons for this were probably as follows: (1) a lack of sufficient samples of twin pregnancies in women with previous CS; (2) most of the twins conceived naturally are monozygotic, while those conceived by ART are dizygotic. Therefore, the risk of adverse obstetric and perinatal outcomes in twin pregnancies conceived by ART compared with spontaneously conceived twins should be further studied, and conclusions should be drawn with caution.

Our findings provide valuable information for estimating and improving the safety of pregnancies in women with a Cesarean scar who seek ART, and might be useful in decision-making for women and clinical doctors to balance the risks and benefits of a Cesarean delivery in the first and subsequent births. With an enlarged sample size (> 10,000), we could adjust for confounders to enhance statistical power and thereby could provide more precise and reliable risk estimates. Our analyzed data were collected from case notes at the time of delivery, which minimized selection and recall bias. Additionally, our study indicated that ART singletons in women with previous CS carry an increased risk of adverse obstetric and perinatal outcomes, which has been poorly investigated to date.

This study had some limitations. The major weakness of this study lies in its retrospective nature and some confounders may be unavailable or unknown for adjustment. Although obstetric and perinatal outcomes during the first delivery may contribute to increased risks of adverse outcomes in the subsequent delivery, this information was only available in aggregated form in the delivery records for the second live birth. Therefore, there may have been residual confounding due to the lack of control for other potential confounding factors. It was difficult to confirm the indications for previous CS from the retrospective data. Further prospective studies are required to reduce the information bias. In addition, our database did not routinely record ultrasonographic features of Cesarean scar defect and therefore, we were unable to separately assess its role on obstetric and perinatal outcomes.

## CONCLUSION

In women with previous CS, clinicians should be aware of the increased risk of adverse obstetric and perinatal outcomes in pregnancies conceived by ART, particularly placental anomalies of implantation. Compared with other types of ART procedures, IVF and blastocyst-stage ET may be relatively safe for the high-risk population of women with previous CS who are undergoing ART.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## AUTHOR CONTRIBUTIONS

FJ: conceptualization and methodology. YL: data curation and writing—original draft preparation. QC: writing—reviewing and editing. XH: supervision. ZW: formal analysis and software. CC and HC: investigation. All authors contributed to the article and approved the submitted version.

## FUNDING

This work was supported by the National Key R&D Program of China (2018YFC1004900) and the National Natural Science Program of China (No. 81771652).

## ACKNOWLEDGMENTS

We are grateful to all members of our ART team and many colleagues in the Department of Obstetrics of the hospital in Wenzhou for their assistance during this study.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.808079/full#supplementary-material

## REFERENCES

Fitzpatrick, K. E., Sellers, S., Spark, P., Kurinczuk, J. J., Brocklehurst, P., and Knight, M. (2012). Incidence and risk factors for placenta accreta/increta/percreta in the UK: a national case-control study. *PLoS One* 7:e52893. doi: 10.1371/journal.pone.0052893

Granfors, M., Sandström, A., Stephansson, O., Belachew, J., Axelsson, O., and Wikström, A. K. (2020). Placental location and risk of retained placenta in women with a previous cesarean section: A population-based cohort study. *Acta Obstet. Gynecol. Scand.* 99, 1666–1673. doi: 10.1111/aogs.13943

Grove, J. S., Zhao, L. P., and Quiaoit, F. (1993). Correlation analysis of twin data with repeated measures based on generalized estimating equations. *Genet. Epidemiol.* 10, 539–544. doi: 10.1002/gepi.1370100635

Hosmer, D. W., and Lemeshow, S. (1992). Confidence interval estimation of interaction. *Epidemiology* 3, 452–456. doi: 10.1097/00001648-199209000-00012

Hu, H. T., Xu, J. J., Lin, J., Li, C., Wu, Y. T., Sheng, J. Z., et al. (2018). Association between first caesarean delivery and adverse outcomes in subsequent pregnancy: a retrospective cohort study. *BMC Preg. Childbirth* 18:273. doi: 10.1186/s12884-018-1895-x

Jauniaux, E., and Burton, G. J. (2018). Pathophysiology of placenta accreta spectrum disorders: a review of current findings. *Clin. Obstet. Gynecol.* 61, 743–754. doi: 10.1097/GRF.0000000000000392

Jauniaux, E., Moffett, A., and Burton, G. J. (2020). Placental implantation disorders. *Obstet. Gynecol. Clin. North Am.* 47, 117–132. doi: 10.1016/j.ogc.2019.10.002

Kangatharan, C., Labram, S., and Bhattacharya, S. (2017). Interpregnancy interval following miscarriage and adverse pregnancy outcomes: systematic review and meta-analysis. *Hum. Reprod. Update* 23, 221–231. doi: 10.1093/humupd/dmw043

Knol, M. J., and VanderWeele, T. J. (2012). Recommendations for presenting analyses of effect modification and interaction. *Int. J. Epidemiol.* 41, 514–520. doi: 10.1093/ije/dyr218

Miller, H. E., Leonard, S. A., Fox, K. A., Carusi, D. A., and Lyell, D. J. (2021). Placenta accreta spectrum among women with twin gestations. *Obstet. Gynecol.* 137, 132–138. doi: 10.1097/AOG.0000000000004204

Ming, L., Liu, P., Qiao, J., Lian, Y., Zheng, X., Ren, X., et al. (2012). Synchronization between embryo development and endometrium is a contributing factor for rescue ICSI outcome. *Reprod. Biomed. Online* 24, 527–531. doi: 10.1016/j.rbmo.2012.02.001

Nakamura, Y., Yaguchi, C., Itoh, H., Sakamoto, R., Kimura, T., Furuta, N., et al. (2015). Morphologic characteristics of the placental basal plate in in vitro fertilization pregnancies: a possible association with the amount of bleeding in delivery. *Hum. Pathol.* 46, 1171–1179. doi: 10.1016/j.humpath.2015.04.007

Qin, J., Liu, X., Sheng, X., Wang, H., and Gao, S. (2016). Assisted reproductive technology and the risk of pregnancy-related complications and adverse pregnancy outcomes in singleton pregnancies: a meta-analysis of cohort studies. *Fertil. Steril.* 105, 73.e–85.e. doi: 10.1016/j.fertnstert.2015.09.007

Simon, C., Domínguez, F., Valbuena, D., and Pellicer, A. (2003). The role of estrogen in uterine receptivity and blastocyst implantation. *Trends Endocrinol. Metab.* 14, 197–199. doi: 10.1016/s1043-2760(03)00084-5

Souvannavong-Vilivong, X., Sitticharoon, C., Klinjampa, R., Keadkraichaiwat, I., Sripong, C., Chatree, S., et al. (2019). Placental expressions and serum levels of adiponectin, visfatin, and omentin in GDM. *Acta Diabetol.* 56, 1121–1131. doi: 10.1007/s00592-019-01355-0

Tarín, J. J., García-Pérez, M. A., Hermenegildo, C., and Cano, A. (2014). Changes in sex ratio from fertilization to birth in assisted-reproductive-treatment cycles. *Reprod. Biol. Endocrinol.* 12:56. doi: 10.1186/1477-7827-12-56

Vahanian, S. A., Lavery, J. A., Ananth, C. V., and Vintzileos, A. (2015). Placental implantation abnormalities and risk of preterm delivery: a systematic review and metaanalysis. *Am. J. Obstet. Gynecol.* 213, S78–S90. doi: 10.1016/j.ajog.2015.05.058

Vannuccini, S., Ferrata, C., Perelli, F., Pinzauti, S., Severi, F. M., Reis, F. M., et al. (2018). Peripartum and postpartum outcomes in uncomplicated term pregnancy following ART: a retrospective cohort study from two Italian obstetric units. *Hum. Reprod. Open* 2018:hoy012. doi: 10.1093/hropen/hoy012

Ventura Laveriano, W. R., and Redondo, C. E. (2013). Obstetric outcomes in the second birth of women with a previous caesarean delivery: a retrospective cohort study from Peru. *Rev. Bras.*

*Ginecol. Obstet.* 35, 148–152. doi: 10.1590/s0100-72032013000400003

Yanaihara, A., Hatakeyama, S., Ohgi, S., Motomura, K., Taniguchi, R., Hirano, A., et al. (2018). Difference in the size of the placenta and umbilical cord between women with natural pregnancy and those with IVF pregnancy. *J. Assist. Reprod. Genet.* 35, 431–434. doi: 10.1007/s10815-017-1084-2

Zhang, N., Chen, H., Xu, Z., Wang, B., Sun, H., and Hu, Y. (2016). Pregnancy, delivery, and neonatal outcomes of in vitro fertilization-embryo transfer in patient with previous Cesarean scar. *Med. Sci. Monit.* 22, 3288–3295. doi: 10.12659/msm.900581

Zhao, L., Zheng, X., Liu, J., Zheng, R., Yang, R., Wang, Y., et al. (2019). The placental transcriptome of the first-trimester placenta is affected by in vitro fertilization and ET. *Reprod. Biol. Endocrinol.* 17:50. doi: 10.1186/s12958-019-0494-7

Zhu, L., Zhang, Y., Liu, Y., Zhang, R., Wu, Y., Huang, Y., et al. (2016). Maternal and Live-birth Outcomes of Pregnancies following Assisted Reproductive Technology: A Retrospective Cohort Study. *Sci. Rep.* 6:35141. doi: 10.1038/srep35141

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor declared a shared affiliation with two of the authors YL and FJ at time of review.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 17 February 2022
doi: 10.3389/fendo.2022.819963

# Non-Assisted Hatching Trophectoderm Biopsy Does Not Increase The Risks of Most Adverse Maternal and Neonatal Outcome and May Be More Practical for Busy Clinics: Evidence From China

*Shuo Li*[1,2,3,4,5], *Shuiying Ma*[1,4], *Jialin Zhao*[1,2,3,4,5], *Jingmei Hu*[1,2,3,4,5], *Hongchang Li*[1,4], *Yueting Zhu*[1,4], *Wenjie Jiang*[1,4], *Linlin Cui*[1,2,3,4,5*], *Junhao Yan*[1,2,3,4,5*] *and Zi-Jiang Chen*[1,2,3,4,5,6,7]

[1] *Center for Reproductive Medicine, Cheeloo College of Medicine, Shandong University, Jinan, China,* [2] *Key Laboratory of Reproductive Endocrinology of Ministry of Education, Shandong University, Jinan, China,* [3] *Shandong Key Laboratory of Reproductive Medicine, Shandong University, Jinan, China,* [4] *Shandong Provincial Clinical Research Center for Reproductive Health, Shandong University, Jinan, China,* [5] *National Research Center for Assisted Reproductive Technology and Reproductive Genetics, Shandong University, Jinan, China,* [6] *Shanghai Key Laboratory for Assisted Reproduction and Reproductive Genetics, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China,* [7] *Center for Reproductive Medicine, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China*

**Objective:** This study was conducted in order to investigate whether non-assisted hatching trophectoderm (TE) biopsy increases the risks of adverse perinatal outcomes in livebirths following elective single cryopreserved-thawed blastocyst transfer.

**Patients and Methods:** A total of 5,412 cycles from 4,908 women who achieved singleton livebirths between 2013 and 2019 were included in this retrospective cohort study. All embryos in this study were fertilized by intracytoplasmic sperm injection (ICSI) and cryopreserved through vitrification. The main intervention is to open the zona pellucida (ZP) of day 5/6 blastocyst immediately for biopsy without pre-assisted hatching. The main outcome measures are the common maternal and neonatal outcomes, including hypertensive disorders of pregnancy (HDPs), gestational diabetes mellitus (GDM), abnormal placentation, abnormalities in umbilical cord and amniotic fluid, preterm birth, cesarean section, low birth weight, postpartum hemorrhage, and prolonged hospital stay (both mothers and infants). The generalized estimation equation (GEE) was used to control the effects of repeated measurements. The non-conditional logistic regression model was used to examine the associations between embryo biopsy status and each adverse perinatal event. Given that the selection bias and changes in learning curve might affect the results, we selected 1,086 similar (matching tolerance = 0.01) cycles from the ICSI group *via* propensity score matching (PSM) for second comparisons and adjustment (conditional logistic regression).

OPEN ACCESS

**Edited by:**
*Yimin Zhu,*
*Zhejiang University, China*

**Reviewed by:**
*Zhiqin Bu,*
*Zhengzhou University, China*
*Zhexin Ni,*
*Second Military Medical University, China*

***Correspondence:**
*Junhao Yan*
*yyy306@126.com*
*Linlin Cui*
*fdclear3@126.com*

**Specialty section:**
*This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology*

**Received:** *22 November 2021*
**Accepted:** *13 January 2022*
**Published:** *17 February 2022*

**Citation:**
*Li S, Ma S, Zhao J, Hu J, Li H, Zhu Y, Jiang W, Cui L, Yan J and Chen Z-J (2022) Non-Assisted Hatching Trophectoderm Biopsy Does Not Increase The Risks of Most Adverse Maternal and Neonatal Outcome and May Be More Practical for Busy Clinics: Evidence From China. Front. Endocrinol. 13:819963. doi: 10.3389/fendo.2022.819963*

**Results:** After adjusting for confounders, we confirmed that the non-assisted hatching protocol did not increase the risks of most adverse maternal and neonatal outcomes. Despite this, there were increased risks of GDM (aOR: 1.522, 95% CI: 1.141–2.031) and umbilical cord abnormalities (aOR: 11.539, 95% CI: 1.199–111.067) in the biopsy group. In the second comparisons after PSM, GDM incidence in the biopsy group was still higher (7.26% vs. 5.16%, $P = 0.042$), yet all measurement outcomes were equally likely to occur in both groups after the second adjustment.

**Conclusions:** The non-assisted hatching TE biopsy does not increase the risks of most adverse perinatal outcomes. However, there is a higher GDM incidence in the biopsy group, and this association warrants further study. Considering its safety and simplicity, the non-assisted hatching protocol has the potential to become the preferred option for TE biopsy, especially in busy clinics and IVF laboratories.

**Keywords: elective single-embryo transfer, gestational diabetes mellitus, non-assisted hatching trophectoderm biopsy, perinatal outcomes, preimplantation genetic testing**

## INTRODUCTION

Preimplantation genetic testing (PGT) is now a widely used tool, and its share of total assisted reproductive technology (ART) cycles nearly doubled in the USA and UK from 2014 to 2017 (1). Possible drivers of such use include the preference to have children after the age of 35, the clinical promotion of new sequencing techniques, the need to block hereditary diseases, and the rising number of unexplained infertilities. Compared with *in-vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI), embryo biopsy is the main technique in the PGT procedure and the most intrusive intervention for the embryo. The method to obtain 4–10 cells from the ectoderm of the blastocyst (5–7 days after fertilization) was defined as trophectoderm (TE) biopsy (2), which significantly reduced the proportion of biopsied cells in the total cell count of an embryo. Due to the little influence on developmental potential and high diagnostic accuracy (3), this gradually replaced the previous polar body (PB) biopsy and blastomere biopsy, becoming the mainstream approach in most centers.

Currently, there are three main strategies for TE biopsy: the day 3 and day 4 hatching-based strategies, the same-day hatching-based strategy, and the simultaneous zona pellucida (ZP) opening and TE cell retrieval strategy (4). The main difference resides on whether the ZP needs to be opened for pre-assisted hatching. The first two require a pre-drilled hole of about 5 μm in the ZP of cleavage/morula/blastula-stage embryos to allow TE cells to herniate for biopsy. The last one, the ZP of day 5/6/7 blastocyst, is open instantly before the biopsy; thus, it was also called the "day 5–7 sequential ZP opening and TE cells retrieval approach" (4). Protocols that require assisted hatching present several issues such as the risk of the early gap thinning the ZP and affecting the normal expansion of blastocyst (5) as it has been reported that laser opening at cleavage might reduce the development potential (6) and alter epigenetic modification (7) of embryos. Additionally, the component of the blastocyst cells that were hatched out might be uncontrollable, and if hatching starts from the inner cell mass (ICM), biopsy could only be performed once the blastocyst had completely hatched out. During this period, the embryos need to be observed more frequently, which not only prolonged the *in-vitro* exposure time of the embryos but also significantly increased the workloads of the embryologist. Finally, the timing required for hatching out is uncertain and the development speed for each embryo is not exactly in sync, requiring the operators to biopsy these embryos in batches. By contrast, the protocol without pre-assisted hatching does not require assisted laser drilling in advance; thus, the embryo can remain undisturbed until the blastocyst stage. Meanwhile, the blastocysts with different characteristics can be handled flexibly and the ICM can be clearly identified during the drilling and aspirating process. It is generally better to vitrify a collapsed blastocyst, and non-assisted hatching protocol exactly has the characteristic of easily inducing embryo collapse. Some reports suggest that cycles adopting the non-assisted hatching protocol achieved better pregnancy outcomes [blastocyst frozen rate (8), thawing survival rate/clinical pregnancy rate (9), and lower mosaic rate (10)], but some uncertainty remains regarding non-assisted hatching biopsy. Hatching-based protocols only require retrieval of cellular components outside the ZP leaving other embryonic components in the ZP less affected, particularly the ICM. Contrastingly, the pulling and aspiration movements are more intense during the non-assisted hatching biopsy, and the manipulations must be adapted to the characteristics of each blastocyst, sometimes requiring extra steps such as injection of culture medium (11).

The debate over the benefits and risks of PGT has persisted. Some studies reported that embryo biopsy did not increase the risks related to abnormal placentation, maternal complications, and neonatal adverse events (12–16). However, others claimed that embryo biopsy increased the incidence of pregnancy-induced hypertension (PIH) or preeclampsia (17, 18), preterm

birth (19), and small-term for gestational age of the baby (20). The differences in biopsied time, position, and method might explain the inconsistency of these observations. Moreover, some IVF pregnancies were also mixed in the control group of previous studies. Since the fertilization method was different from that of the PGT group, we believe the conclusions of these studies are still open to discussion. Some embryologists have proposed that the TE biopsy protocols can affect pregnancy outcomes (9, 10), but in previous studies made by clinicians, they either adopted based day-3 hatching protocol or did not control for protocol types.

The invasive activity and syncytial degree of TE during the early stages of pregnancy directly determined the structure and function of mature placenta in late pregnancy. Considering that abnormal placentation could lead to a series of adverse perinatal events and higher long-term risks of chronic diseases in women and their offspring with obstetric complication histories (21), it is necessary to clarify the association between TE biopsy and each adverse perinatal event. Our goal is to provide definitive evidence with a large sample size to assess the safety of non-assisted hatching TE biopsy and PGT.

# MATERIAL AND METHODS

## Study Populations

A total of 22,754 cryopreserved-thawed cycles that achieved livebirths at the Center for Reproductive Medicine, Shandong University from 2011 to 2019 were included in our screening range. After excluding IVF cycles, transfers of mosaic embryo, multiple pregnancies, PB and blastomere biopsy, hatching-based TE biopsy, and others (stillbirth, premature death, vanishing twin syndrome, multifetal pregnancy reduction surgery), 5,412 cycles from 4,908 women were included in this retrospective study (a detailed flowchart is shown in **Figure 1**). The subjects in the ICSI group ($n$ = 4,324) were transferred to a vitrified cryopreserved-thawed blastocyst fertilized by ICSI from 2013 to 2018. The subjects in the biopsy group ($n$ = 1,088) received a biopsied blastocyst following general PGT procedure from 2014 to 2019. To keep the fertilization method consistent, the couples in the ICSI group underwent ART mainly due to male factor infertility, which might cause the basic physical conditions of women in the ICSI group to be better than those of the biopsy group. The non-assisted hatching TE biopsy was used for all transfer cycles in the biopsy group, which eliminated the interference of biopsy protocols.

This study was approved by the Ethics Committee of Reproductive Medicine Center of Shandong University, and all participants consented to their information being used for scientific research anonymously.

## PGT Procedure

Controlled ovarian hyperstimulation (COH) was performed according to the clinical routines of our center, monitoring serum sex hormones and ultrasonography, and the dosage of gonadotropins was timely adjusted based on the response of the ovary. When at least two follicles reached 18 mm in diameter, exogenous recombinant human chorionic gonadotropin (rhCG) was injected and the oocytes were retrieved 24–36 h later. Oocytes that develop to metaphase II (MII) were fertilized by ICSI as previously described (8). Zygotes were cultured *in vitro* to the blastocyst stage (5–7 days) and cryopreserved through vitrification using the Mukaida protocol with cryoloop (22). For the embryos requiring PGT, the ZP was opened immediately and 4–10 TE cells were removed from high-quality blastocysts (>4BC) (23) through laser-mediated drilling (RI, England, Saturn Active) before freezing. TE cells were then rinsed three times in 1% polyvinyl pyrrolidone (PVP,

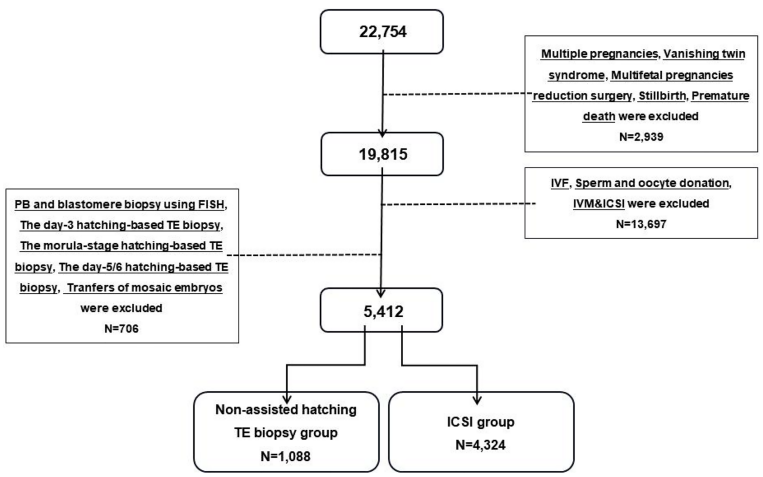



**FIGURE 1** | Flowchart of the study populations. IVF, *in-vitro* fertilization; ICSI, intracytoplasmic sperm injection; IVM, *in-vitro* maturation; PB, polar body; TE, trophectoderm.

Scandinavian IVF Science, Sweden, 10111) and enclosed into PCR tubes containing 2 μl phosphate-buffered saline (PBS, Solarbio, USA, P1020). The concrete experimental operation was conducted by equally skilled embryologists and the blastocysts were scored in accordance with the Gardner standards (24). **Figure 2** illustrates the common procedure for non-assisted hatching TE biopsy. DNA was extracted from the isolated blastula cells and amplified (SurePlex whole genome amplification kit, Illumina, San Diego, CA, USA) to meet the sample requirements for the subsequent next-generation sequencing (NGS) or comparative genomic hybridization (CGH). The genetic diagnoses were made by a panel of professional geneticists.

At the second (or later) menstrual cycles after oocyte retrieval, endometrial preparation was performed, and the clinicians decided on the most appropriate implantation protocol based on the endometrial status of the patients and the previous embryo transfer history. Only one qualified blastocyst was selected for transplantation based on Gardner scoring and the advice of geneticists. The data of maternal and neonatal outcomes were collected 42 days after childbirth through clinical medical records and telephone follow-up.

## Diagnostic Criteria

The diagnosis of polycystic ovarian syndrome (PCOS) was based on the consensus issued by the European Society of Human Reproduction and Embryology (ESHRE)/American Society for Reproductive Medicine (ASRM) in 2004 (25) and the Chinese Ministry of Health in 2011 (http://hbba.sacinfo.org.cn/stdDetail/78020832ca41940e1d0665507a75b539). The diagnoses of diabetes were based on the standard released by the American Diabetes Association (ADA) in 2010 (26). Thyroid disorders involved in this study included hyperthyroidism, hypothyroidism, chronic thyroiditis, and history of surgery or iodine treatment, the diagnoses of which were referred to the guideline recommended by the American Thyroid Association (ATA) in 2011 (27).

The diagnoses of HDPs were based on the guidelines issued by the American College of Obstetricians and Gynecologists (ACOG) in 2013 (28) and the Chinese Ministry of Health in 2012 (http://hbba.sacinfo.org.cn/stdDetail/d74604a6950738b4faf7e9ee34aa7b99). The diagnosis of gestational diabetes mellitus (GDM) was based on the criteria published by the International Association of Diabetes and Pregnancy Study Groups (IADPSG) in 2010 (29) and the Chinese Ministry of Health in 2011 (http://hbba.sacinfo.org.cn/stdDetail/97f630da575d4db3e9eee2e6ca3d1f45).

Other covariates were defined as follows: uterine congenital anomalies (infantile, unicornous, rudimentary horn, didelphic, bicornuate, arcuate), abnormal placentation (abruption, previa, increta, percreta, accreta), umbilical cord abnormalities (knot, torsion, polyp, vessel malformation), abnormal amniotic fluid (oligohydramnios, polyhydramnios, 3-degree contamination), preterm birth (<37 weeks), low birth weight (<2,500 g), postpartum hemorrhage (>500 ml for vaginal delivery or >1,000 ml for cesarean section), prolonged stay for mothers (>3 days for vaginal delivery or >5 days for cesarean section), and prolonged stay for infants (>3 days for vaginal delivery or >5 days for cesarean section among children >35 weeks of gestational age).

## Statistical Analyses

To avoid the influence of blood sampling time, the values of hCG were transformed to hCG ratio (serum hCG concentration/gestational day at sampling). Because one woman might contribute to multiple transfer cycles in this study, a generalized

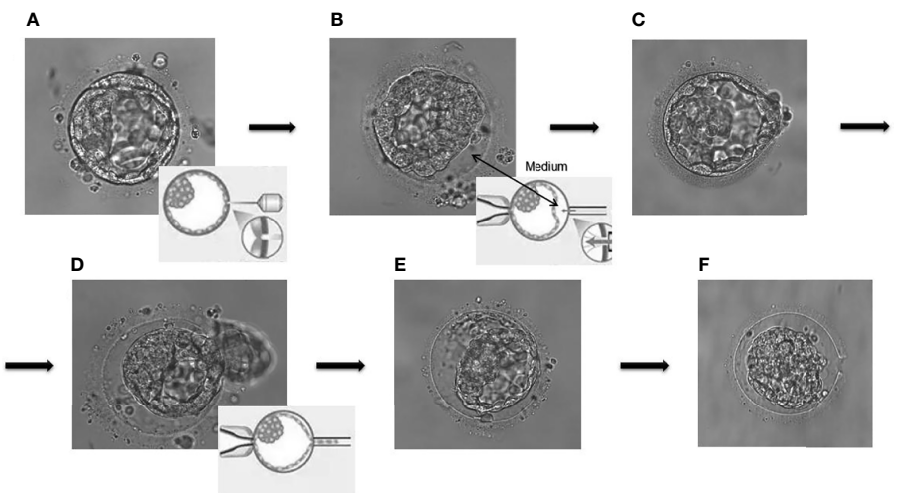



**FIGURE 2** | The common procedure for non-assisted hatching trophectoderm biopsy. **(A)** Laser drilling; **(B)** artificial separation by injection of culture medium; **(C, D)** pulling and suction; **(E, F)** the remaining blastocyst components collapsed in the zone pellucida.

estimation equation (GEE) was used to control the effects of repeated measurements. In GEE, the adjusted odds ratios (aORs) and 95% confidence intervals (CIs) derived from the non-conditional logistic regression model were used to describe the associations between embryo biopsy status and each adverse perinatal event. Considering the selection bias and the changes in learning curve might impact the results, we selected 1,086 similar (matching tolerance: 0.01) cycles from the ICSI group through propensity score matching (PSM) for second comparisons. The predictive variables included the maternal birth date, maternal age, maternal BMI, date of embryo biopsy, date of embryo transfer, date of delivery, parity, neonatal weight, and neonatal sex. A conditional logistic regression model was used for the second adjustment of the matched data.

Statistical tests were two-sided and *P*-value <0.05 was considered statistically significant. Due to the low percentage (<2%) of missing data, we supplemented the missing data through mean imputation. All analyses were conducted with software SPSS (version 25.0, Chicago, IL, USA).

## RESULTS

**Table 1** summarizes the demographic characteristics of subjects in the two groups. There were statistical differences in age (31 vs. 30, $P < 0.001$), BMI (22.66 vs. 22.27, $P < 0.001$), times of previous miscarriages ($P < 0.001$), parity ($P = 0.026$), endometrial preparation protocols ($P < 0.001$), endometrial thickness of transfer day (0.9 vs. 1.0, $P < 0.001$), and hCG ratio (56.36 vs. 60.53, $P < 0.001$) between the groups. As for maternal basic diseases, there were statistical differences in thyroid disorders (11.76% vs. 14.43%, $P = 0.023$), chronic hypertension (1.29% vs. 2.43%, $P = 0.022$), family history of hypertension (15.81% vs. 11.22%, $P < 0.001$), family history of diabetes (6.71% vs. 4.35%, $P = 0.001$), and history of uterine surgery (13.05% vs. 16.93%, $P = 0.002$) between the groups.

Comparisons of maternal and neonatal outcomes are shown in **Table 2**. Women in the biopsy group had a higher incidence of GDM (7.26% vs. 5.04%, $P = 0.004$) than those in the ICSI group. There were no statistical differences in PIH and preeclampsia ($P = 0.784$), preeclampsia with severe features and eclampsia ($P = 0.517$), HDP with GDM ($P = 0.608$), abnormal placentation ($P = 0.341$), umbilical cord abnormalities ($P = 0.059$), abnormal amniotic fluid ($P = 0.804$), preterm birth ($P = 0.809$), delivery mode ($P = 0.782$), neonatal sex ($P = 0.099$), low birth weight ($P = 0.372$), postpartum hemorrhage ($P = 0.310$), and prolonged stay for mothers ($P = 0.188$) and infants ($P = 0.103$).

Increased risks of GDM (aOR: 1.522, 95% CI: 1.414–2.031, $P = 0.004$) and umbilical cord abnormalities (aOR: 11.539, 95% CI: 1.199–111.067, $P = 0.034$) were observed in the biopsy group when using the ICSI group as the reference after adjusting for confounding factors such as maternal age, maternal BMI, parity, times of previous miscarriages, endometrial preparation protocols, endometrial thickness of transfer day, hCG ratio, PCOS, thyroid disorders, chronic hypertension, family history

**TABLE 1** | Comparisons of demographic characteristics between the non-assisted hatching biopsy group and the ICSI group.

| | Non-assisted hatching biopsy group (*n* = 1,088) | ICSI group (*n* = 4,324) | *P*-value |
|---|---|---|---|
| Age (years)* | 31 (3.5) | 30 (3) | <0.001[a] |
| BMI (kg/m²)* | 22.66 (2.23) | 22.27 (2.39) | 0.004[a] |
| Times of previous miscarriages* | None: 32.81% (357) | None: 76.48% (3,307) | <0.001[b] |
| | Once: 19.67% (214) | Once: 18.94% (819) | |
| | Twice or more: 47.52% (517) | Twice or more: 4.58% (198) | |
| Parity* | Primiparous: 72.24% (786) | Primiparous:75.51% (3,265) | 0.026[b] |
| | Multiparous: 27.76% (302) | Multiparous: 24.49% (1,059) | |
| Endometrial preparation protocols* | NC: 49.63% (540) | NC: 58.00% (2,508) | <0.001[b] |
| | HRT: 37.04% (403) | HRT: 31.06% (1,343) | |
| | OI: 11.58% (126) | OI: 9.53% (412) | |
| | Others: 1.75% (19) | Others: 1.41% (61) | |
| Endometrial thickness of transfer day (cm)* | 0.9 (0.1) | 1.0 (0.1) | <0.001[a] |
| Serum hCG level (IU/L)/gestational day at sampling (days)* | 56.36 (23.71) | 60.53 (24.22) | <0.001[a] |
| PCOS | 16.64% (181) | 17.55% (759) | 0.475[b] |
| Uterine congenital anomalies | 2.21% (24) | 1.50% (65) | 0.103[b] |
| Untreated uterine fibroid | 3.49% (38) | 3.45% (149) | 0.940[b] |
| Thyroid disorders* | 11.76% (128) | 14.43% (624) | 0.023[b] |
| Chronic hypertension* | 1.29% (14) | 2.43% (105) | 0.022[b] |
| Family history of hypertension* | 15.81% (172) | 11.22% (485) | <0.001[b] |
| Type 1 or type 2 diabetes | 3.68% (40) | 2.59% (112) | 0.053[b] |
| Family history of diabetes* | 6.71% (73) | 4.35% (188) | 0.001[b] |
| History of uterine surgery* | 13.05% (142) | 16.93% (732) | 0.002[b] |

*Data are shown as median (quartile deviation, QD) and (%) (number of positive cases).*

*ICSI, intracytoplasmic sperm injection; BMI, body mass index; NC, nature cycle; HRT, hormone replacement treatment; OI, ovulation induction; hCG, human chorionic gonadotropin; PCOS, polycystic ovarian syndrome.*

[a]*Mann–Whitney U test.*

[b]*Pearson's chi-squared test.*

**Statistically significant.*

**TABLE 2 |** Comparisons of maternal and neonatal outcomes between the non-assisted hatching biopsy group and the ICSI group.

| | Non-assisted hatching biopsy group (*n* = 1,088) | ICSI group (*n* = 4,324) | *P*-value |
|---|---|---|---|
| PIH + preeclampsia | 4.96% (54) | 4.76% (206) | 0.784[a] |
| Preeclampsia with severe features + eclampsia | 0.64% (7) | 0.49% (21) | 0.517[a] |
| GDM* | 7.26% (79) | 5.04% (218) | 0.004[a] |
| HDP + GDM | 0.55% (6) | 0.42% (18) | 0.608[b] |
| Abnormal placentation | 1.38% (15) | 1.04% (45) | 0.341[a] |
| Umbilical cord abnormalities | 0.28% (3) | 0.05% (2) | 0.059[b] |
| Abnormal amniotic fluid | 1.10% (12) | 1.02% (44) | 0.804[a] |
| Preterm birth | 6.43% (70) | 6.64% (287) | 0.809[a] |
| Delivery mode | Vaginal: 28.03% (305) | Vaginal: 27.61% (1,194) | 0.782[a] |
| | Abdominal: 71.97% (783) | Abdominal: 72.39% (3,130) | |
| Neonatal sex ratio | Female: 46.78% (509) | Female: 49.58% (2,144) | 0.099[a] |
| | Male: 53.22% (579) | Male: 50.42% (2,180) | |
| Low birth weight | 4.50% (49) | 3.91% (169) | 0.372[a] |
| Postpartum hemorrhage | 0.37% (4) | 0.21% (9) | 0.310[b] |
| Prolonged stay for mothers | 1.93% (21) | 1.39% (60) | 0.188[a] |
| Prolonged stay for infants | 5.61% (61) | 4.44% (192) | 0.103[a] |

*Data are shown as (%) (number of positive cases).*

*ICSI, intracytoplasmic sperm injection; PIH, pregnancy-induced hypertension; GDM, gestational diabetes; HDP, hypertensive disorders of pregnancy.*

[a]*Pearson's chi-squared test.*

[b]*Fisher's exact test.*

**Statistically significant.*

of hypertension, diabetes, family history of diabetes, history of uterine surgery, and neonatal sex. The remaining 11 outcome variables did not show increased risks (**Figure 3**, see **Supplementary Table 1** for details).

After PSM, the results of the second comparisons are shown in **Table 3**. Because the positive cases of umbilical cord abnormalities and postpartum hemorrhage in the matched ICSI group were zero, we were unable to compare and adjust these two outcome variables. Compared with the counterparts in the matched ICSI group, there were still more women with GDM in the biopsy group (7.26% vs. 5.16%, $P = 0.042$). **Figure 4** (details are available in **Supplementary Table 3**) displays the results from the conditional logistic regression model that none of the 11 perinatal outcomes in the biopsy group showed an additional risk.

## DISCUSSION

Our study demonstrates the safety of non-assisted hatching TE biopsy and provides a reference for embryologists when selecting protocols for TE biopsy. Due to its simplicity and fewer effects on embryo development potential, non-assisted hatching TE biopsy might be a better option for the busy IVF laboratories with large PGT cycles or countries with large populations.

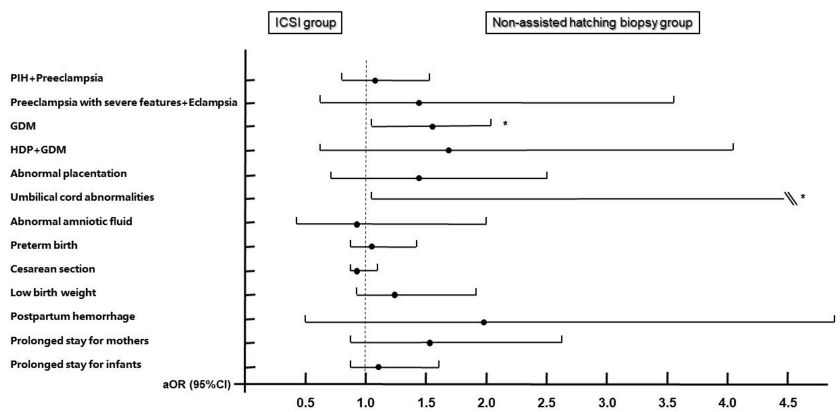



**FIGURE 3** | Adjusted odds ratios and 95% confidence intervals for maternal and neonatal outcomes by embryo biopsy status. Adjusted for maternal age, maternal BMI, times of previous miscarriages, parity, endometrial preparation protocols, endometrial thickness of transfer day, hCG ratio, PCOS, thyroid disorders, chronic hypertension, family history of hypertension, diabetes, family history of diabetes, history of uterine surgery, and neonatal sex. ICSI group is the reference group. PIH, pregnancy-induced hypertension; GDM, gestational diabetes; HDP, hypertensive disorders of pregnancy. * Statistically significant.

**TABLE 3 |** Comparisons of maternal and neonatal outcomes between the non-assisted hatching biopsy group and the matched ICSI group.

| | Non-assisted hatching biopsy group (*n* = 1,088) | Matched ICSI group (*n* = 1,086) | *P*-value |
|---|---|---|---|
| PIH + preeclampsia | 4.96% (54) | 4.88% (53) | 0.929[a] |
| Preeclampsia with severe features + eclampsia | 0.64% (7) | 0.55% (6) | 0.783[a] |
| GDM* | 7.26% (79) | 5.16% (56) | 0.042[a] |
| HDP + GDM | 0.55% (6) | 0.37% (4) | 0.753[b] |
| Abnormal placentation | 1.38% (15) | 1.29% (14) | 0.856[a] |
| Umbilical cord abnormalities | 0.28% (3) | 0% (0) | – |
| Abnormal amniotic fluid | 1.10% (12) | 0.64% (7) | 0.251[a] |
| Preterm birth | 6.43% (70) | 6.54% (71) | 0.922[a] |
| Delivery mode | Vaginal: 28.03% (305)<br>Abdominal: 71.97% (783) | Vaginal: 24.59% (267)<br>Abdominal: 75.41% (819) | 0.068[a] |
| Neonatal sex ratio | Female: 46.78% (509)<br>Male: 53.22% (579) | Female: 43.46% (472)<br>Male: 56.54% (614) | 0.120[a] |
| Low birth weight | 4.50% (49) | 3.22% (35) | 0.121[a] |
| Postpartum hemorrhage | 0.37% (4) | 0% (0) | – |
| Prolonged stay for mothers | 1.93% (21) | 1.20% (13) | 0.168[a] |
| Prolonged stay for infants | 5.61% (61) | 3.96% (43) | 0.072[a] |

*Data are shown as (%) (number of positive cases).*

*ICSI, intracytoplasmic sperm injection; PIH, pregnancy-induced hypertension; GDM, gestational diabetes; HDP, hypertensive disorders of pregnancy.*

[a]*Pearson's chi-squared test.*

[b]*Fisher's exact test.*

**Statistically significant.*

Our conclusions are consistent with that of Swanson and her colleagues (30). In their previous study, PGT was not associated with the risks of most perinatal adverse events but GDM. However, this was based on IVF pregnancies in the control group and neglected the effects of TE biopsy protocols. With a much larger sample size and more controlled and homogeneous groups, our study reinforced these findings.

Except for the GDM incidence, our results are in general agreement with the conclusions by Sites et al. (16). They aggregated the perinatal data of ART cycles at multiple clinics through the state health system and concluded that embryo biopsy for PGT did not increase the odds for diagnoses related to abnormal placentation, maternal complications (including GDM), and prolonged stay (both mothers and infants). Similar to our study, to allow the roles of biopsy to be more clearly apparent, only singleton livebirths were included in their study, but some IVF cycles were also incorporated in their control group, and there was no adjustment for fertilization methods and biopsy protocol types. The applicable indications of PGT in east USA and the selection for biopsy protocols between different IVF laboratories might explain the difference in our observations. Moreover, our study included the data of endometrial preparations, a significant limitation they mentioned in their study. Another meta-analysis (31) reported 785,445 participants from different countries enrolled over an 11-year period and found a lower rate of "very low birth weight" and "cesarean section" and a higher rate of "preterm birth" and "intrauterine growth retardation" in PGT pregnancies, compared with those of

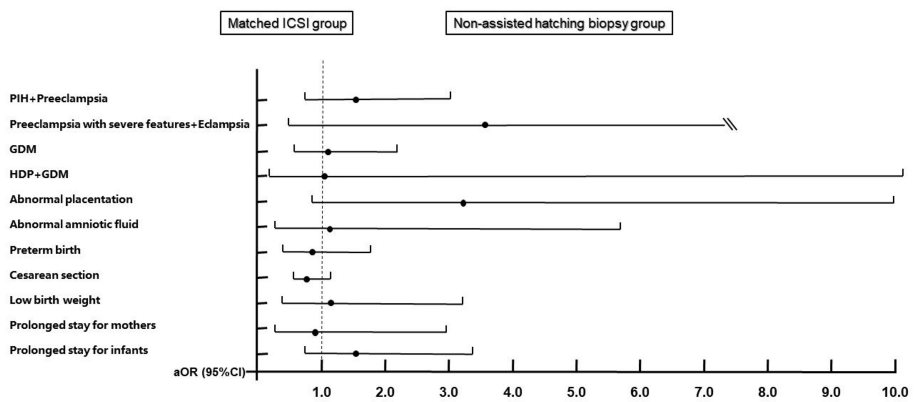



**FIGURE 4** | Adjusted odds ratios and 95% confidence intervals for maternal and neonatal outcomes after matching. Adjusted for maternal age, maternal BMI, times of previous miscarriages, parity, endometrial preparation protocols, endometrial thickness of transfer day, hCG ratio, PCOS, thyroid disorders, chronic hypertension, family history of hypertension, diabetes, family history of diabetes, and history of uterine surgery. Matched ICSI group is the reference group. PIH, pregnancy-induced hypertension; GDM, gestational diabetes; HDP, hypertensive disorders of pregnancy.

IVF/ICSI pregnancies. Over such a longtime period and with such a large sample size, some associations can simply be due to chance. Non-standardization of experimental procedures and changes in learning curve could both impact the final conclusions. The differences in race and region might also be another confounding factor.

Some scholars advocated that PGT was associated with the risk of preeclampsia. Zhang et al. found that PGT increased the risk of preeclampsia (17); however, their report was based on a small sample size and included multiple pregnancies that might account for the discrepancy with our report. Makhijani et al. (18) also only included singleton births, but they adjusted the number of embryos transferred as a confounding factor, suggesting the cycles in their study were not all elective single-embryo transfers (eSET). Additionally, the biopsy protocols between us differed, and they adopted the day 3 hatching-based TE biopsy. A randomized controlled trial (RCT) has already shown that frozen-thawed embryo transfers had higher risks of preeclampsia (32). Considering the embryos in the PGT cycle are almost frozen-thawed, it is necessary to clarify whether the increased risk of preeclampsia is caused by freeze-thaw or biopsy. A reasonable physiological explanation for increased preeclampsia risk was not described in previous studies. Sunkara et al. (14) and Li et al. (19) concluded that PGT was not associated with adverse neonatal outcomes (though Sunkara found a slightly higher risk of preterm birth in the PGT group), which were basically consistent with our findings with neonates.

By comparison, each of the three biopsy strategies has its own advantages and disadvantages. However, limited by several disadvantages we mentioned in the *Introduction*, the hatching-based strategies are not particularly practical for some large clinics or IVF labs (2), especially in countries with a large population such as China or India. The changes in the population policy of the Chinese government increased the pressure over IVF laboratories with a large number of cycles, and many laboratories have gradually abandoned the hatching-based protocols which are more time-consuming and require a constant check. Nonetheless, this is only the choice under the stress of workloads, not based on evidence. As Rubino et al. (9) proposed, it is high time to focus on the blastocyst biopsy protocols. Because clinicians are not the ones performing the biopsy, the impact of different protocols for TE biopsy on the clinical outcomes is often ignored in their previous studies. Similarly, the evidence provided by embryologists focuses mostly on embryo quality and laboratory parameters, rarely involving maternal and neonatal conditions throughout the perinatal period. Because the biopsy protocol was well controlled, our research makes a powerful complement to previous observations.

In terms of the increased risk of GDM, we believe it could be attributed to many reasons. Lower hCG ratio in the biopsy group at the first gestational month might play an important part in the development of GDM (33). hCG can lead to thyroid-stimulating hormone (TSH) activity and induce free thyroxine (FT4) surge (34, 35), facilitate early placentation to indirectly affect insulin resistance (IR) derived by placental endocrine (36), and play as an immune modulator to alleviate pancreatic autoimmunity (37). Liu et al. (38) demonstrated that higher hCG levels in early pregnancy were associated with a lower risk of GDM and maternal FT4 which may act as an important mediator (24%) in this association. While some scholars (39, 40) claim that TE biopsy might reduce the level of serum beta-hCG in early pregnancies, this is still controversial (41). In our study, hCG values were measured within 1 month of embryo transfer; at a stage when serum hCG concentration doubles rapidly, we were unable to convert the concentration values into the median of multiples (MoMs) and perform further mediation analysis. Furthermore, it was not clear whether this decrease in hCG levels occurred only in the first month of pregnancy or continued into the first trimester. Future studies will collect longitudinal serum samples for hCG, FT4, and TSH measurement, to assess the difference between the biopsy group and the no-biopsy group. Secondly, considering chromosomal abnormality is one of the indications for PGT, we thought the couples in the biopsy group had more complex genetic backgrounds, such as translocation or inversion, than their counterparts in the ICSI group. Numerous single nucleotide polymorphisms (SNPs) in susceptibility genes associated with both glucose metabolism and placental development, such as ADIPOQ (42), IL1B (43, 44), and ABCC8 (45), might go undetected due to the absence of carrier screening (20). Thirdly, the percentage of Asian women (46) included in our study might also contribute to some discrepancies with previous studies. The risk of GDM is significantly higher in Asian women, whose BMI was lower than that of women in the general population. In contrast, these were the least likely ethnic group to receive recommended diabetes screening (47–49). In fact, this might explain why our results align with the study of Swanson et al. (30) which also included a high percentage of Asians.

However, our study presents several limitations. The particularity of PGT populations leads to significant demographic differences which cannot be controlled outside of an RCT. Besides, some data of perinatal outcomes were obtained through telephone follow-up, which inevitably resulted in recall bias. Furthermore, we could not include HDP or GDM history as some patients with childbearing histories forgot the details of their last obstetric experiences.

Overall, our study demonstrated that despite the increased risk of GDM, non-assisted hatching TE biopsy is not associated with the increased risks of HDPs, abnormal placentation, preterm birth, postpartum hemorrhage, and prolonged stay (both mothers and infants), compared with ICSI pregnancies. Despite the findings of our study, further study and validation need to be conducted.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Center for Reproductive Medicine, Shandong University. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

JY and Z-JC conceived and designed this study. SL conducted the data collection and analyses and wrote the manuscript. LC revised the manuscript and the other authors reviewed the manuscript. All authors were involved in interpreting the data and approved the final version.

## FUNDING

This study was generously funded by the Ministry of Science and Technology, PRC (2021YFC2700604, 2018YFC1002804).

## ACKNOWLEDGMENTS

We appreciated Jingfu Yang for his invaluable help in the export of electronic medical records.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.819963/full#supplementary-material

## REFERENCES

1. Theobald R, SenGupta S, Harper J. The Status of Preimplantation Genetic Testing in the UK and USA. *Hum Reprod* (2020) 35:986–98. doi: 10.1093/humrep/deaa034
2. Cimadomo D, Rienzi L, Capalbo A, Rubio C, Innocenti F, García-Pascual CM, et al. The Dawn of the Future: 30 Years From the First Biopsy of a Human Embryo. The Detailed History of an Ongoing Revolution. *Hum Reprod Update* (2020) 26:453–73. doi: 10.1093/humupd/dmaa019
3. Scott RT, Upham KM, Forman EJ, Zhao T, Treff NR. Cleavage-Stage Biopsy Significantly Impairs Human Embryonic Implantation Potential While Blastocyst Biopsy Does Not: A Randomized and Paired Clinical Trial. *Fertil Steril* (2013) 100:624–30. doi: 10.1016/j.fertnstert.2013.04.039
4. ESHRE PGT Consortium and SIG-Embryology Biopsy Working Group, Kokkali G, Coticchio G, Bronet F, Celebi C, Cimadomo D, et al. ESHRE PGT Consortium and SIG Embryology Good Practice Recommendations for Polar Body and Embryo Biopsy for PGT. *Hum Reprod Open* (2020) 2020: hoaa020. doi: 10.1093/hropen/hoaa020
5. Davidson LM, Liu Y, Griffiths T, Jones C, Coward K. Laser Technology in the ART Laboratory: A Narrative Review. *Reprod BioMed Online* (2019) 38:725–39. doi: 10.1016/j.rbmo.2018.12.011
6. Honguntikar SD, Uppangala S, Salian SR, Kalthur G, Kumar P, Adiga SK. Laser-Assisted Hatching of Cleavage-Stage Embryos Impairs Developmental Potential and Increases DNA Damage in Blastocysts. *Lasers Med Sci* (2015) 30:95–101. doi: 10.1007/s10103-014-1625-1
7. Honguntikar SD, Salian SR, D'Souza F, Uppangala S, Kalthur G, Adiga SK. Epigenetic Changes in Preimplantation Embryos Subjected to Laser Manipulation. *Lasers Med Sci* (2017) 32:2081–7. doi: 10.1007/s10103-017-2334-3
8. Zhao H, Tao W, Li M, Liu H, Wu K, Ma S. Comparison of Two Protocols of Blastocyst Biopsy Submitted to Preimplantation Genetic Testing for Aneuploidies: A Randomized Controlled Trial. *Arch Gynecol Obstet* (2019) 299:1487–93. doi: 10.1007/s00404-019-05084-1
9. Rubino P, Tapia L, Ruiz de Assin Alonso R, Mazmanian K, Guan L, Dearden L, et al. Trophectoderm Biopsy Protocols can Affect Clinical Outcomes: Time to Focus on the Blastocyst Biopsy Technique. *Fertil Steril* (2020) 113:981–9. doi: 10.1016/j.fertnstert.2019.12.034
10. Xiong S, Liu W, Wang J, Liu J, Gao Y, Wu L, et al. Trophectoderm Biopsy Protocols may Impact the Rate of Mosaic Blastocysts in Cycles With Pre-Implantation Genetic Testing for Aneuploidy. *J Assist Reprod Genet* (2021) 38:1153–62. doi: 10.1007/s10815-021-02137-w
11. Capalbo A, Rienzi L, Cimadomo D, Maggiulli R, Elliott T, Wright G, et al. Correlation Between Standard Blastocyst Morphology, Euploidy and Implantation: An Observational Study in Two Centers Involving 956 Screened Blastocysts. *Hum Reprod* (2014) 29:1173–81. doi: 10.1093/humrep/deu033
12. Eldar-Geva T, Srebnik N, Altarescu G, Varshaver I, Brooks B, Levy-Lahad E, et al. Neonatal Outcome After Preimplantation Genetic Diagnosis. *Fertil Steril* (2014) 102:1016–21. doi: 10.1016/j.fertnstert.2014.06.023
13. Hasson J, Limoni D, Malcov M, Frumkin T, Amir H, Shavit T, et al. Obstetric and Neonatal Outcomes of Pregnancies Conceived After Preimplantation Genetic Diagnosis: Cohort Study and Meta-Analysis. *Reprod BioMed Online* (2017) 35:208–18. doi: 10.1016/j.rbmo.2017.05.003
14. Sunkara SK, Antonisamy B, Selliah HY, Kamath MS. Pre-Term Birth and Low Birth Weight Following Preimplantation Genetic Diagnosis: Analysis of 88 010 Singleton Live Births Following PGD and IVF Cycles. *Hum Reprod* (2017) 32:432–8. doi: 10.1093/humrep/dew317
15. Sites C, Bachilova S, Gopal D, Cabral H, Coddington C, Stern J. Associations Between Embryo Biopsy and Obstetrical and Neonatal Outcomes Following Frozen-Thawed Single Embryo Transfer. *Fertil Steril* (2020) 114:e151. doi: 10.1016/j.fertnstert.2020.08.437
16. Sites CK, Bachilova S, Gopal D, Cabral HJ, Coddington CC, Stern JE. Embryo Biopsy and Maternal and Neonatal Outcomes Following Cryopreserved-Thawed Single Embryo Transfer. *Am J Obstet Gynecol* (2021) 225:285.e1–285.e7. doi: 10.1016/j.ajog.2021.04.235
17. Zhang WY, von Versen-Höynck F, Kapphahn KI, Fleischmann RR, Zhao Q, Baker VL. Maternal and Neonatal Outcomes Associated With Trophectoderm Biopsy. *Fertil Steril* (2019) 112:283–90.e2. doi: 10.1016/j.fertnstert.2019.03.033
18. Makhijani R, Bartels CB, Godiwala P, Bartolucci A, DiLuigi A, Nulsen J, et al. Impact of Trophectoderm Biopsy on Obstetric and Perinatal Outcomes Following Frozen-Thawed Embryo Transfer Cycles. *Hum Reprod* (2021) 36:340–8. doi: 10.1093/humrep/deaa316
19. Li M, Kort J, Baker VL. Embryo Biopsy and Perinatal Outcomes of Singleton Pregnancies: An Analysis of 16,246 Frozen Embryo Transfer Cycles Reported in the Society for Assisted Reproductive Technology Clinical Outcomes Reporting System. *Am J Obstet Gynecol* (2021) 224:500.e1-500.e18. doi: 10.1016/j.ajog.2020.10.043
20. Feldman B, Orvieto R, Weisel M, Aizer A, Meyer R, Haas J, et al. Obstetric and Perinatal Outcomes in Pregnancies Conceived After Preimplantation Genetic Testing for Monogenetic Diseases. *Obstet Gynecol* (2020) 136:782–91. doi: 10.1097/AOG.0000000000004062
21. Burton GJ, Fowden AL, Thornburg KL. Placental Origins of Chronic Disease. *Physiol Rev* (2016) 96:1509–65. doi: 10.1152/physrev.00029.2015
22. Mukaida T, Nakamura S, Tomiyama T, Wada S, Kasai M, Takahashi K. Successful Birth After Transfer of Vitrified Human Blastocysts With Use of a Cryoloop Containerless Technique. *Fertil Steril* (2001) 76(3):618–20. doi: 10.1016/s0015-0282(01)01968-9
23. Puissant F, Van Rysselberge M, Barlow P, Deweze J, Leroy F. Embryo Scoring as a Prognostic Tool in IVF Treatment. *Hum Reprod* (1987) 2:705–8. doi: 10.1093/oxfordjournals.humrep.a136618
24. Gardner DK, Lane M, Stevens J, Schlenker T, Schoolcraft WB. Reprint of: Blastocyst Score Affects Implantation and Pregnancy Outcome: Towards a Single Blastocyst Transfer. *Fertil Steril* (2019) 112:e81–4. doi: 10.1016/j.fertnstert.2019.08.077

25. Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group. Revised 2003 Consensus on Diagnostic Criteria and Long-Term Health Risks Related to Polycystic Ovary Syndrome (PCOS). *Hum Reprod* (2004) 19:41–7. doi: 10.1093/humrep/deh098
26. American Diabetes Association. Standards of Medical Care in Diabetes–2010. *Diabetes Care* (2010) 33:S11–61. doi: 10.2337/dc10-S011
27. Stagnaro-Green A, Abalovich M, Alexander E, Azizi F, Mestman J, Negro R, et al. Guidelines of the American Thyroid Association for the Diagnosis and Management of Thyroid Disease During Pregnancy and Postpartum. *Thyroid* (2011) 21:1081–125. doi: 10.1089/thy.2011.0087
28. Hypertension in pregnancy. Report of the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy. *Obstet Gynecol* (2013) 122:1122–31. doi: 10.1097/01.AOG.0000437382.03963.88
29. International Association of Diabetes and Pregnancy Study Groups. Recommendations on the Diagnosis and Classification of Hyperglycemia in Pregnancy. *Diabetes Care* (2010) 33:676–82. doi: 10.2337/dc09-1848
30. Swanson K, Wijekoon A, Huang D, Rosenstein M, Blat C, Mok-Lin E, et al. 197: Does Preimplantation Genetic Testing Increase the Risk of Abnormal Placentation in IVF Pregnancies? *Am J Obstetr Gynecol* (2020) 222:S136–7. doi: 10.1016/j.ajog.2019.11.213
31. Hou W, Shi G, Ma Y, Liu Y, Lu M, Fan X, et al. Impact of Preimplantation Genetic Testing on Obstetric and Neonatal Outcomes: A Systematic Review and Meta-Analysis. *Fertil Steril* (2021) 116:990–1000. doi: 10.1016/j.fertnstert.2021.06.040
32. Wei D, Liu J-Y, Sun Y, Shi Y, Zhang B, Liu J-Q, et al. Frozen Versus Fresh Single Blastocyst Transfer in Ovulatory Women: A Multicentre, Randomised Controlled Trial. *Lancet* (2019) 393:1310–8. doi: 10.1016/S0140-6736(18)32843-5
33. Donovan BM, Nidey NL, Jasper EA, Robinson JG, Bao W, Saftlas AF, et al. First Trimester Prenatal Screening Biomarkers and Gestational Diabetes Mellitus: A Systematic Review and Meta-Analysis. *PLoS One* (2018) 13:e0201319. doi: 10.1371/journal.pone.0201319
34. Yang S, Shi F-T, Leung PCK, Huang H-F, Fan J. Low Thyroid Hormone in Early Pregnancy Is Associated With an Increased Risk of Gestational Diabetes Mellitus. *J Clin Endocrinol Metab* (2016) 101:4237–43. doi: 10.1210/jc.2016-1506
35. Korevaar TIM, Medici M, Visser TJ, Peeters RP. Thyroid Disease in Pregnancy: New Insights in Diagnosis and Clinical Management. *Nat Rev Endocrinol* (2017) 13:610–22. doi: 10.1038/nrendo.2017.93
36. Desoye G, Shafrir E. Placental Metabolism and Its Regulation in Health and Diabetes. *Mol Aspects Med* (1994) 15:505–682. doi: 10.1016/0098-2997(94)90004-3
37. Khil L-Y, Jun H-S, Kwon H, Yoo JK, Kim S, Notkins AL, et al. Human Chorionic Gonadotropin Is an Immune Modulator and can Prevent Autoimmune Diabetes in NOD Mice. *Diabetologia* (2007) 50:2147–55. doi: 10.1007/s00125-007-0769-y
38. Liu Y, Guo F, Maraka S, Zhang Y, Zhang C, Korevaar TIM, et al. Associations Between Human Chorionic Gonadotropin, Maternal Free Thyroxine, and Gestational Diabetes Mellitus. *Thyroid* (2021) 31:1282–8. doi: 10.1089/thy.2020.0920
39. Lu M-M, Wen Y-X, Liu Y-L, Ding C-H, Zhou C-Q, Xu Y-W. Trophectoderm Biopsy Reduces the Level of Serum β-Human Chorionic Gonadotropin in Early Pregnancy. *Fertil Steril* (2020) 114:801–8. doi: 10.1016/j.fertnstert.2020.05.015
40. Gordon CE, Racowsky C. Trophectoderm Biopsy-Perhaps Not Such a Benign Intervention. *Fertil Steril* (2020) 114:748–9. doi: 10.1016/j.fertnstert.2020.06.027
41. Wu Y, Ying Y, Cao M, Liu J, Liu H. Trophectoderm Biopsy of Blastocysts for a Preimplantation Genetic Test Does Not Affect Serum β-hCG Levels in Early Pregnancy: A Study Using Propensity Score Matching. *J Ovarian Res* (2021) 14:78. doi: 10.1186/s13048-021-00824-x
42. Dendana M, Bahia W, Finan RR, Al-Mutawa M, Almawi WY. Association of Adiponectin Gene Variants With Idiopathic Recurrent Miscarriage According to Obesity Status: A Case-Control Study. *J Transl Med* (2018) 16:76. doi: 10.1186/s12967-018-1453-3
43. Stefanidis I, Kreuer K, Dardiotis E, Arampatzis S, Eleftheriadis T, Hadjigeorgiou GM, et al. Association Between the Interleukin-1β Gene (IL1B) C-511T Polymorphism and the Risk of Diabetic Nephropathy in Type 2 Diabetes: A Candidate-Gene Association Study. *DNA Cell Biol* (2014) 33:463–8. doi: 10.1089/dna.2013.2204
44. Ma J, Zhang X, He G, Yang C. Association Between TNF, IL1B, IL6, IL10 and IFNG Polymorphisms and Recurrent Miscarriage: A Case Control Study. *Reprod Biol Endocrinol* (2017) 15:83. doi: 10.1186/s12958-017-0300-3
45. Hoffman TL, Blanco E, Lane A, Galvin-Parton P, Gadi I, Santer R, et al. Glucose Metabolism and Insulin Secretion in a Patient With ABCC8 Mutation and Fanconi-Bickel Syndrome Caused by Maternal Isodisomy of Chromosome 3. *Clin Genet* (2007) 71:551–7. doi: 10.1111/j.1399-0004.2007.00802.x
46. Spracklen CN, Horikoshi M, Kim YJ, Lin K, Bragg F, Moon S, et al. Identification of Type 2 Diabetes Loci in 433,540 East Asian Individuals. *Nature* (2020) 582:240–5. doi: 10.1038/s41586-020-2263-3
47. Tung EL, Baig AA, Huang ES, Laiteerapong N, Chua K-P. Racial and Ethnic Disparities in Diabetes Screening Between Asian Americans and Other Adults: BRFSS 2012-2014. *J Gen Intern Med* (2017) 32:423–9. doi: 10.1007/s11606-016-3913-x
48. Chi C, Loy SL, Chan S-Y, Choong C, Cai S, Soh SE, et al. Impact of Adopting the 2013 World Health Organization Criteria for Diagnosis of Gestational Diabetes in a Multi-Ethnic Asian Cohort: A Prospective Study. *BMC Pregnancy Childbirth* (2018) 18:69. doi: 10.1186/s12884-018-1707-3
49. Read SH, Rosella LC, Berger H, Feig DS, Fleming K, Ray JG, et al. BMI and Risk of Gestational Diabetes Among Women of South Asian and Chinese Ethnicity: A Population-Based Study. *Diabetologia* (2021) 64:805–13. doi: 10.1007/s00125-020-05356-5


**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 04 March 2022
doi: 10.3389/fendo.2022.852620

# Application of Two Blastocyst Biopsy Strategies in Preimplantation Genetic Testing Treatment and Assessment of Their Effects

*Han Yang[1,2,3,4†], Dandan Yang[2,3,4†], Qi Zhu[1,2,3,4], Kaijuan Wang[2,3,4], Chao Zhang[2,3,4], Beili Chen[2,3,4], Weiwei Zou[2,3,4], Yan Hao[2,3,4], Ding Ding[2,3,4], Zhaojuan Yu[2,3,4], Dongmei Ji[2,3,4], Dawei Chen[2,3,4], Yunxia Cao[2,3,4*], Huijuan Zou[2,3,4*] and Zhiguo Zhang[1,2,3,4*]*

[1] Department of Biomedical Engineering, Anhui Medical University, Hefei, China, [2] Reproductive Medicine Center, Department of Obstetrics and Gynecology, The First Affiliated Hospital of Anhui Medical University, Hefei, China, [3] National Health Commission (NHC) Key Laboratory of Study on Abnormal Gametes and Reproductive Tract (Anhui Medical University), Hefei, China, [4] Biopreservation and Artificial Organs, Anhui Provincial Engineering Research Center, Anhui Medical University, Hefei, China

**Background:** Blastocyst biopsy has become the most mainstream biopsy method. Currently, there are two blastocyst biopsy strategies. Many studies have compared the advantages and disadvantages between blastomere and blastocyst biopsy, but fewer articles have compared the two blastocyst biopsy strategies. For the moment, no published studies have explored the entire set of information on embryo development, next-generation sequencing results, and clinical outcomes, including the baby's health status with the two blastocyst biopsy strategies.

**Methods:** A total of 323 preimplantation genetic testing cycles from April 2018 to May 2020, including 178 cycles with Strategy A and 145 cycles with Strategy B. Strategy A was to create a laser-assisted zona pellucid opening for cleavage embryo on the third day after insemination, but Strategy B was not. Strategy A performed a biopsy for artificially assisted hatching blastocysts, while Strategy B performed a biopsy for expanded blastocysts on day 5 or 6. In this study, embryo development, next-generation sequencing results, pregnancy outcomes, and offspring health of the two strategies were compared and analyzed.

**Results:** There were no statistical differences between the two groups in the rate of fertilization, blastocyst and abortion. The rate of cleavage from Strategy A was slightly higher than Strategy B, and the rate of high-quality cleavage embryo was lower than Strategy B, while the rate of high-quality blastocyst was higher than Strategy B. The rate of no-results blastocyst was significantly lower than Strategy B. In particular, the rate of biochemical pregnancy, clinical pregnancy, and live birth of Strategy A were significantly

OPEN ACCESS

**Edited by:**
Yimin Zhu,
Zhejiang University, China

**Reviewed by:**
Iman Halvaei,
Tarbiat Modares University, Iran
Caixia Lei,
Fudan University, China

***Correspondence:**
Zhiguo Zhang
zzg_100@163.com
Huijuan Zou
hienjoyshine@aliyun.com
Yunxia Cao
caoyunxia6@126.com

†These authors have contributed equally to this work and share first authorship

**Specialty section:**
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

**Received:** 11 January 2022
**Accepted:** 11 February 2022
**Published:** 04 March 2022

**Citation:**
Yang H, Yang D, Zhu Q, Wang K, Zhang C, Chen B, Zou W, Hao Y, Ding D, Yu Z, Ji D, Chen D, Cao Y, Zou H and Zhang Z (2022) Application of Two Blastocyst Biopsy Strategies in Preimplantation Genetic Testing Treatment and Assessment of Their Effects. Front. Endocrinol. 13:852620. doi: 10.3389/fendo.2022.852620

lower than those of Strategy B. The average Apgar scores of newborns were ≥8 in both groups, and there was no significant difference in average height and weight. In Strategy A, a baby was born with thumb syndactyly, and Strategy B had no congenital disabilities.

**Conclusions:** Blastocyst biopsy strategy without laser-assisted zona pellucid drilling on day 3 achieves better clinical treatment effects. Therefore, Strategy B is an optimal treatment regime for PGT.

**Keywords: blastocyst biopsy, clinical outcomes, embryo development, human-assisted reproductive technology, next-generation sequencing, preimplantation genetic testing**

# 1 INTRODUCTION

Preimplantation genetic testing (PGT) is one of the essential techniques in human-assisted reproductive technology (ART), which contributes to reducing the transmission of genetic diseases. With the growth of women's age, especially after 40 years, the probability of embryo aneuploidy increases dramatically, which easily leads to implantation failure or miscarriage (1, 2). PGT is to identify embryos with normal chromosomes for transfer, containing Preimplantation genetic testing for aneuploidy (PGT-A), Preimplantation genetic testing for monogenic/single gene defects (PGT-M), and Preimplantation genetic testing for structural rearrangements (PGT-SR). It can significantly increase the success rate of ART. The first PGT baby was born in 1990, in which to avoid the transmission of recessive X chromosome disease to male offspring, DNA amplification was used to screen out female embryos for transfer, and finally, healthy female twins were delivered successfully (3).

Currently, the biopsy methods used in the clinic mainly include cleavage embryo biopsy, blastocyst biopsy, and polar body biopsy. Cleavage embryo biopsy extracts 1-2 blastomeres from the embryo containing 6 cells or more on the third day after insemination. Blastocyst biopsy is a dissection method of trophectoderm (TE) cells from blastocysts, usually performed on day 5 or 6. Polar body biopsy is to analyze the first polar body of mature oocytes or the second polar body of fertilized eggs. It is a diagnostic method for maternally derived genetic defects, but it cannot assess paternal factors (4). Recent studies have extracted DNA and blastocoel fluid from the conditioned blastocyst culture medium to verify the euploidy of chromosomes. In this way, non-invasive preimplantation genetic screening can be realized (5, 6). However, more research data are still insufficient for its application in clinical practice.

Many embryologists have studied the effect of biopsy methods on embryo safety and clinical outcomes. Kalma et al. demonstrated that blastomere biopsy performed 15-20 hours after the embryo develops to 8 cells is less harmful to the embryo (7). Chen Linjun et al. have shown that the blastocyst biopsy on day 5 after insemination has a higher embryo implantation rate and live birth rate than the day 6 (8). There are also many studies comparing cleavage stage biopsy and blastocyst stage biopsy, proving that blastomere biopsy significantly reduces the probability of embryo implantation and live birth, while blastocyst biopsy is relatively safer and has better clinical outcomes (9, 10). A growing number of reproductive medicine centers are using blastocyst biopsy. There are two blastocyst biopsy strategies currently. However, which method is more effective among the two blastocyst biopsy methods? This study compared the embryo development, NGS results, and clinical pregnancy outcomes of the two blastocyst stage biopsy strategies, hoping to provide a reference for this question.

In this research, the blastocyst biopsy method was applied to all PGT treatments. The cleavage embryos underwent laser hatching on the third day after insemination and left a hole, and then the laser was used again to biopsy TE cells herniating through that hole on day 5 or 6 (11, 12), which was called Strategy A here. The blastocysts reaching a morphologic grade of 4 with AA, AB, BA, or BB (also called as an expanded blastocyst) (13) were biopsied for TE cells on day 5 or 6 called Strategy B (14). The main difference between the two biopsy strategies is that Strategy A is to create a laser-assisted zona pellucida (ZP) opening for the cleavage embryo on the third day after insemination, while Strategy B does not. All biopsy samples in this study were assessed using next-generation sequencing (NGS). We analyzed the embryo development, NGS results, and clinical outcomes of the two strategies to determine which is safer and more effective.

# 2 MATERIALS AND METHODS

## 2.1 Study Population

We practiced a total of 323 preimplantation genetic testing-thawed embryo transfer (PGT-TET) cycles at Reproductive Medicine Center of the First Affiliated Hospital of Anhui Medical University, from April 2018 to May 2020. Each patient was randomly assigned into one of the two strategies by lottery. There were 178 cycles of Strategy A and 145 cycles of Strategy B included, with 1187 embryos undergoing biopsy in Strategy A and 902 embryos in Strategy B (**Table 3**). Most patients were diagnosed with chromosomal abnormalities in one or both partners; recurrent miscarriage; abnormal gestation and birth or teratozoospermia.

## 2.2 Ethics Statement

The biological sample study was approved by the Medical Ethics Committee of Anhui Medical University (Ethics approval number: 2017002). All patients in this study had signed informed consent before PGT therapy cycles.

## 2.3 Oocyte Retrieval and ICSI

The female patients received classic controlled ovarian hyperstimulation program to promote ovulation. The specific drugs and procedures have been reported (15). Through the transvaginal ultrasound-guided oocyte retrieval, the cumulus-oocyte complexes were picked up from the follicular fluid under an inverted microscope. After ovum pick-up, the cumulus and corona cells were removed through the action of hyaluronidase solution (VitroLife, Gotebor, Sweden). Then, the mature oocytes were selected for intracytoplasmic sperm injection (ICSI). Operators with many years of experience performed all ICSI processes. The ICSI oocytes were cultured for three days in the environment containing 5% $O_2$ and 6% $CO_2$ at 37°C in a microdroplet (20-30 μL) of cleavage culture medium (COOK, Sydney, Australia), covered with mineral oil (VitroLife, Gotebor, Sweden). During the period, fertilization was evaluated 16 - 18 hours after ICSI. In this study, high-quality embryos refer to those reach 7–9 cells by day 3, with <15% fragmentation and no multinucleation, and have cleaved during the preceding 24 h (16). High-quality blastocysts refer to embryos which were ≥3BB on day 5 or ≥4BB on day 6 (13).

## 2.4 The Operation of Two Biopsy Strategies

### 2.4.1 Strategy A

Around 10:00 am on the third day, a ~10 μm hole in the ZP was made with a series of 500-μs laser pulses (Hamilton Thorne LYKOS, Beverly, MA, USA). Then the embryos were placed into a microdroplet of blastocyst medium (COOK, Sydney, Australia) covered with mineral oil and cultured to day 5 or 6 until the blastocyst was hatching. Embryo biopsy was performed around 11-12 am in a petri dish (Life Sciences, Durham, USA) containing 7.5 μL blastocyst medium. The holding pipette (Sunlight Medical Inc, Jacksonville, USA) was used to fix the hatching blastocyst at the 9 o'clock position. Then, 8-10 herniating TE cells were aspirated through the biopsy pipette (Sunlight Medical Inc, Jacksonville, USA) at the 3 o'clock position. The TE cells were disconnected with lasers along the flat mouth of biopsy pipette (**Figure 1** and **Video 1**). The cells to be detected were transferred into a 200 μL PCR tube containing 2 μL phosphate buffered saline (ThermoFisher Biochemical Products Co., Ltd, Beijing, China). All of the operations were performed on the heated micromanipulator (Nikon, eclipse Ti2, Japan). The post-biopsy blastocysts were cryopreserved by vitrification in liquid nitrogen for future TET.

### 2.4.2 Strategy B

Around 10:00 am on the third day, the embryos were directly transferred to the microdroplet blastocyst medium covered with mineral oil and continued to be cultured until they reached the blastocyst stage with a morphologic grade of 4 (4AA, 4AB, 4BA, or 4BB) on day 5 or 6. The biopsy was performed around 11-12 am. Firstly, the expanded blastocyst was fixed with a holding pipette at the 9 o'clock position and then a small hole was left on the ZP with the assist of a laser. Secondly, the biopsy pipette was used to

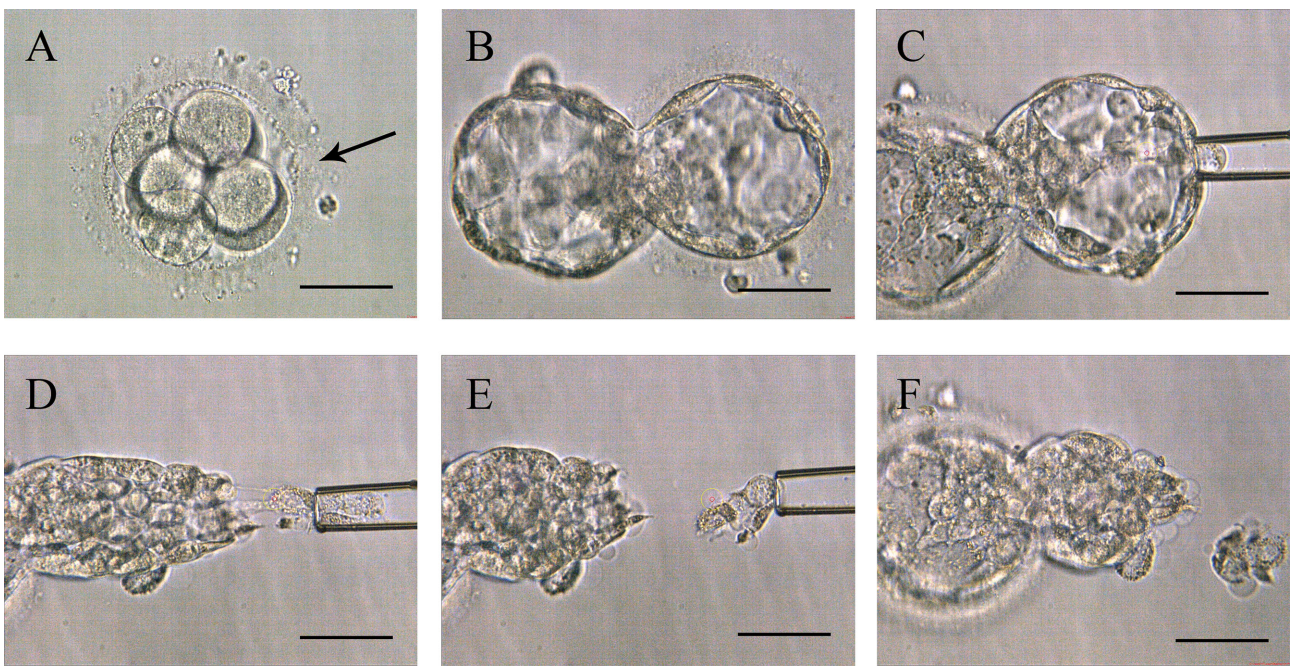



**FIGURE 1** | Procedures of Strategy A. **(A)** The zona pellucida was opened a 10-15 μm hole (pointed by the black arrow in the picture) by lasers to assist hatching on day 3. **(B)** Expanding blastocyst with trophectoderm cells herniating from the artificial opening on day 5 or 6. **(C)** The biopsy pipette sucked the herniating cells. **(D)** Disconnected the junction in front of the biopsy pipette with lasers. **(E)** Trophectoderm cell samples isolated from the embryo's body. **(F)** Blastocyst morphology after biopsy. Scale bar = 50 μm.

continuously press the blastocyst's periphery until the TE shrunk and separated from the inner surface of the ZP. Subsequently, the laser was used again to drill an about 5 μm hole by multiple pulses, and the hole was far away from the inner cell mass (ICM). Finally, the biopsy pipette was inserted into the blastocyst through the hole, and the contracted TE was sucked tightly through the negative pressure, and then some of the TE cells are pulled out of the hole at the same time, the laser is emitted to cut TE cells along the flat mouth of the pipette (**Figure 2** and **Video 2**). The following steps were as described in Strategy A.

## 2.5 Embryo Selection and Transfer

The NGS was performed with an Ion Proton Sequencing (Life Technologies, Grand Island, NY, USA). High-quality blastocysts with normal chromosomes tested by PGT would be firstly recommended to thaw and transfer. If there was no euploid embryo with patients, mosaic embryos with mosaicism <30% could be transferred in our center. Single embryo transfer was used for all statistical cycles. A positive hCG value (≥25 IU/L) on day 14 after transplantation is a sign of biochemical pregnancy. The appearance of a pregnancy sac by ultrasound scanning is regarded as clinical pregnancy.

## 2.6 Statistical Analysis

Continuous variables were expressed as the mean ± SD (standard deviation), and categorical variables were evaluated by the Chi-square test ($\chi^2$). We used the $\chi^2$ test for the data of embryo development, NGS results, and clinical outcomes. The two-sample t-test for age, hormone values and neonatal health, including Apgar score, height, and weight. GraphPad Prism 8.0 software (GraphPad Software, San Diego, USA) was used for statistical analysis. *P*-values < 0.05 were considered statistically significant.

# 3 RESULTS

In these cycles, the average age of the women in the two groups was about 30 years old, and the men was about 32 years old. In addition, the majority of the basal characteristics for all patients in the two groups are listed in **Table 1**. There were no significant differences in the baseline data between the two groups of patients.

We analyzed the embryo development, NGS results, and clinical outcomes of all PGT-TET with complete information from April 2018 to May 2020. The fertilization rate of Strategy A was slightly lower than that of Strategy B, with no statistical difference (82.74% vs 83.37%; *P*=0.547). Strategy A had a higher cleavage rate (98.73%) than Strategy B (97.69%). Although there was a statistical difference (*P*=0.01), both were above 97%, which may be related to the quality of patients' oocytes and other factors. There were no significant differences in the blastocyst rate of the two strategies, both are above 50% (53.51% vs 52.56%; *P*=0.546). However, the high-quality blastocyst rate was

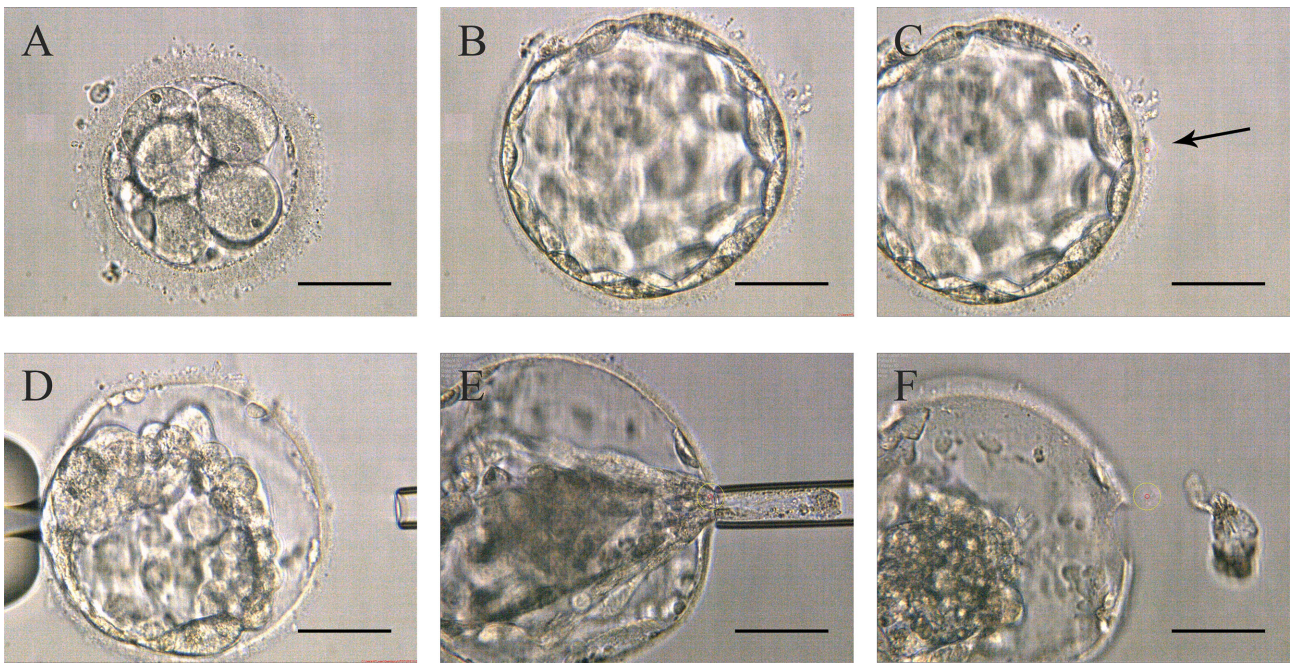



**FIGURE 2** | Procedures of Strategy B **(A)** Embryos on day 3 without zona pellucida opening. **(B)** High-quality blastocyst with an expansion grade of 4 with AA, AB, BA, or BB on day 5 or 6. **(C)** The zona pellucida was opened a small hole (pointed by the black arrow in the picture) to flow out blastocyst fluid. **(D)** After the trophectoderm cells shrunk, the lasers were used to make a hole in the zona pellucida. **(E)** The biopsy pipette entered the inner blastocyst to suck the cells out of the zona pellucida, and disconnected the junction in front of the biopsy pipette with lasers. **(F)** Trophectoderm cell samples isolated from the embryo's body. Scale bar = 50 μm.

**TABLE 1 |** Baseline level of patients from the two biopsy strategies.

| | Strategy A | Strategy B | t value | *P*-value |
|---|---|---|---|---|
| Female age (years) | 30.59 ± 4.45 | 30.73 ± 4.54 | 0.280 | 0.779 |
| Advanced female (≥35 years old) (%) | 19.66% (35/178) | 22.07% (32/145) | – | 0.596 |
| Male age (years) | 32.39 ± 4.66 | 32.48 ± 5.81 | 0.152 | 0.880 |
| BMI (kg/m$^2$) | 22.27 ± 2.81 | 22.06 ± 4.25 | 0.543 | 0.588 |
| Basal FSH (IU/L) | 6.78 ± 1.74 | 6.94 ± 2.06 | 0.783 | 0.434 |
| Basal $E_2$ (IU/L) | 151.40 ± 92.47 | 155.80 ± 138.80 | 0.339 | 0.735 |
| Basal P (IU/L) | 3.00 ± 6.51 | 3.09 ± 8.16 | 0.110 | 0.912 |
| Basal PRL (IU/L) | 24.29 ± 46.66 | 35.90 ± 86.11 | 1.543 | 0.124 |
| Basal LH (IU/L) | 5.28 ± 3.32 | 6.28 ± 9.24 | 1.348 | 0.179 |
| Basal T (IU/L) | 1.95 ± 4.78 | 2.98 ± 7.80 | 1.453 | 0.147 |
| PGT-A (%) | 41.57% (74/178) | 46.21% (67/145) | – | 0.404 |
| PGT-SR (%) | 48.31% (86/178) | 46.90% (68/145) | – | 0.800 |
| PGT-M (%) | 10.11% (18/178) | 6.90% (10/145) | – | 0.307 |

*BMI, body mass index; FSH, follicle-stimulating hormone; $E_2$, estrogenic hormone; P, progestational hormone; PRL, prolactin; LH, luteinizing hormone; T, testosterone.*

obviously higher in Strategy A groups (47.73% vs 42.31%; *P*<0.001) (**Table 2**).

The euploidy rate of Strategy A was significantly higher than that of Strategy B (35.80% vs 25.28%; *P*<0.001). While the aneuploidy rate (63.35% vs 68.40%; *P*=0.016), mosaic rate (10.45% vs 22.95%; *P*<0.001) and no-results rate (0.93% vs 5.65%; *P*<0.001) were significantly lower than those of Strategy B (**Table 3**).

In terms of clinical pregnancy outcomes, Strategy A's biochemical pregnancy rate (48.31% vs 68.97%), clinical pregnancy rate (43.26% vs 66.21%), and live birth rate (38.76% vs 57.24%) were significantly lower than Strategy B (*P*<0.001). There was no difference in abortion rate between the two groups (10.39% vs 13.54%; *P*=0.528) (**Table 2**). Infants born with the two biopsy strategies were similar in Apgar score, height, and

**TABLE 2 |** Embryo development and clinical outcomes.

| | Strategy A | Strategy B | *P*-value |
|---|---|---|---|
| Fertilization (%) | 82.74% (2364/2857) | 83.37% (1817/2179) | 0.547 |
| Cleavage (%) | 98.73% (2334/2364) | 97.69% (1775/1817) | 0.010 |
| High-quality embryo on day 3 (%) | 48.46% (1131/2334) | 52.06% (924/1775) | 0.022 |
| Blastocyst (%) | 53.51% (1249/2334) | 52.56% (933/1775) | 0.546 |
| High-quality blastocyst (%) | 47.73% (1114/2334) | 42.31% (751/1775) | <0.001 |
| Biochemical pregnancy (%) | 48.31% (86/178) | 68.97% (100/145) | <0.001 |
| Clinical pregnancy (%) | 43.26% (77/178) | 66.21% (96/145) | <0.001 |
| Abortion (%) | 10.39% (8/77) | 13.54% (13/96) | 0.528 |
| Live birth (%) | 38.76% (69/178) | 57.24% (83/145) | <0.001 |

*Rate of fertilization: the number of fertilized oocytes/the number of matured oocytes.*
*Rate of cleavage: the number of cleaved embryos/the number of fertilized oocytes.*
*Rate of high-quality embryo: the number of high-quality embryos/the number of cleaved embryos.*
*Rate of blastocyst: the number of blastocysts/the number of cleavage embryos.*
*Rate of high-quality blastocyst: the number of high-quality blastocysts/the number of cleavage embryos.*
*Rate of biochemical pregnancy: the number of biochemical pregnancies/the number of TET cycles.*
*Rate of clinical pregnancy: the number of clinical pregnancies/the number of TET cycles.*
*Rate of abortion: the number of abortions/the number of clinical pregnancies.*
*Rate of live birth: the number of deliveries with live births/the number of TET cycles.*

**TABLE 3 |** NGS results.

| | Strategy A | Strategy B | *P*-value |
|---|---|---|---|
| PGT-TET cycles (n) | 178 | 145 | – |
| No. of blastocysts biopsied (n) | 1187 | 902 | – |
| Euploid blastocyst (%) | 35.80% (425/1187) | 25.28% (228/902) | <0.001 |
| Aneuploid blastocyst (%) | 63.35% (752/1187) | 68.40% (617/902) | 0.016 |
| Mosaic blastocyst (%) | 10.45% (124/1187) | 22.95% (207/902) | <0.001 |
| No-results blastocyst (%) | 0.93% (11/1187) | 5.65% (51/902) | <0.001 |

*Rate of euploid blastocyst: the number of euploid blastocysts/the number of blastocysts biopsied.*
*Rate of aneuploid blastocyst: the number of aneuploid blastocysts/the number of blastocysts biopsied.*
*Rate of mosaic blastocyst: the number of mosaic blastocysts/the number of blastocysts biopsied.*
*Rate of no-results blastocyst: the number of no-results blastocysts/the number of blastocysts biopsied.*

weight. The average length and weight of newborns in Strategy A were 50.03cm and 3346g, respectively, 50.04cm and 3290g in Strategy B (**Table 4**). Among them, Strategy A had 10 premature babies, including a pair of monozygotic twin daughters. Strategy B had 7 premature babies, including a pair of monozygotic twin daughters and sons. Strategy A had a baby girl born with thumb syndactyly, and Strategy B had no babies with congenital disabilities. The follow-up survey showed that these children were in good health.

## 4 DISCUSSION

In the number of cycles we counted, 35 elderly females (≥35 years old) from Strategy A, accounting for 19.67% of the total number of cycles, while 32 elderly females from Strategy B, accounting for 22.07% of the total number of cycles. Advanced age may affect oocyte quality, embryo development potential, ovarian function, hormone level, embryo implantation, and pregnancy (17, 18), which may be why Strategy B had a higher abortion rate than Strategy A, but there was no statistically significant difference.

In terms of embryo development in both groups, it is noteworthy that the rate of high-quality embryos of Strategy A was significantly lower than that of Strategy B, but the rate of high-quality blastocyst was higher. This is very interesting and worth thinking about. We suspected that this might be because Strategy A perforated the ZP on day 3, which was more conducive to embryo hatching. Some hatching blastocysts may actually didn't fully expand to a morphologic grade of 5 or greater with AA, AB, BA, or BB on day 5 or 6, but the ZP was opened, and the embryos were "squeezed" out, thus increasing the so-called "high-quality hatching blastocyst rate" in Strategy A. Study demonstrated that higher-quality blastocysts could achieve better implantation and live birth rates (8). This may explain why Strategy A had a higher-quality blastocyst rate but unsatisfactory pregnancy outcomes.

As for the biochemical pregnancy rate, clinical pregnancy rate, and live birth rate of Strategy A were far lower than Strategy B, several reasons may explain this phenomenon. Embryonic genome activation (EGA) mainly occurs at the stage of division from 4 to 8 cells (19). Both Dobson and Vassena's teams demonstrated that the major wave of EGA in human occurs on the third day regardless of the number of cells (20, 21). On the third day, the ZP perforation caused trauma to the embryo during the cleavage stage, and the frequent manipulation of the embryo made the culture environment unstable, which may adversely affect the EGA and be detrimental to the growth of embryos (22). Furthermore, although drilling of the ZP with laser pulses on day 3 could promote early blastocyst hatching, the phenomenon of complete expansion of the blastocyst cavity and thinning of the ZP would not occur during the development of the blastocyst. Additionally, the number of TE cells would also be less than that of non-intervened blastocysts, which could only reach 60-80 cells in total, while non-assisted hatching blastocysts could reach 60-100 cells (23). Sufficient cell numbers could alleviate the negative impact of further reduction of cell numbers caused by biopsy on the results of embryo transfer. Another important reason may have to do with the ICM. Some studies have shown that the natural incubation site of human embryos is near the ICM, so that the embryos are more accessible to implant (24). Due to the randomness of the placement that zona breached by laser, the auxiliary incubation site may be far away from the ICM, thus reducing the chance of embryo implantation after TET. Moreover, if the ICM hatched out, in order to avoid hurting the ICM during the biopsy, a double zona drilling method for ICM incarceration may be used (25). Repeated laser stimulation would inevitably cause adverse effects on the embryo, thereby reducing the embryo quality and affecting the development potential of the blastocyst.

In contrast, Strategy B didn't damage the embryos during the cleavage stage, which could effectively avoid the potential danger of warming by laser and exposure to a suboptimal environment for a long time. In addition, it was safer that ZP remained intact, which could prevent premature hatching of embryos when the number of cells was small, thus ensuring the normal development of embryos.

The NGS data showed that Strategy A had a higher euploidy rate, while the rate of aneuploidy, mosaic and non-result were lower. Why the NGS analysis results of Strategy A is better? We suspect that the possible reason is that the biopsy subject of Strategy B is contracted TE, which increases the operation difficulty of biopsy, thereby resulting in that the number of TE cells biopsied by Strategy B is generally smaller than that by Strategy A. Therefore, the no-results rate of Strategy B is higher. Contrary to Shun Xiong et al. (26), our results showed that Strategy A had a lower rate of mosaic blastocyst. Mosaic embryos with mosaicism <50% transplantation could lead to a healthy pregnancy, it may be related to reduced implantation rate, increased miscarriage rate, and increased risk of fetal 229 abnormalities (27, 28). There was a significant difference in the rate of mosaic embryo transfer in this study. Strategy A transferred 2 mosaic embryos, accounting for 1.11% of the cycles, while strategy B transferred 10 mosaic embryos, accounting for 6.45% of the cycles. However, the clinical pregnancy outcome of Strategy B was still more ideal than that of Strategy A, consistent with previous studies (29, 30). It demonstrated that Strategy B may be less harmful to the embryos. The study is reporting an experience of a single center, which may be limited in some aspects. More PGT cycles are needed for further exploration.

In a word, the embryos biopsied by Strategy B were more likely to implant and maintain the pregnancy, and the rate of

**TABLE 4** | Health of newborns.

| | Strategy A | Strategy B | t value | *P*-value |
|---|---|---|---|---|
| No. of births (n) | 70 | 85 | – | – |
| Apgar score | 10.00 ± 0.00 | 9.95 ± 0.27 | 1.505 | 0.134 |
| Weight (g) | 3346 ± 529.20 | 3290 ± 612.00 | 0.607 | 0.545 |
| Height (cm) | 50.03 ± 2.37 | 50.04 ± 2.32 | 0.037 | 0.971 |

biochemical pregnancy, clinical pregnancy, live birth were much higher than those of Strategy A (about 20%), showing better clinical outcomes. Therefore, based on the above results, Strategy B is an optimal treatment regime for PGT.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

## ETHICS STATEMENT

The biological sample study was approved by the Medical Ethics Committee of Anhui Medical University (Ethics approval number: 2017002). The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

ZZ designed and supervised the study. HY and DY analyzed the data and wrote the main manuscript. HZ and YC performed part of the experiments and revised the manuscript. QZ, KW, and CZ prepared all the figures and videos. BC, WZ, DD, WZ, and DJ performed part of the experiments. YH and DC provided the NGS reports. All authors read and approved the final manuscript.

## FUNDING

This research was supported by National Natural Science Foundation of China (No.82071724, 32000642 and 82001631).

## ACKNOWLEDGMENTS

We thank the physicians, technicians, and related staff of the Reproductive Medicine Center, the First Affiliated Hospital of Anhui Medical University for their help and support. We also appreciate all the patients who participated in this study.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.852620/full#supplementary-material

## REFERENCES

1. Hassold T, Chiu D. Maternal Age-Specific Rates of Numerical Chromosome Abnormalities With Special Reference to Trisomy. *Hum Genet* (1985) 70 (1):11–7. doi: 10.1007/BF00389450
2. Pellestor F, Andréo B, Anahory T, Hamamah S. The Occurrence of Aneuploidy in Human: Lessons From the Cytogenetic Studies of Human Oocytes. *Eur J Med Genet* (2006) 49(2):103–16. doi: 10.1016/j.ejmg.2005.08.001
3. Handyside AH, Kontogianni EH, Hardy K, Winston RM. Pregnancies From Biopsied Human Preimplantation Embryos Sexed by Y-Specific DNA Amplification. *Nature* (1990) 344(6268):768–70. doi: 10.1038/344768a0
4. Montag M, van der Ven K, Rösing B, van der Ven H. Polar Body Biopsy: A Viable Alternative to Preimplantation Genetic Diagnosis and Screening. *Reprod BioMed Online* (2009) 18(Suppl 1):6–11. doi: 10.1016/S1472-6483(10)60109-5
5. Kuznyetsov V, Madjunkova S, Antes R, Abramov R, Motamedi G, Ibarrientos Z, et al. Evaluation of a Novel non-Invasive Preimplantation Genetic Screening Approach. *PloS One* (2018) 13(5):e0197262. doi: 10.1371/journal.pone.0197262
6. Simon C. Noninvasive Preimplantation Genetic Testing for Aneuploidy in Spent Blastocyst Media Will Substitute for Trophectoderm Biopsy. *Fertil Steril* (2021) 115(4):840. doi: 10.1016/j.fertnstert.2021.02.046
7. Kalma Y, Bar-El L, Asaf-Tisser S, Malcov M, Reches A, Hasson J, et al. Optimal Timing for Blastomere Biopsy of 8-Cell Embryos for Preimplantation Genetic Diagnosis. *Hum Reprod* (2018) 33(1):32–8. doi: 10.1093/humrep/dex343
8. Chen L, Diao Z, Wang J, Xu Z, Zhang N, Fang J, et al. The Effects of the Day of Trophectoderm Biopsy and Blastocyst Grade on the Clinical and Neonatal Outcomes of Preimplantation Genetic Testing-Frozen Embryo Transfer Cycles. *Zygote* (2022) 30(1):132–7. doi: 10.1017/S0967199421000435
9. Scott RTJr, Upham KM, Forman EJ, Zhao T, Treff NR. Cleavage-Stage Biopsy Significantly Impairs Human Embryonic Implantation Potential While Blastocyst Biopsy Does Not: A Randomized and Paired Clinical Trial. *Fertil Steril* (2013) 100(3):624–30. doi: 10.1016/j.fertnstert.2013.04.039
10. Coll L, Parriego M, Boada M, Devesa M, Arroyo G, Rodríguez I, et al. Transition From Blastomere to Trophectoderm Biopsy: Comparing Two Preimplantation Genetic Testing for Aneuploidies Strategies. *Zygote* (2018) 26(3):191–8. doi: 10.1017/S0967199418000084
11. McArthur SJ, Leigh D, Marshall JT, de Boer KA, Jansen RP. Pregnancies and Live Births After Trophectoderm Biopsy and Preimplantation Genetic Testing of Human Blastocysts. *Fertil Steril* (2005) 84(6):1628–36. doi: 10.1016/j.fertnstert.2005.05.063
12. Schoolcraft WB, Fragouli E, Stevens J, Munne S, Katz-Jaffe MG, Wells D. Clinical Application of Comprehensive Chromosomal Screening at the Blastocyst Stage. *Fertil Steril* (2010) 94(5):1700–6. doi: 10.1016/j.fertnstert.2009.10.015
13. Gardner DK, Vella P, Lane M, Wagley L, Schlenker T, Schoolcraft WB. Culture and Transfer of Human Blastocysts Increases Implantation Rates and Reduces the Need for Multiple Embryo Transfers. *Fertil Steril* (1998) 69 (1):84–8. doi: 10.1016/S0015-0282(97)00438-X
14. Capalbo A, Rienzi L, Cimadomo D, Maggiulli R, Elliott T, Wright G, et al. Correlation Between Standard Blastocyst Morphology, Euploidy and Implantation: An Observational Study in Two Centers Involving 956 Screened Blastocysts. *Hum Reprod* (2014) 29(6):1173–81. doi: 10.1093/humrep/deu033
15. Ding D, Wang Q, Li X, Chen B, Zou W, Ji D, et al. Effects of Different Polyvinylpyrrolidone Concentrations on Intracytoplasmic Sperm Injection. *Zygote* (2020) 28(2):148–53. doi: 10.1017/S0967199419000820
16. Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology. The Istanbul Consensus Workshop on Embryo Assessment: Proceedings of an Expert Meeting. *Hum Reprod* (2011) 26(6):1270–83. doi: 10.1093/humrep/der037
17. Bernstein LR, Treff NR. Editorial: Causes of Oocyte Aneuploidy and Infertility in Advanced Maternal Age and Emerging Therapeutic Approaches. *Front Endocrinol* (2021) 12:652990. doi: 10.3389/fendo.2021.652990
18. Ntostis P, Swanson G, Kokkali G, Iles D, Huntriss J, Pantou A, et al. The Effects of Aging on Molecular Modulators of Human Embryo Implantation. *iScience* (2021) 24(7):102751. doi: 10.1016/j.isci.2021.102751
19. Braude P, Bolton V, Moore S. Human Gene Expression First Occurs Between the Four- and Eight-Cell Stages of Preimplantation Development. *Nature* (1988) 332(6163):459–61. doi: 10.1038/332459a0

20. Dobson AT, Raja R, Abeyta MJ, Taylor T, Shen S, Haqq C, et al. The Unique Transcriptome Through Day 3 of Human Preimplantation Development. *Hum Mol Genet* (2004) 13(14):1461–70. doi: 10.1093/hmg/ddh157
21. Vassena R, Boué S, González-Roca E, Aran B, Auer H, Veiga A, et al. Waves of Early Transcriptional Activation and Pluripotency Program Initiation During Human Preimplantation Development. *Development* (2011) 138(17):3699–709. doi: 10.1242/dev.064741
22. Niakan KK, Han J, Pedersen RA, Simon C, Pera RA. Human Pre-Implantation Embryo Development. *Development* (2012) 139(5):829–41. doi: 10.1242/dev.060426
23. Aoyama N, Kato K. Trophectoderm Biopsy for Preimplantation Genetic Test and Technical Tips: A Review. *Reprod Med Biol* (2020) 19(3):222–31. doi: 10.1002/rmb2.12318
24. Gonzales DS, Jones JM, Pinyopummintr T, Carnevale EM, Ginther OJ, Shapiro SS, et al. Trophectoderm Projections: A Potential Means for Locomotion, Attachment and Implantation of Bovine, Equine and Human Blastocysts. *Hum Reprod* (1996) 11(12):2739–45. doi: 10.1093/oxfordjournals.humrep.a019201
25. Rubino P, Cotarelo RP, Alteri A, Rega E, Viganò P, Giannini P. Trophectoderm Biopsy Performed by a Double Laser Zona Drilling: First Safety Evidence. *Eur J Obstet Gynecol Reprod Biol* (2014) 181:344–5. doi: 10.1016/j.ejogrb.2014.08.019
26. Xiong S, Liu W, Wang J, Liu J, Gao Y, Wu L, et al. Trophectoderm Biopsy Protocols may Impact the Rate of Mosaic Blastocysts in Cycles With Pre-Implantation Genetic Testing for Aneuploidy. *J Assist Reprod Genet* (2021) 38 (5):1153–62. doi: 10.1007/s10815-021-02137-w
27. Spinella F, Fiorentino F, Biricik A, Bono S, Ruberti A, Cotroneo E, et al. Extent of Chromosomal Mosaicism Influences the Clinical Outcome of In Vitro Fertilization Treatments. *Fertil Steril* (2018) 109(1):77–83. doi: 10.1016/j.fertnstert.2017.09.025
28. Greco E, Minasi MG, Fiorentino F. Healthy Babies After Intrauterine Transfer of Mosaic Aneuploid Blastocysts. *N Engl J Med* (2015) 373(21):2089–90. doi: 10.1056/NEJMc1500421
29. Zhao H, Tao W, Li M, Liu H, Wu K, Ma S. Comparison of Two Protocols of Blastocyst Biopsy Submitted to Preimplantation Genetic Testing for Aneuploidies: A Randomized Controlled Trial. *Arch Gynecol Obstet* (2019) 299(5):1487–93. doi: 10.1007/s00404-019-05084-1
30. Rubino P, Tapia L, Ruiz de Assin Alonso R, Mazmanian K, Guan L, Dearden L, et al. Trophectoderm Biopsy Protocols can Affect Clinical Outcomes: Time to Focus on the Blastocyst Biopsy Technique. *Fertil Steril* (2020) 113(5):981–9. doi: 10.1016/j.fertnstert.2019.12.034

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 08 March 2022
doi: 10.3389/fendo.2022.830414

# Impact of Maternal Age on Singleton Birthweight in Frozen Embryo Transfer Cycles

*Zhe-xin Ni*[1†], *Kun-ming Wan*[1†], *Zhi-hao Zhou*[1†], *Yan-ping Kuang*[2*] *and Chao-qin Yu*[1*]

[1] *Department of Traditional Chinese Medicine Gynecology, Changhai Hospital, Naval Medical University, Shanghai, China,*
[2] *Department of Assisted Reproduction, Shanghai Ninth People's Hospital, Shanghai JiaoTong University School of Medicine, Shanghai, China*

**Background:** Previous studies have investigated the effect of maternal age on assisted reproductive technology success rates. However, little is known about the relationship between maternal age and neonatal birthweight in frozen embryo transfer (FET) cycles. Whether maternal age influences singleton birthweight in FET cycles remains to be elucidated.

**Methods:** This study was conducted at a tertiary care center, involving singleton live births born to women undergoing frozen–thawed embryo transfer during the period from January 2010 to December 2017. A total of 12,565 women who fulfilled the inclusion criteria were enrolled and grouped into four groups according to the maternal age: <30, 30–34, 35–39, and ≥40 years old. A multivariable linear regression analysis was conducted to reveal the relationship between maternal age and neonatal birthweight with controlling for a number of potential confounders.

**Results:** The highest proportions of low birthweight (LBW, 4.1%), high birthweight (1.2%), preterm birth (PTB, 5.9%), and very PTB (0.9%) were found in the group over 40 years old, but no significant difference was observed among the four groups. Additionally, the 35–39-year-old group had the highest rate of very LBW (0.6%), whereas the 30–34-year-old group had the lowest rate of small for gestational age (SGA, 2.7%). However, multivariate analyses revealed that neonatal outcomes including PTB, LBW, and SGA were similar between the different maternal age groups.

**Conclusion:** Grouping with different maternal age was not associated with mean birthweight and Z-scores of singletons resulting from FET.

**Keywords: assisted reproductive technology, frozen–thawed embryo transfer, vitrification, maternal age, birthweight**

OPEN ACCESS

***Edited by:***
*Yimin Zhu,*
*Zhejiang University, China*

***Reviewed by:***
*Silvia Vannuccini,*
*University of Florence, Italy*
*Fan Jin,*
*Zhejiang University, China*

***Correspondence:**
*Chao-qin Yu*
*chqyu81@163.com*
*Yan-ping Kuang*
*kuangyp@sh9hospital.org*

[†]*These authors have contributed equally to this work*

***Specialty section:***
*This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology*

***Received:*** *07 December 2021*
***Accepted:*** *07 February 2022*
***Published:*** *08 March 2022*

***Citation:***
*Ni Z-x, Wan K-m, Zhou Z-h, Kuang Y-p and Yu C-q (2022) Impact of Maternal Age on Singleton Birthweight in Frozen Embryo Transfer Cycles. Front. Endocrinol. 13:830414. doi: 10.3389/fendo.2022.830414*

## BACKGROUND

In the past decades, many women delay childbearing until after the age of 40. The decline of fertility of elderly women forced them to seek the help of assisted reproductive technology (ART). According to statistics by the European IVF-monitoring Consortium, the proportion of women over 40 years of age that became pregnant through *in vitro* fertilization or intracytoplasmic sperm

injection (IVF/ICSI) in Europe is increasing annually. For example, in the UK, the proportion increased from 12.7% to 26% in 1997 to 2015, and the same trend was observed in other high-income countries (1, 2).

Having a healthy baby is the ultimate goal of ART treatment. Increased risk of adverse neonatal outcomes is believed to appear in IVF/ICSI-conceived babies, even singletons, such as preterm birth (PTB), low birthweight (LBW), and small for gestational age (SGA), when compared with babies that are conceived spontaneously (3). Maternal age is a frequently used predictor of PTB, LBW, and perinatal mortality in women who have conceived naturally (4–7), and a number of studies have well revealed the negative correlation between maternal age and neonatal outcomes (8, 9). Women who have a baby through ART are, on average, older than those who conceive spontaneously as these techniques are often applied in response to age-related infertility problems (10). However, little is known regarding the influence of maternal age on birthweight in vitrified–thawed embryo transfer cycles.

To our knowledge, only few studies have examined the effect of maternal age on birthweight with ART, included different kinds of treatments, and considered sufficient confounders (11–14). Furthermore, the published data, except for Lin's study (14), exclusively focused on fresh IVF cycles, without ruling out the possibility of adverse fetal growth caused by a hypoestrogenic milieu. Of note, supraphysiological estrogen levels during ovarian stimulation can create a suboptimal peri-implantation environment for implantation and placentation, thus causing abnormal fetal growth including LBW and SGA (15, 16). Unlike fresh ovarian stimulation cycles, frozen embryo transfer (FET) seems to provide a more physiological uterine environment for early fetal development (17). Thus, the current study aims to explore the effect of maternal age on the birthweight of newborns conceived through embryo transfers during FET cycles.

# METHODS

## Study Design and Population

This retrospective study involved women who had undergone FET during the period from January 2010 to December 2017, which was performed at the Department of Assisted Reproduction of the Ninth People's Hospital of Shanghai Jiao Tong University School of Medicine. Women who met the following inclusion criteria were included in the study: BMI <30 kg/m$^2$ and transfer of embryos resulting in a live singleton birth. In all FET cycles, no more than two embryos can be transferred. Furthermore, only the first live birth IVF/ICSI cycles were retained for women who had more than one delivery during the study period. The exclusion criteria were as follows: vanishing twin syndrome, congenital uterine malformations, and presence of submucosal fibroids or polyps and intramural fibroids >4 cm determined by ultrasound or hysteroscopy. Women with gestational diabetes, pregnancy-induced hypertension, and preeclampsia were excluded as these pregnancy-related factors may have a negative effect on intrauterine fetal growth. This study was approved by the institutional review board of the hospital and was conducted in accordance with the Helsinki Declaration. Informed consent was not required due to the retrospective nature of the study, and patients' data were used anonymously.

## Laboratory Protocols

The procedures of ovarian stimulation, oocyte retrieval, and IVF/ICSI have been described in previous studies (18, 19). In brief, IVF or ICSI was conventionally performed according to semen parameters and previous fertilization histories. For IVF, oocytes were inseminated in human tubal fluid (HTF, Irvine Scientific), which was supplemented with 10% serum substitute supplement (SSS, Irvine Scientific) and ~300,000 progressively motile spermatozoa. For ICSI, oocytes were transferred into dishes immediately after microinjection with HTF+10% SSS. The assessment of fertilization was performed 16–18 h after insemination/injection. A dish containing pre-equilibrated culture medium was then prepared for the transfer of zygotes. Before 2013, embryos were cultured in Early Cleavage Medium (Irvine Scientific) before day 3 and then in MultiBlast Medium (Irvine Scientific). However, a continuous single culture medium (Irvine Scientific) was introduced after January 2013. All embryos were cultured under mineral oil and grown in an incubator at 37°C under 5% $O_2$ and 6% $CO_2$ concentration (the balance gas was nitrogen). Except for the change of culture medium types, no change was made for other laboratory conditions and IVF protocols throughout the study period.

## Endometrial Preparation and Vitrification

The endometrial preparation protocols for FET have been previously described (20). Briefly, a natural cycle FET was suitable for women having regular menstrual cycles with the use of HCG for triggering ovulation. Artificial cycles were offered for women with irregular cycles according to the discretion of treating physicians. The procedure of vitrification and thawing have been previously described (19). In short, the Cryotop carrier system with dimethyl sulfoxide–ethylene glycol–sucrose was used as cryoprotectants for embryo vitrification. Dilution solution in a sequential manner (1 mol/L to 0.5 mol/L to 0 mol/L sucrose) was used for thawing embryos. All embryos were thawed on the day of transfer.

## Maternal Age

Maternal age at the birth of the child was the key explanatory variable, which was divided into the following categories: <30, 30–34, 35–39, and ≥40 years old. The age group <30 years old was set as the reference category in our analyses.

## Outcome Measures

The primary neonatal outcomes focused on live singleton birthweight, including Z-scores, birthweight categories, and birthweight percentiles. Secondary outcome measures were associated with neonatal outcomes, including GA at birth and newborn gender. The definition for live singleton birth was a delivery of a singleton viable infant after the 24th gestational week. GA in FET cycles was calculated from the day of embryo transfer (day 17 for cleavage-stage embryo transfer and day 19 for blastocyst embryo transfer) (21). The definitions for PTB and

very PTB were live births at <37 and <32 completed gestational weeks, respectively. Z-scores were calculated according to GA and newborn gender on birthweight based on the national birthweight reference as previously described (22, 23). A birth weight <2500 g was defined as LBW, and that <1500 g was defined as very LBW. The birthweight of infant was divided as follows: LBW (<2500 g), very LBW (<1500 g), high birthweight (HBW, >4500 g), and normal birthweight. Birthweight percentiles were also based on the national birthweight reference (23) and were divided into the following categories: SGA defined as birthweight <10th percentile, very SGA defined as birthweight <3rd percentile, large for gestational age (LGA) defined as birthweight >90th percentile, and very LGA defined as birthweight >97th percentile.

## Statistical Analysis

One-way analysis of variance was performed for continuous data, whereas Pearson's chi-squared test or Fisher's exact test was applied for categorical data. A *post hoc* Bonferroni correction was performed for multiple comparisons. The association between maternal age and neonatal outcomes was detected by multivariable logistic regression analysis, and the independent effect of maternal age on neonatal outcomes was analyzed by multiple linear regression.

The multivariable analyses included the following confounders: maternal BMI, paternal age, parity, infertility cause and duration, insemination method, type of endometrial preparation, endometrial thickness, year of treatment, and newborn gender. The continuous covariates (maternal BMI, paternal age, infertility duration, endometrial thickness, and year of treatment) and categorical covariates in multivariable models are listed in **Table 1**. Maternal age <30 years old was taken as a reference group in multivariable analyses. For the development of IVF techniques over time (24), a sensitivity analysis was performed on treating the year of treatment as a categorical variable. All analyses were conducted with SPSS Statistics (version 21.0), and $P < 0.05$ was considered to be statistically significant.

# RESULTS

The final dataset included 12,565 women who fulfilled the inclusion criteria, with no loss to follow-up. Baseline demographic and cycle characteristics are presented in **Table 1**. Comparison between the reference group and the other three groups revealed significant difference for maternal BMI, infertility cause, parity, infertility duration, FET cycle rank, fertilization method, number of embryo transferred, FET endometrial preparation, endometrial thickness, and year of treatment. Infertility duration and embryo developmental stage at transfer did not differ significantly among maternal age categories.

Neonatal outcomes stratified by maternal age are listed in **Table 2**. No significant difference was observed on GA and mean birthweight, and the gestation-adjusted Z-scores varied significantly according to maternal age categories. The 30–34-year-old group had the highest birthweight and Z-scores (3355.8 ± 483.3 g, 0.38 ± 1.03). With the increase of maternal age, a modest decrease of birthweight was observed, and the group over 40 years old had the lowest birthweight values (3321.6 ± 503.9 g). Additionally, no significant differences were found between any two groups by *post hoc* analysis on birthweight and Z-scores. The highest proportions of LBW (4.1%), HBW (1.2%), PTB (5.9%), and very PTB (0.9%) were found in the group over 40 years old, but no difference was observed among the four groups. Interestingly, the 35–39-year-old group had the highest rate of very LBW (0.6%), whereas the 30–34-year-old group had the lowest rate of SGA (2.7%). Furthermore, no difference was observed in very LGA, LGA, very SGA, and newborn gender between groups.

In multivariate analyses (**Table 3**), the neonatal outcomes including PTB, LBW, HBW, SGA, and LGA were similar between the different maternal age groups. The odds of PTB and LBW were lower in the group over 40 years old compared with the reference group, which did not reach a significant difference. Although the analysis of very PTB (<32 weeks) and very LBW (<1500 g) was performed, the number of cases in the two categories was too small to make any meaningful comparisons. In addition, no significant difference was found on birthweight percentile categories between the reference group and the other three groups.

Multiple linear regression analyses were conducted to assess the relationship between maternal age and birthweight (**Table 4**). Even after correction for a number of potential confounders, no significant correlation was found between maternal age and neonatal birthweight. Moreover, maternal BMI ($P < 0.001$), parity ($P < 0.001$), FET cycle rank ($P = 0.001$), number of embryos transferred ($P = 0.034$), embryo developmental stage at transfer ($P < 0.001$), endometrial thickness (8–11 mm, $P = 0.018$; >11 mm, $P = 0.001$), year of treatment ($P < 0.001$), GA ($P < 0.001$), and newborn gender ($P < 0.001$) were all independent predictors for birthweight.

# DISCUSSION

In recent years, much attention has been paid to the impact of maternal age on ART success rates, and several studies have revealed that a high maternal age (over 40 years old) has a negative effect on pregnancy outcomes (11, 13). However, our study showed that no significant association existed between maternal age and singleton birthweight in FET cycles with consideration for related confounders. Furthermore, linear regression indicated that maternal age was not an independent predictor of singleton birthweight in FET cycles.

To our knowledge, four studies have analyzed the potential relationship between maternal age and neonatal birthweight. Wennberg *et al.* investigated the influence of maternal age on adverse maternal and neonatal outcomes following ART treatment and found that the risk of LBW and very LBW was significantly higher in ART than in spontaneous conception (SC) singletons in all ages up to maternal age of 40 years (LBW: aORs

**TABLE 1** | Patient treatment and demographic characteristics according to maternal age.

| | <30 y n=3586 | 30-34 y n=5461 | 35-39 y n=2861 | ≥40 y n=657 | P value[a] | P value[b] | P value[c] |
|---|---|---|---|---|---|---|---|
| **Maternal Age (years)** | 27.29 ± 1.63 | 31.93 ± 1.40 | 36.47 ± 1.34 | 41.31 ± 1.53 | <0.001 | <0.001 | <0.001 |
| **Maternal BMI (kg/m2)** | | | | | <0.001 | <0.001 | <0.001 |
| **<18.5** | 534 (14.9) | 615 (11.3) | 270 (9.4) | 37 (5.6) | | | |
| **18.5-22.9** | 2218 (61.9) | 3448 (63.1) | 1791 (62.6) | 414 (63.0) | | | |
| **23-27.4** | 729 (20.3) | 1254 (23.0) | 719 (25.1) | 194 (29.5) | | | |
| **≥27.5** | 105 (2.9) | 144 (2.6) | 81 (2.8) | 12 (1.8) | | | |
| **Paternal Age (years)** | 29.69 ± 3.53 | 33.74 ± 3.68 | 38.03 ± 4.29 | 42.62 ± 5.15 | <0.001 | <0.001 | <0.001 |
| **Infertility cause** | | | | | <0.001 | <0.001 | <0.001 |
| Tubal factors | 2126 (59.3) | 3226 (59.1) | 1788 (62.5) | 321 (48.9) | | | |
| PCOS | 391 (10.9) | 425 (7.8) | 97 (3.4) | 12 (1.8) | | | |
| Endometriosis | 255 (7.1) | 466 (8.5) | 280 (9.8) | 57 (8.7) | | | |
| Diminished ovarian reserve. | 61 (1.7) | 121 (2.2) | 117 (4.1) | 142 (21.6) | | | |
| Uterine factors | 52 (1.5) | 112 (2.1) | 65 (2.3) | 17 (2.6) | | | |
| Male | 513 (14.3) | 675 (12.4) | 294 (10.3) | 67 (10.2) | | | |
| Unexplained | 188 (5.2) | 436 (8.0) | 220 (7.7) | 41 (6.2) | | | |
| **Parity** | | | | | <0.001 | <0.001 | <0.001 |
| 0 | 3489 (97.3) | 5159 (94.5) | 2478 (86.6) | 471 (71.7) | | | |
| >0 | 97 (2.7) | 302 (5.5) | 383 (13.4) | 186 (28.3) | | | |
| **Infertility duration (years)** | 2.53 ± 1.82 | 3.29 ± 2.42 | 4.11 ± 3.41 | 4.70 ± 4.55 | 0.058 | 0.067 | 0.11 |
| **FET cycle rank** | | | | | <0.001 | <0.001 | <0.001 |
| First | 2332 (65.0) | 3112 (57.0) | 1444 (50.5) | 298 (45.4) | | | |
| High order | 1254 (35.0) | 2349 (43.0) | 1417 (49.5) | 359 (54.6) | | | |
| **Fertilization method** | | | | | 0.118 | <0.001 | <0.001 |
| IVF | 2210 (61.6) | 3425 (62.7) | 1871 (65.4) | 414 (63.0) | | | |
| ICSI | 990 (27.6) | 1407 (25.8) | 762 (26.6) | 225 (34.2) | | | |
| IVF+ICSI | 386 (10.8) | 629 (11.5) | 228 (8.0) | 18 (2.7) | | | |
| **Number of embryos transferred** | | | | | 0.002 | <0.001 | <0.001 |
| 1 | 555 (15.5) | 985 (18.0) | 569 (19.9) | 145 (22.1) | | | |
| ≥2 | 3031 (84.5) | 4476 (82.0) | 2292 (80.1) | 512 (77.9) | | | |
| **Embryo developmental stage at transfer** | | | | | 0.815 | 0.607 | 0.200 |
| Day 3 | 3010 (83.9) | 4573 (83.7) | 2415 (84.4) | 565 (86.0) | | | |
| Day 5/6 | 576 (16.1) | 888 (16.3) | 446 (15.6) | 92 (14.0) | | | |
| **FET endometrial preparation** | | | | | <0.001 | <0.001 | <0.001 |
| Natural cycle | 737 (20.6) | 1338 (24.5) | 750 (26.2) | 176 (26.8) | | | |
| Artificial cycle | 2849 (79.4) | 4123 (75.5) | 2111 (73.8) | 481 (73.2) | | | |
| **Endometrial thickness (mm)** | | | | | 0.040 | <0.001 | <0.001 |
| <8 | 232 (6.5) | 399 (7.3) | 270 (9.4) | 83 (12.6) | | | |
| 8-11 | 1958 (54.6) | 3067 (56.2) | 1610 (56.3) | 369 (56.2) | | | |
| >11 | 1396 (38.9) | 1995 (36.5) | 981 (34.3) | 205 (31.2) | | | |
| **Year of treatment** | | | | | 0.380 | 0.001 | <0.001 |
| 2010–2012 | 276 (7.7) | 465 (8.5) | 218 (7.6) | 33 (5.0) | | | |
| 2013–2014 | 1693 (47.2) | 2561 (46.9) | 1219 (42.6) | 250 (38.1) | | | |
| 2015-2017 | 1617 (45.1) | 2435 (44.6) | 1424 (49.8) | 375 (56.9) | | | |

[a]*30-34 years old vs. <30 years old.*
[b]*35-39 years old vs. <30 years old.*
[c]*≥40 years old vs. <30 years old.*
*Data are presented as mean ± SD for continuous variables and n (%) for dichotomous variables.*

1.44–2.35; VLBW: aORs 1.67–3.44). Additionally, when the analysis was restricted to maternal age >35 years, an increased risk of LBW existed for SC pregnancies, but not for ART pregnancies (11). Due to medical, educational, and socioeconomic reasons, women aged >35 years who conceive through ART may pay more attention to their state of health and seek medical assistance more often than SC women, which could result in increased detection of complications and decreased risk of LBW. Second, Moaddab *et al.* found that maternal age did not predict newborns' birthweight in pregnancies with maternal age grouping as <40, 40–44, 45–49, and ≥50 years old (12). However, the analysis on birthweight in the maternal age group <40 years old per se is inadequate, and important information is missing. There may be some interesting findings among groups at ages <25, 26–30, 31–34, and 35–40 years old, and the increased risk of LBW may appear at the age of 35 years old. However, this study seemed to miss this potential information. Many studies have set up more detailed groups with maternal age under 40 years old to assess the influence of maternal age on neonatal outcomes and gained more credible results (13, 14). Another study reported that the risk of LBW was increased only at maternal ages over 40 years old (6 percentage points, 95% CI: 0.2, 12) with medically assisted reproduction (MAR) compared with the risk of LBW at ages 30–34 years old (13). However, a limited number of

**TABLE 2 |** Neonatal outcomes of live born singletons by maternal age.

| | <30 y n=3586 | 30-34 y n=5461 | 35-39 y n=2861 | ≥40 y n=657 | P value[a] | P value[b] | P value[c] |
|---|---|---|---|---|---|---|---|
| **Gestational age** | | | | | 0.779 | 0.324 | 0.487 |
| ≥37 weeks | 3367 (93.9) | 5117 (93.7) | 2694 (94.2) | 612 (93.2) | | | |
| preterm birth (<37 weeks) | 198 (5.5) | 316 (5.8) | 143 (5.0) | 39 (5.9) | | | |
| very preterm birth (<32 weeks) | 21 (0.6) | 28 (0.5) | 24 (0.8) | 6 (0.9) | | | |
| **Birthweight (g)** | 3352.2 ± 485.7 | 3355.8 ± 483.3 | 3334.2 ± 493.6 | 3321.6 ± 503.9 | 0.976 | 0.336 | 0.335 |
| **Z-scores** | 0.36 ± 1.04 | 0.38 ± 1.03 | 0.35 ± 1.06 | 0.38 ± 1.10 | 0.754 | 0.984 | 0.939 |
| **Birthweight categories** | | | | | 0.761 | 0.464 | 0.240 |
| Very low birthweight (<1500 g) | 12 (0.3) | 19 (0.3) | 17 (0.6) | 2 (0.3) | | | |
| Low birthweight (<2500 g) | 107 (3.0) | 181 (3.3) | 90 (3.1) | 27 (4.1) | | | |
| High birthweight (>4500 g) | 27 (0.8) | 48 (0.9) | 22 (0.8) | 8 (1.2) | | | |
| **Birthweight percentiles** | | | | | 0.186 | 0.498 | 0.937 |
| Very small for gestational age (<3rd percentile) | 48 (1.3) | 83 (1.5) | 35 (1.2) | 9 (1.4) | | | |
| Small for gestational age (<10th percentile) | 126 (3.5) | 150 (2.7) | 100 (3.5) | 20 (3.0) | | | |
| Large for gestational age (>90th percentile) | 369 (10.3) | 583 (10.7) | 326 (11.4) | 74 (11.3) | | | |
| Very large of gestational age (>97th percentile) | 258 (7.2) | 354 (6.5) | 183 (6.4) | 46 (7.0) | | | |
| **Newborn gender** | | | | | 0.667 | 0.564 | 0.393 |
| Female | 1881 (52.5) | 2839 (52.0) | 1522 (53.2) | 349 (53.1) | | | |
| Male | 1705 (47.5) | 2622 (48.0) | 1339 (46.8) | 308 (46.9) | | | |

[a]*30-34 years old vs. <30 years old.*
[b]*35-39 years old vs. <30 years old.*
[c]*≥40 years old vs. <30 years old.*
*Data are presented as mean ± SD for continuous variables and n (%) for dichotomous variables.*

**TABLE 3 |** Odds ratios for gestational age and birth weights by maternal age.

| | <30 y n=3586 | 30-34 y n=5461 | 35-39 y n=2861 | ≥40 y n=657 |
|---|---|---|---|---|
| **Gestational age between < 37 weeks** | | | | |
| Crude OR | Reference | 0.952 (0.793-1.143) | 1.108 (0.888-1.382) | 0.923 (0.648-1.315) |
| Adjusted OR | Reference | 0.951 (0.749-1.209) | 1.096 (0.779-1.542) | 0.947 (0.586-1.530) |
| **Gestational age < 32 weeks** | | | | |
| Crude OR | Reference | 1.140 (0.646-2.010) | 0.700 (0.389-1.260) | 0.636 (0.256-1.583) |
| Adjusted OR | Reference | 1.015 (0.476-2.163) | 0.825 (0.301-2.257) | 0.741 (0.206-2.668) |
| **Very low birthweight (<1500 g)** | | | | |
| Crude OR | Reference | 0.957 (0.464-1.974) | 0.561 (0.267-1.176) | 1.081 (0.241-4.844) |
| Adjusted OR | Reference | 1.132 (0.470-2.728) | 0.701 (0.222-2.217) | 1.211 (0.203-7.228) |
| **Low birthweight (<2500 g)** | | | | |
| Crude OR | Reference | 0.896 (0.703-1.142) | 0.944 (0.710-1.255) | 0.715 (0.465-1.100) |
| Adjusted OR | Reference | 0.959 (0.703,1.309) | 1.029 (0.662-1.599) | 0.787 (0.435-1.423) |
| **High birthweight (>4500 g)** | | | | |
| Crude OR | Reference | 0.836 (0.521-1.339) | 0.975 (0.554-1.716) | 0.610 (0.276-1.350) |
| Adjusted OR | Reference | 0.961 (0.537-1.716) | 0.965 (0.413-2.256) | 0.846 (0.256-2.791) |
| **Very small for gestational age (<3rd percentile)** | | | | |
| Crude OR | Reference | 0.891 (0.623-1.275) | 1.092 (0.704-1.694) | 0.973 (0.474-1.995) |
| Adjusted OR | Reference | 0.890 (0.571-1.388) | 0.824 (0.432-1.570) | 0.653 (0.253-1.685) |
| **Small for gestational age (<10th percentile)** | | | | |
| Crude OR | Reference | 1.294 (1.016-1.648) | 1.003 (0.767-1.312) | 1.149 (0.710-1.859) |
| Adjusted OR | Reference | 1.217 (0.877-1.260) | 1.015 (0.655-1.573) | 1.066 (0.564-2.017) |
| **Large for gestational age (>90th percentile)** | | | | |
| Crude OR | Reference | 0.975 (0.849-1.120) | 0.901 (0.769-1.056) | 0.910 (0.696-1.188) |
| Adjusted OR | Reference | 1.051 (0.877-1.260) | 1.026 (0.799-1.316) | 1.034 (0.721-1.483) |
| **Very large of gestational age (>97th percentile)** | | | | |
| Crude OR | Reference | 1.123 (0.950-1.328) | 1.122 (0.921-1.367) | 1.023 (0.737-1.420) |
| Adjusted OR | Reference | 1.071 (0.856-1.339) | 1.052 (0.769-1.439) | 0.983 (0.623-1.549) |

*Data are Odds Ratios (OR) with 95% confidence interval (CI). Analyses were adjusted for maternal BMI, paternal age, infertility cause, parity, infertility duration, FET cycle rank, infertility cause, fertilization method, number of embryos transferred, embryo developmental stage at transfer, the type of endometrial preparation, endometrial thickness, year of treatment, and newborn sex.*

**TABLE 4** | Results of multiple regression analysis of singleton birthweight.

| | Unstandardized coefficients | Std. error | Standardized coefficients | t | P value |
|---|---|---|---|---|---|
| | B | | Beta | | |
| **(Constant)** | -3532.855 | 99.754 | | -35.416 | <0.001 |
| **Maternal age** | | | | | |
| <30 (reference) | | | | | |
| 30-34 | -4.313 | 9.513 | -0.004 | -0.453 | 0.650 |
| 35-39 | -14.879 | 13.020 | -0.013 | -1.143 | 0.253 |
| ≥40 | -13.387 | 21.706 | -0.006 | -0.617 | 0.537 |
| **Maternal BMI (kg/m$^2$)** | | | | | |
| **<18.5** | -109.739 | 11.516 | -0.072 | -9.530 | <0.001 |
| **18.5-22.9 (reference)** | | | | | |
| **23-27.4** | 86.312 | 8.825 | 0.075 | 9.781 | <0.001 |
| **≥27.5** | 157.905 | 22.417 | 0.053 | 7.044 | <0.001 |
| **Paternal age** | 1.556 | 0.932 | 0.017 | 1.669 | 0.095 |
| **Parity, high order (versus 0)** | 62.961 | 13.955 | 0.034 | 4.512 | <0.001 |
| **Infertility duration (years)** | 1.282 | 1.364 | 0.007 | 0.940 | 0.347 |
| **FET cycle rank, High order (versus First)** | 26.116 | 7.516 | 0.027 | 3.474 | 0.001 |
| **Infertility cause** | | | | | |
| Tubal factors (reference) | | | | | |
| PCOS | 9.216 | 14.497 | 0.005 | 0.636 | 0.525 |
| Endometriosis | 2.194 | 13.273 | 0.001 | 0.165 | 0.869 |
| Diminished ovarian reserve. | -10.895 | 20.651 | -0.004 | -0.528 | 0.598 |
| Uterine factors | -10.179 | 26.186 | -0.003 | -0.389 | 0.697 |
| Male | -14.409 | 12.591 | -0.010 | -1.144 | 0.252 |
| Unexplained | -7.991 | 14.833 | -0.004 | -0.539 | 0.590 |
| **Fertilization method** | | | | | |
| IVF (reference) | | | | | |
| ICSI | -18.255 | 9.320 | -0.017 | -1.959 | 0.050 |
| VF+ICSI | 4.260 | 12.769 | 0.003 | 0.334 | 0.739 |
| **Number of embryos transferred, ≥2 (versus 1)** | 22.384 | 10.540 | 0.018 | 2.124 | 0.034 |
| **Embryo developmental stage at transfer, Day 5/6 (versus Day 3)** | 71.030 | 11.139 | 0.053 | 6.377 | <0.001 |
| **FET endometrial preparation, Artificial cycle (versus Natural cycle)** | -15.143 | 8.558 | -0.013 | -1.769 | 0.077 |
| **Endometrial thickness (mm)** | | | | | |
| <8 (reference) | | | | | |
| 8-11 | 32.475 | 13.757 | 0.033 | 2.361 | 0.018 |
| >11 | 45.989 | 14.243 | 0.045 | 3.229 | 0.001 |
| **Year of treatment** | | | | | |
| 2010–2012 (reference) | | | | | |
| 2013–2015 | -61.556 | 13.940 | -0.063 | -4.416 | <0.001 |
| 2016-2017 | -77.498 | 14.127 | -0.079 | -5.486 | <0.001 |
| **Gestational age (week)** | 178.687 | 2.429 | 0.548 | 73.556 | <0.001 |
| **Newborn gender, female (versus male)** | -137.516 | 7.200 | -0.141 | -19.098 | <0.001 |

confounders were included in the study, and the effect of different kinds of MAR treatments could not be reliably distinguished, which included less invasive treatments such as ovulation induction only that were less strongly associated with adverse birth outcomes (25). A recent study based on 4958 infertile women using a freeze-all strategy observed that maternal age grouping was not related with increased risks of LBW, very LBW, preterm LBW, and macrosomia (14). However, only 1450 singleton live births were involved for the analysis of LBW, and the 44–50-year-old group of singleton live births was very small (n = 9), which may limit the power of statistical analyses between groups.

Our study aimed to improve on the flaws of the abovementioned studies and focused on the exact role of maternal age in singleton birthweight after FET cycles. The current study based on 12,565 singleton newborns born after FET cycles demonstrated that maternal age itself had no impact on singleton birthweight, and neonatal outcomes including PTB, LBW, and SGA were similar between the different maternal age groups. Due to several confounding factors, direct comparability across different age groups has very limited clinical significance in **Table 2**. Because of the strict exclusion criteria, the generality of this finding may be, to some extent, restricted. The reason why no significant correlation existed between maternal age and birthweight in our study is likely complex. It is generally known that with aging comes a reduction in ovarian function, resulting in the decrease of ovarian response to ovulation-promoting drugs and the low number of oocytes retrieved (26). Additionally, the decreased quality of oocytes (27, 28), abnormal endometrial function, and degeneration of multiple organ function will appear in women with advanced maternal age (22, 29). All the above-mentioned factors would affect the

development of the embryo and cause adverse effects on the newborn, leading to LBW. However, with the popularization of education, many women tend to choose late marriage and late childbearing and enjoy a simple single life before marriage. In this kind of life, they are less stressed and have more opportunities to get in touch with life than women who are married and have children. Meanwhile, these knowledgeable women tend to choose a healthy and regular life and possess good habits, physical quality, and economic conditions, thus having a better choice on ART treatment (11). Aging leads to an irreversible decline in fertility, forcing older women to pay more attention to pregnancy and to seek medical help more actively than young women. In addition, the spouse's income increases with age to guarantee maternity. Most importantly, the development of ART has well fulfilled the reproductive needs of women with different ages to improve the quality of newborns.

In this study, the results of multiple linear regression analysis indicated that maternal BMI, embryo developmental stage at transfer, parity, number of embryos transferred, endometrial thickness, year of treatment, GA, and newborn gender were independent predictors for neonatal birthweight, which was consistent with previous results (22, 30, 31). Z-scores were calculated and compared across the four groups to reduce bias caused by newborn gender and GA, and no significant difference on Z-scores was found among different maternal age groups. In addition, significant differences were observed between the maternal age groups in baseline and cycle characteristics including infertility duration, infertility cause, and fertilization method. However, these confounders had no impact on neonatal birthweight based on the linear regression model.

This study has several limitations. The biggest one is its retrospective design, so we strictly checked the database with strict criteria. Second, due to personal privacy restrictions, we were unable to obtain the education and economic background of patients. Third, many confounding factors were strongly associated with birthweight in linear regression analysis. Data bias during the experimental design cannot be all corrected by regression equations. Furthermore, embryo quality and paternal BMI were important factors that may affect neonatal outcomes, yet these data were missed in this study. However, the large number of singleton live births from a single center can assure the practice consistency, which is the main strength of the current study. Additionally, aside from the change of culture medium types, all other laboratory conditions and protocols remained invariant throughout the study period. Furthermore, maternal age was recorded according to the identification card, and endometrial thickness was measured by the same trained sonographers, reducing recorder variability. Importantly, a number of potential confounders were included in our study, which may minimize their impact on the findings.

## CONCLUSIONS

This study expands the current knowledge about the association between maternal age and neonatal outcomes and shows that maternal age is not associated with mean birthweight and Z-scores. This important finding should be adequately applied for women over 40 years old prior to FET and strengthen their confidence. A large prospective study, of course, is needed to verify our findings in the future.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

## ETHICS STATEMENT

This study was approved by the institutional review board of the Ninth People's Hospital of Shanghai Jiao Tong University School of Medicine (reference number 2017-211) and the Changhai Hospital of Naval Medical University (reference number CHEC 2019-100), and was carried out in accordance with the Helsinki Declaration. Due to the retrospective nature, informed consent was not required, and patients' data were used anonymously.

## AUTHOR CONTRIBUTIONS

C-qY and Y-pK conceived and designed this study. Z-xN, K-mW, and Z-hZ contributed to data acquisition, analysis and interpretation, and drafted the manuscript. K-mW and Z-xN were responsible for the collection of data. All authors interpreted the data. All authors contributed to the article and approved the submitted version.

## FUNDING

This work was supported by the National Natural Science Foundation of China (81774352, 81703874).

## ACKNOWLEDGMENTS

The authors thank the nurses and laboratory staff of the Department of Assisted Reproduction for their contribution to this work. Moreover, the authors thank the infertile couples who participated in this study.

## REFERENCES

1. Nygren KG, Andersen AN. Assisted Reproductive Technology in Europe, 1997. Results Generated From European Registers by ESHRE. European IVF-Monitoring Programme (EIM), for the European Society of Human Reproduction and Embryology (ESHRE). *Hum Reprod* (2001) 16(2):384–91. doi: 10.1093/humrep/16.2.384
2. De Geyter C, Calhaz-Jorge C, Kupka MS, Wyns C, Mocanu E, Motrenko T, et al. European IVF-Monitoring Consortium (EIM) for the European Society of Human Reproduction and Embryology (ESHRE). ART in Europe, 2015:

Results Generated From European Registries by ESHRE. *Hum Reprod Open* (2020) 2020(1):hoz038. doi: 10.1093/hropen/hoz038
3. Berntsen S, Söderström-Anttila V, Wennerholm UB, Laivuori H, Loft A, Oldereid NB, et al. The Health of Children Conceived by ART: 'The Chicken or the Egg? *Hum Reprod Update* (2019) 25(2):137–58. doi: 10.1093/humupd/dmz001
4. Kawwass JF, Badell ML. Maternal and Fetal Risk Associated With Assisted Reproductive Technology. *Obstet Gynecol* (2018) 132(3):763–72. doi: 10.1097/AOG.0000000000002786
5. Pinheiro RL, Areia AL, Mota Pinto A, Donato H. Advanced Maternal Age: Adverse Outcomes of Pregnancy, A Meta-Analysis. *Acta Med Port* (2019) 32 (3):219–26. doi: 10.20344/amp.11057
6. Leader J, Bajwa A, Lanes A, Hua X, Rennicks White R, Rybak N, et al. The Effect of Very Advanced Maternal Age on Maternal and Neonatal Outcomes: A Systematic Review. *J Obstet Gynaecol Can* (2018) 40(9):1208–18. doi: 10.1016/j.jogc.2017.10.027
7. Lean SC, Derricott H, Jones RL, Heazell AEP. Advanced Maternal Age and Adverse Pregnancy Outcomes: A Systematic Review and Meta-Analysis. *PloS One* (2017) 12(10):e0186287. doi: 10.1371/journal.pone.0186287
8. Wu Y, Chen Y, Shen M, Guo Y, Wen SW, Lanes A, et al. Adverse Maternal and Neonatal Outcomes Among Singleton Pregnancies in Women of Very Advanced Maternal Age: A Retrospective Cohort Study. *BMC Pregnancy Childbirth* (2019) 19(1):3. doi: 10.1186/s12884-018-2147-9
9. Koshida S, Arima H, Fujii T, Ito Y, Murakami T, Takahashi K. Impact of Advanced Maternal Age on Adverse Infant Outcomes: A Japanese Population-Based Study. *Eur J Obstet Gynecol Reprod Biol* (2019) 242:178–81. doi: 10.1016/j.ejogrb.2019.08.011
10. Luke B, Brown MB. Elevated Risks of Pregnancy Complications and Adverse Outcomes With Increasing Maternal Age. *Hum Reprod* (2007) 22(5):1264–72. doi: 10.1093/humrep/del522
11. Wennberg AL, Opdahl S, Bergh C, Aaris Henningsen AK, Gissler M, Romundstad LB, et al. Effect of Maternal Age on Maternal and Neonatal Outcomes After Assisted Reproductive Technology. *Fertil Steril* (2016) 106 (5):1142–9.e14. doi: 10.1016/j.fertnstert.2016.06.021
12. Moaddab A, Chervenak FA, Mccullough LB, Sangi-Haghpeykar H, Shamshirsaz AA, Schutt A, et al. Effect of Advanced Maternal Age on Maternal and Neonatal Outcomes in Assisted Reproductive Technology Pregnancies. *Eur J Obstet Gynecol Reprod Biol* (2017) 216:178–83. doi: 10.1016/j.ejogrb.2017.07.029
13. Barbuscia A, Martikainen P, Myrskylä M, Remes H, Somigliana E, Klemetti R, et al. Maternal Age and Risk of Low Birth Weight and Premature Birth in Children Conceived Through Medically Assisted Reproduction. Evidence From Finnish Population Registers. *Hum Reprod* (2020) 35(1):212–20. doi: 10.1093/humrep/dez275
14. Lin J, Huang J, Zhu Q, Kuang Y, Cai R, Wang Y. Effect of Maternal Age on Pregnancy or Neonatal Outcomes Among 4,958 Infertile Women Using a Freeze-All Strategy. *Front Med (Lausanne)* (2020) 6:316. doi: 10.3389/fmed.2019.00316
15. Pereira N, Elias RT, Christos PJ, Petrini AC, Hancock K, Lekovich JP, et al. Supraphysiologic Estradiol is an Independent Predictor of Low Birth Weight in Full-Term Singletons Born After Fresh Embryo Transfer. *Hum Reprod* (2017) 32(7):1410–7. doi: 10.1093/humrep/dex095
16. Imudia AN, Awonuga AO, Doyle JO, Kaimal AJ, Wright DL, Toth TL, et al. Peak Serum Estradiol Level During Controlled Ovarian Hyperstimulation is Associated With Increased Risk of Small for Gestational Age and Preeclampsia in Singleton Pregnancies After *In Vitro* Fertilization. *Fertil Steril* (2012) 97(6):1374–9. doi: 10.1016/j.fertnstert.2012.03.028
17. Järvelä IY, Pelkonen S, Uimari O, Mäkikallio K, Puukka K, Ruokonen A, et al. Controlled Ovarian Hyperstimulation Leads to High Progesterone and Estradiol Levels During Early Pregnancy. *Hum Reprod* (2014) 29(11):2393–401. doi: 10.1093/humrep/deu223
18. Kuang Y, Chen Q, Hong Q, Lyu Q, Ai A, Fu Y, et al. Double Stimulations During the Follicular and Luteal Phases of Poor Responders in IVF/ICSI Programmes (Shanghai Protocol). *Reprod BioMed Online* (2014) 29(6):684–91. doi: 10.1016/j.rbmo.2014.08.009
19. Kuang Y, Chen Q, Fu Y, Wang Y, Hong Q, Lyu Q, et al. Medroxyprogesterone Acetate is an Effective Oral Alternative for Preventing Premature Luteinizing Hormone Surges in Women Undergoing Controlled Ovarian Hyperstimulation for *In Vitro* Fertilization. *Fertil Steril* (2015) 104(1):62–70.e3. doi: 10.1016/j.fertnstert.2015.03.022
20. Du T, Chen H, Fu R, Chen Q, Wang Y, Mol BW, et al. Comparison of Ectopic Pregnancy Risk Among Transfers of Embryos Vitrified on Day 3, Day 5, and Day 6. *Fertil Steril* (2017) 108(1):108–16.e1. doi: 10.1016/j.fertnstert.2017.05.027
21. Nelissen EC, Van Montfoort AP, Coonen E, Derhaag JG, Geraedts JP, Smits LJ, et al. Further Evidence That Culture Media Affect Perinatal Outcome: Findings After Transfer of Fresh and Cryopreserved Embryos. *Hum Reprod* (2012) 27(7):1966–76. doi: 10.1093/humrep/des145
22. Zhang J, Liu H, Mao X, Chen Q, Si J, Fan Y, et al. Effect of Endometrial Thickness on Birthweight in Frozen Embryo Transfer Cycles: An Analysis Including 6181 Singleton Newborns. *Hum Reprod* (2019) 34(9):1707–15. doi: 10.1093/humrep/dez103
23. Dai L, Deng C, Li Y, Zhu J, Mu Y, Deng Y, et al. Birth Weight Reference Percentiles for Chinese. *PloS One* (2014) 9(8):e104779. doi: 10.1371/journal.pone.0104779
24. Castillo CM, Horne G, Fitzgerald CT, Johnstone ED, Brison DR, Roberts SA. The Impact of IVF on Birthweight From 1991 to 2015: A Cross-Sectional Study. *Hum Reprod* (2019) 34(5):920–31. doi: 10.1093/humrep/dez025
25. De Geyter C, Calhaz-Jorge C, Kupka MS, Wyns C, Mocanu E, Motrenko T, et al. European IVF-Monitoring Consortium (EIM) for the European Society of Human Reproduction and Embryology (ESHRE). ART in Europe, 2014: Results Generated From European Registries by ESHRE: The European IVF-Monitoring Consortium (EIM) for the European Society of Human Reproduction and Embryology (ESHRE). *Hum Reprod* (2018) 33(9):1586–601. doi: 10.1093/humrep/dey242
26. Yang X, Zhang J, Wu J, Huang J, Chen Q, Lu X, et al. Association Between the Number of Oocytes Retrieved and Neonatal Outcomes After Freeze-All IVF Cycles. *Hum Reprod* (2019) 34(10):1937–47. doi: 10.1093/humrep/dez137
27. Pacella L, Zander-Fox DL, Armstrong DT, Lane M. Women With Reduced Ovarian Reserve or Advanced Maternal Age Have an Altered Follicular Environment. *Fertil Steril* (2012) 98(4):986–94.e1-2. doi: 10.1016/j.fertnstert.2012.06.025
28. Pella R, Suarez-Cunza S, Orihuela P, Escudero F, Perez Y, Garcia M, et al. Oxidative Balance in Follicular Fluid of ART Patients of Advanced Maternal Age and Blastocyst Formation. *JBRA assisted Reprod* (2020) 24(3):296–301. doi: 10.5935/1518-0557.20200012
29. Gibson DA, Simitsidellis I, Kelepouri O, Critchley HOD, Saunders PTK. Dehydroepiandrosterone Enhances Decidualization in Women of Advanced Reproductive Age. *Fertil Steril* (2018) 109(4):728–34.e2. doi: 10.1016/j.fertnstert.2017.12.024
30. Zhang J, Wang Y, Liu H, Mao X, Chen Q, Fan Y, et al. Effect of *In Vitro* Culture Period on Birth Weight After Vitrified-Warmed Transfer Cycles: Analysis of 4,201 Singleton Newborns. *Fertil Steril* (2019) 111(1):97–104. doi: 10.1016/j.fertnstert.2018.10.006
31. Zhang J, Liu H, Mao X, Chen Q, Fan Y, Xiao Y, et al. Effect of Body Mass Index on Pregnancy Outcomes in a Freeze-All Policy: An Analysis of 22,043 First Autologous Frozen-Thawed Embryo Transfer Cycles in China. *BMC Med* (2019) 17(1):114. doi: 10.1186/s12916-019-1354-1

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 16 March 2022
doi: 10.3389/fendo.2022.837731

# Comparison of Perinatal Outcomes of Letrozole-Induced Ovulation and Hormone Replacement Therapy Protocols in Patients With Abnormal Ovulation Undergoing Frozen-Thawed Embryo Transfer: A Propensity Score Matching Analysis

*Wenjuan Zhang*[†], *Zhaozhao Liu*[†], *Junwei Zhang*, *Bingnan Ren*, *Manman Liu*, *Jiaheng Li*, *Wen Zhang and Yichun Guan*[*]

*Reproduction Center, The Third Affiliated Hospital of ZhengZhou University, ZhengZhou, China*

**Background:** With the increasing use of frozen embryo transfer (FET), the best endometrial preparation protocol is continuously being discussed. The hormone replacement therapy (HRT) cycle and letrozole-induced ovulation (L-OI) cycle are available protocols for patients with abnormal ovulation. Previous comparisons of the two protocols have focused on pregnancy outcomes, with less attention to perinatal outcomes, and population heterogeneity was large; thus, convincing conclusions about which protocol is more appropriate could not be drawn.

**Methods:** We performed a retrospective cohort study using propensity score matching (PSM) analysis for a population of patients undergoing FET cycles in the reproductive center of the Third Affiliated Hospital of Zhengzhou University from January 2016 to September 2020. The main outcome measures were clinical pregnancy rate, live birth rate, very preterm delivery (VPTD), preterm delivery (PTD), low birth weight (LBW), macrosomia, small for gestational age (SGA), large for gestational age (LGA), hypertensive disorders of pregnancy (HDP), gestational diabetes mellitus (GDM), premature rupture of membranes (PROM), placenta previa, and congenital abnormality.

**Results:** A total of 8010 women were enrolled. Due to the large heterogeneity among the patients, we conducted 1:1 PSM, and 1461 women matched in each group. Compared with the HRT group, the L-OI group had a smaller proportion of thin endometrium (27.38% vs. 41.07%) and thicker endometrium on the day of embryo transfer (9.63 ± 1.82 vs. 8.91 ± 1.38). There were no significant differences in clinical pregnancy rate, early abortion rate or live birth rate between the groups. There was no significant difference in perinatal outcomes of singleton live birth, including VPTD, PTD, postterm delivery, LBW, macrosomia, SGA, LGA, GDM, HDP, placenta previa, and congenital malformation.

OPEN ACCESS

***Edited by:***
*Yimin Zhu,*
*Zhejiang University, China*

***Reviewed by:***
*Kok-Min Seow,*
*Shin Kong Wu Ho-Su Memorial Hospital, Taiwan*
*Bo Sun,*
*First Affiliated Hospital of Zhengzhou University, China*

****Correspondence:***
*Yichun Guan*
*guanyichunmay@163.com*

*[†]These authors have contributed equally to this work*

***Specialty section:***
*This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology*

***Received:*** *17 December 2021*
***Accepted:*** *17 February 2022*
***Published:*** *16 March 2022*

***Citation:***
*Zhang W, Liu Z, Zhang J, Ren B, Liu M, Li J, Zhang W and Guan Y (2022) Comparison of Perinatal Outcomes of Letrozole-Induced Ovulation and Hormone Replacement Therapy Protocols in Patients With Abnormal Ovulation Undergoing Frozen-Thawed Embryo Transfer: A Propensity Score Matching Analysis. Front. Endocrinol. 13:837731. doi: 10.3389/fendo.2022.837731*

**Conclusion:** For women with abnormal ovulation, the pregnancy and perinatal outcomes of HRT and L-OI protocols are reassuring. It seems that both protocols are safe and effective for endometrial preparation in frozen-thawed embryo transfer in the clinic.

**Keywords: frozen-thawed embryo transfer, pregnancy outcomes, perinatal outcomes, letrozole-induced ovulation protocol, hormone replacement therapy protocol**

## INTRODUCTION

Since the first successful live birth following human frozen embryo transfer (FET) reported by Zeilmaker's team (1), the number of FET cycles has increased steadily worldwide due to improvements in laboratory technology, especially vitrification technology, and an increase in the number of available embryos (2, 3). In addition, the "whole-embryo freezing" strategy, i.e., selective freezing of all embryos before FET, has become a suitable option, especially for patients with a high risk of ovarian hyperstimulation syndrome (OHSS), preimplantation genetic testing (PGT) and double ovarian stimulation (DuoStim), as it reduces complications while simultaneously enhancing the live birth rate (4, 5).

Recently, many studies have shown that the outcome of frozen embryo transfer cycles was not inferior to that of FET cycles (6, 7), and some studies have even suggested that FET was associated with a higher pregnancy rate and lower complication rate (8, 9). Nevertheless, a recent meta-analysis showed that FET was associated with an increased risk of hypertensive disorders of pregnancy (HDP), postterm delivery, macrosomia and large for gestational age (LGA) (10, 11), but with reduced risk of preterm birth (PTD), low birth weight (LBW) and small for gestational age (SGA) (12, 13).

Endometrial preparation protocols optimize the success rate of FET by synchronizing endometrial receptivity and embryonic development stage. Multiple protocols for endometrial preparation for FET have been explored. A natural cycle (NC), an artificial cycle with hormone replacement therapy (HRT), and a cycle with ovulation induction (OI) are the most common protocols. All three protocols are suitable for patients with normal ovulation, and the latter two are also appropriate for patients with ovulatory disorders. Several recent retrospective studies found that NC was the best choice for women with normal ovulation (14, 15); however, there is no unified conclusion on the optimal choice for patients with abnormal ovulation (16, 17).

Clomiphene (CC) and letrozole (LE) are commonly used drugs in the OI cycle. In recent years, LE has been most widely used in OI for patients with polycystic ovary syndrome (PCOS), and it is the first-line OI drug for PCOS patients (18, 19). LE, a third-generation aromatase inhibitor, is commonly used in the clinic because it does not consume estrogen receptors, maintains a normal central feedback system, and promotes normal follicular growth, and it has no negative impact on the endometrium (20, 21) or pregnancy or fetal development (22). Because of its convenience, low cost and time controllability, HRT cycles have been widely applied for patients with abnormal ovulation (2, 23).

Recent studies have demonstrated that by using exogenous estrogen and progesterone to prepare the endometrium and inhibit ovulation, the HRT protocol in FET affected maternal and neonatal outcomes, resulting in the loss of the corpus luteum (CL), which can lead to adverse perinatal outcomes (14, 24). However, at present, there are few studies on the specific population of patients with abnormal ovulation, and heterogeneity in this population is large. Furthermore, as most comparisons between HRT and OI cycles have focused on the clinical pregnancy rate or live birth rate and paid little attention to maternal and neonatal outcomes, convincing conclusions cannot be drawn.

Therefore, this study aimed to explore the relationship between exposure of patients with abnormal ovulation to different endometrial preparation protocols and pregnancy and perinatal outcomes, including pregnancy rate, live birth rate, adverse obstetric complications and neonatal outcomes, to further optimize maternal and infant health after FET in patients with abnormal ovulation.

## MATERIALS AND METHODS

### Patients

A total of 8010 women who were undergoing FET cycles from January 2016 to September 2020 at our center were enrolled. We included FET cycles of oligoanovulation (menstrual cycle>37 d), anovulation with letrozole-induced ovulation (L-OI) or HRT after *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI). The following exclusion criteria were applied: 1) maternal age >40 years; 2) adenomyosis, uterine malformations or recurrent miscarriage; and 3) use of donor oocytes and PGT cycles.

### Endometrial Preparation Protocols

For HRT cycles, patients were prescribed 2 mg of estradiol valerate (Bayer Co., Germany) to be taken orally three times daily, starting at days 2-4 of menstruation for 7 days. Then, the drug dose was adjusted according to the thickness of the endometrium (up to 9 mg per day). Endometrial transformation was performed when the medication was taken for more than 12 days and endometrial thickness was ≥7 mm; the cycle was cancelled if endometrial thickness was less than 7 mm.

LE (2.5 mg/5 mg) was administered orally for 5 days on the 3rd-5th days of menstruation, and the follicular development speed was monitored by ultrasound. If follicular development was poor, HMG (Lizhu Pharmaceutical Trading Co., China) (37.5-75 IU daily) was added as appropriate to aid in the development of follicles. When the dominant follicle developed to 14 mm, the serum luteinizing hormone (LH) level indicated that ovulation was about to occur, the estradiol (E2) level was more than 150 pg/mL, and the endometrium thickness was more than 7 mm, 10,000 IU urinary

hCG was injected (Lizhu Pharmaceutical Trading Co., China). Endometrial transformation was then performed. The cycle was cancelled if follicular dysplasia occurred.

For HRT or L-OI cycles, oral dydrogesterone (2 times daily, 10 mg once) (Abbott Co. USA) and intravaginal administration of 90 mg of a progesterone sustained-release vaginal gel (Merck Co. Germany) were given as luteal phase support until the 12th week of pregnancy. The same dose of estrogen valerate as before transformation was taken until 14 days after embryo transfer. In the case of pregnancy, the drug was continued until clinical pregnancy, which was defined as the presence of an intrauterine gestational sac by ultrasonography at 7–8 weeks of gestation.

## Data Collection and Outcome Definition

Patient characteristics, such as age, body mass index (BMI), type of infertility, indication for IVF, duration of infertility, basal serum follicle stimulating hormone (FSH), basal antral follicle count (AFC), the number of previous FET failures, endometrial thickness, number of transferred embryos, developmental stage of embryo, pregnancy or live birth, and singleton or twins, were collected through the electronic case system of our center.

For patients with a gestational sac echo and singleton live birth after embryo transfer, pregnancy complications were collected during a telephone follow-up and recorded by a designated nurse in our center. Maternal and neonatal outcomes were recorded and classified according to the information provided by the patients.

Early spontaneous abortion was defined as a clinical pregnancy that failed to reach the 12th gestational week. Live birth was defined as the birth of a live child after 28 weeks of gestation per embryo transfer cycle. Very preterm delivery (VPTD), preterm delivery (PTD), term birth and postterm delivery were defined as a baby born after <32 weeks, <37 weeks of gestation, ≤37 weeks ≤ 41 weeks and >41 weeks of gestation, respectively. The neonatal birth weight of singleton live births was as follows: LBW (<2500 g), SGA (<10th percentile for gestational age) (25), macrosomia (≥4000 g), and LGA (>90th percentile for gestational age) (25).

## Statistical Analysis

All statistical management and analyses were performed using SPSS software, version 22.0.

Because there was obvious heterogeneity in basic characteristics, the data were analyzed after 1:1 propensity score matching (PSM).

The one-sample K-S test was used to check for normality. Continuous variables with abnormal distributions are expressed as the mean ± SD, and Student's t test was used to assess between-group differences. Categorical variables are represented as the number of cases (n) and percentage (%).

Means from chi-square analyses were used to assess differences between the groups. Multiple logistic regression was applied to further analyze different items. Unadjusted odds ratios and adjusted odds ratios with 95% confidence intervals (CIs) were calculated. Statistical significance was set at $P<0.05$.

# RESULTS

## Study Population

From January 2016 to September 2020, 8010 FET cycles were evaluated according to the inclusion and exclusion criteria. There were 6549 patients in the HRT group and 1461 patients in the L-OI group. We separately analyzed the patients with a gestational sac echo and singleton live birth after embryo transfer, with 395 patients in the HRT group and 457 in the L-OI group.

## Baseline Characteristics

When comparing basic characteristics between the two groups, we found that there were differences in female and male age, type of infertility, indication for IVF, duration of infertility, basal serum FSH, and basal AFC (**Table 1**). Therefore, based on these

**TABLE 1** | Patient clinical characteristics.

| Characteristics | HRT (6549) | L-OI (1461) | P value |
|---|---|---|---|
| Female age (y) | 31.14 ± 4.40 | 30.31 ± 4.06 | 0.000 |
| Male age (y) | 32.20 ± 5.34 | 31.25 ± 4.56 | 0.000 |
| Body mass index (kg/m2) | 23.98 ± 3.29 | 24.02 ± 3.16 | 0.634 |
| Type of infertility | | | 0.012 |
| Primary infertility | 42.80% (2803/6549) | 46.41% (678/1461) | |
| Secondary infertility | 57.20% (3746/6549) | 53.59% (783/1461) | |
| Indication for IVF | | | |
| Tubal factor | 36.11% (2365/6549) | 31.35% (458/1461) | 0.001 |
| Endometriosis | 0.37% (24/6549) | 0.55% (8/1461) | 0.321 |
| Ovulatory dysfunction | 12.98% (850/6549) | 14.37% (210/1461) | 0.155 |
| Male factor | 17.50% (1146/6549) | 21.01% (308/1461) | 0.001 |
| Others | 5.08% (333/6549) | 5.61% (82/1461) | 0.410 |
| Mixed factors | 27.96% (1831/6549) | 27.04% (395/1461) | 0.477 |
| Duration of Infertility (y) | 3.47 ± 2.84 | 3.34 ± 2.67 | 0.000 |
| Basal serum FSH level (IU/L) | 7.20 ± 19.05 | 6.15 ± 2.25 | 0.000 |
| Basal antral follicle count | 17.76 ± 8.15 | 20.14 ± 7.22 | 0.000 |

*Data are presented as the mean ± SD for continuous variables and % (n/N) for categorical variables. Student's t test was used for continuous variables, and the Pearson's chi-squared test was used for categorical variables with Fisher's exact test when necessary.*

**TABLE 2** | Patient clinical characteristics after PSM.

| Characteristics | HRT (1461) | L-OI (1461) | P value |
|---|---|---|---|
| Female age (y) | 30.14 ± 4.04 | 30.31 ± 4.06 | 0.250 |
| Male age (y) | 31.12 ± 4.83 | 31.25 ± 4.56 | 0.479 |
| Body mass index (kg/m2) | 24.03 ± 3.38 | 24.02 ± 3.16 | 0.773 |
| Type of infertility | | | 0.251 |
| Primary infertility | 48.53% (709/1461) | 46.41% (678/1461) | |
| Secondary infertility | 51.47% (752/1461) | 53.59% (783/1461) | |
| Indication for IVF | | | |
| Tubal factor | 34.84% (509/1461) | 31.35% (458/1461) | 0.045 |
| Endometriosis | 0.41% (6/1461) | 0.55% (8/1461) | 0.592 |
| Ovulatory dysfunction | 12.80% (187/1461) | 14.37% (210/1461) | 0.214 |
| Male factor | 18.89% (276/1461) | 21.08% (308/1461) | 0.139 |
| Others | 4.93% (72/1461) | 5.61% (82/1461) | 0.408 |
| Mixed factors | 28.13% (411/1461) | 27.04% (395/1461) | 0.508 |
| Duration of Infertility (y) | 3.40 ± 2.61 | 3.34 ± 2.67 | 0.533 |
| Basal serum FSH level (IU/L) | 6.15 ± 2.58 | 6.15 ± 2.25 | 0.986 |
| Basal antral follicle count | 19.85 ± 7.35 | 20.14 ± 7.22 | 0.295 |

*Data are presented as the mean ± SD for continuous variables and % (n/N) for categorical variables. Student's t test was used for continuous variables, and the Pearson's chi-squared test was used for categorical variables with Fisher's exact test when necessary.*

differences, we conducted 1:1 PSM, and 1461 women were matched in each group. After matching, there were no significant differences in basic characteristics between the groups (**Table 2** and **Figure 1**).

We found that the number of previous FET failures was higher in the L-OI group than that of the HRT group. In terms of clinical data, the endometrium was thicker and the proportion of thin endometrium lower in the L-OI group (**Table 3**).

## Clinical Outcomes

In terms of clinical outcome, there were no significant differences in clinical pregnancy rate, early abortion rate or live birth rate between the two groups, but the twin rate was higher in the HRT group, which may be because the number of transferred embryos was greater than that in the L-OI group (**Table 4**).

Regarding the main outcome measures, we conducted a multiple logistic regression analysis to adjust for the influence of confounding factors. The included factors were female age, number of previous FET failures, BMI, AFC, endometrial thickness on the day of embryo transfer, thin endometrium and number of transferred embryos. After adjustments for confounding factors, the clinical pregnancy rate, early spontaneous abortion rate, live birth rate and twin rate were not significantly different between the groups (**Table 5**).

We mainly analyzed maternal and neonatal outcomes and observed no significant differences in perinatal outcomes, including VPTD, PTD, postterm delivery, LBW, macrosomia, SGA, LGA, GDM, HDP, placenta previa, and congenital malformation, between the groups (**Table 6**). The same

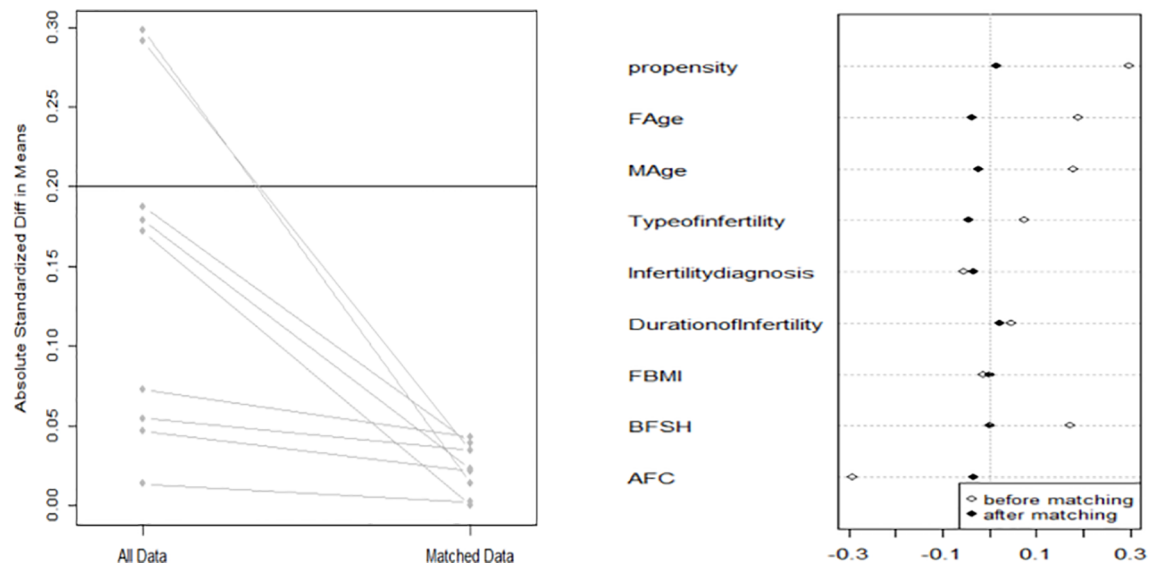



**FIGURE 1** | After 1:1 PSM, the data heterogeneity between the two groups was significantly reduced.

**TABLE 3** | Patient clinical and embryological characteristics.

| Characteristics | HRT (1461) | L-OI (1461) | P value |
|---|---|---|---|
| Number of previous FET failures | 0.11 ± 0.330 | 0.16 ± 0.422 | 0.000 |
| Endometrial thickness on the day of embryo transfer (mm) | 8.91 ± 1.38 | 9.63 ± 1.82 | 0.000 |
| Thin endometrium | 41.07% (60/1461) | 27.38% (40/1461) | 0.042 |
| Number of transferred embryos | 1.47 ± 0.50 | 1.42 ± 0.49 | 0.002 |
| One | 52.57% (768/1461) | 58.38% (853/1461) | |
| Two | 47.43% (693/1461) | 41.62% (608/1461) | |
| Development stage of the embryo | | | 0.604 |
| D3 | 31.69% (463/1461) | 30.80% (450/1461) | |
| D5/D6 | 68.31% (998/1461) | 69.20% (1011/1461) | |

*Data are presented as the mean ± SD for continuous variables and % (n/N) for categorical variables. Student's t test was used for continuous variables, and the Pearson's chi-squared test was used for categorical variables with Fisher's exact test when necessary.*
*FET, Frozen embryo transfer.*

conclusion was reached after further multiple logistic regression analysis (**Table 7**).

## DISCUSSION

Our study showed no difference in pregnancy rate, live birth rate or abortion rate between HRT and L-OI cycles for patients with abnormal ovulation. Moreover, there was no difference between the two groups regarding perinatal outcomes.

For patients with abnormal ovulation, both HRT and L-OI are common endometrial preparation protocols in the clinic. The safety and OI effect of LE have also been generally recognized (26). Previous studies have suggested that L-OI cycles resulted in a higher live birth rate than HRT cycles; nevertheless, these studies did not examine maternal and neonatal outcomes (16, 27). Interestingly, some studies reached the same conclusions as in our study. A prospective study including 116 PCOS patients reported similar clinical pregnancy rates for HRT and L-OI protocols (28). Another randomized controlled study including 100 patients found that the L-OI protocol did not improve pregnancy outcomes compared with HRT (17). However, these studies did not focus on maternal and infant outcomes.

Despite no difference in pregnancy outcomes between the two groups, endometrial thickness on the day of embryo transfer in the L-OI group was greater than that in the HRT group in our study, which was consistent with a previous study from our center (29). The reason may be that the proportion of patients with thin endometrium was relatively high in the HRT group. In addition, LE has no negative effects on endometrial or cervical mucus. LE can enable full endometrial pinopode expression and increase integrin αvβ3 expression in the endometrium during implantation (18, 30). LE further decreases intraovarian and serum estrogen levels by blocking conversion of androgens to estrogens in ovarian granulosa cells (20). Subsequently, low estrogen levels reduce ubiquitination of estrogen receptors. This process leads to faster endometrial proliferation and increased blood levels in the uterus and endometrium, with positive effects on pregnancy outcomes (31, 32). Thus, L-OI can be used to prepare endometrial tissue for FET for patients with thin endometrial tissue. However, a previous study in our center reported that L-OI cycles were associated with a higher live birth rate than HRT cycles. Although the live birth rate was increased in our study by using the L-OI protocol, there was no significant differences between the groups possibly due to a large difference in the number of cases included in the L-OI (502) and HRT (2280) groups. There was also heterogeneity in basic characteristics; a previous study adopted regression analysis for correction (29), which was different from the 1:1 PSM in our study.

Moreover, our study found no significant difference in maternal or infant health between the two groups. A large retrospective cohort study in Japan in 2017 that included 110,772 FET cycles, which were divided into an LE-induced ovulation group, NC group and HRT group according to the endometrial preparation protocol used, found that neonatal outcomes of the different treatment schemes were basically similar, consistent with our results. A previous study in our center also reached similar conclusions (29).

There have been few studies on the perinatal complications and infant safety of the two protocols. Studies have shown that newborns were likely to have LBW and macrosomia after HRT cycles (14, 33) while pregnant women had an increased risk of

**TABLE 4** | Clinical outcomes.

| | HRT (1461) | L-OI (1461) | P value |
|---|---|---|---|
| Clinical pregnancy rate | 50.17% (733/1461) | 49.01% (716/1461) | 0.529 |
| Early spontaneous abortion rate | 10.95% (160/1461) | 9.38% (137/1461) | 0.159 |
| Live birth rate | 36.41% (532/1461) | 37.99% (555/1461) | 0.379 |
| Singletons | 78.01% (415/532) | 85.05% (472/555) | 0.003 |
| Twin | 21.99% (117/532) | 14.95% (83/555) | |

*Data are presented as % (n/N) for categorical variables. The Pearsonc2 test was used for categorical variables with Fisher's exact test when necessary.*

**TABLE 5 |** Unadjusted and adjusted odds ratios of pregnancy outcomes following L-OI versus HRT cycles.

| | Unadjusted OR (95%CI) | Adjusted OR (95%CI) |
|---|---|---|
| Clinical pregnancy rate | 1.048 (0.906-1.211) | 1.086 (0.93-1.267) |
| Early spontaneous abortion rate | 1.189 (0.934-1.512) | 1.248 (0.973-1.602) |
| Live birth rate | 0.932 (0.802-1.083) | 0.951 (0.808-1.120) |
| Twins | 1.606 (1.177-2.191) | 1.431 (0.994-2.060) |

*The analysis was adjusted for female age, number of previous FET failures, BMI, AFC, endometrial thickness on the day of embryo transfer, thin endometrium and number of transferred embryos. CI, Confidence interval; FET, Frozen-thawed embryo transfer.*

HDP and cesarean section (15, 34). Saito et al.'s study suggested that the HRT cycles were associated with a higher risk of HDP and placental implantation and a lower risk of GDM (35). Another meta-analysis demonstrated that compared with the NC protocol, the OI protocol was associated with an increased incidence of PTD and LBW (36). However, the studies mentioned above compared three protocols, and the study population was not limited.

Recent studies have shown that the HRT protocol lacks CL, which is a crucial hormone for embryo implantation, placenta and pregnancy maintenance. Recent studies emphasized that loss of CL is associated with altered vascular health and insufficient cardiovascular adaptation in early pregnancy, leading to the occurrence of preeclampsia, affecting placental formation and causing placental hyperplasia (37, 38), with impacts on the mother and newborn. CL not only provides estrogen and progesterone but also vasoactive substances, such as relaxin and vascular endothelial growth factor, which may be important for placental formation. These substances are not available in the HRT cycle, which may increase the incidence of obstetric complications (39, 40). In our study, there was no difference between the two groups with regard to singleton delivery. The reason may be due to the different doses and types of luteal support after FET.

In FET cycles, it is necessary to add progesterone to obtain sufficient corpus luteum support to obtain a good pregnancy outcome due to the lack of endogenous progesterone production. In our study, corpus luteum support was provided by a combination of oral and vaginal administration, and the dose was sufficient. In Hu et al.'s study, only oral dydrogesterone (20 mg/d) was applied as luteal support in HRT cycles (14). Previous studies have suggested that dydrogesterone alone was likely not effective as a monotherapy in FET (41) but that the combination of oral and vaginal administration increased the concentration of progesterone in the serum and endometrium and improved the reproductive outcome (42, 43). In the study of Zong et al. dydrogesterone (40 mg/d) and progesterone capsules (Utrogestan, Capsugel) (200 mg/d) were given as luteal-phase support in HRT and OI cycles (33). This was not consistent with our study, in which oral dydrogesterone (60 mg/d) and intravaginal administration of 90 mg of a progesterone sustained-release vaginal gel were given as luteal-phase support. A recent meta-analysis suggested that once-daily Crinone gel or micronized progesterone (200 mg) three times per day is the most suitable luteal support dose (44).

Another reason may be that in some studies, when there were significant differences in basic characteristics and obvious differences in the number of included populations between the groups, logistic regression was used to correct confounding

**TABLE 6 |** Perinatal and neonatal outcomes of singleton live birth.

| | HRT (395) | L-OI (457) | P value |
|---|---|---|---|
| VPTD | 0.25% (1/395) | 0.66% (3/457) | 0.390 |
| PTD | 5.82% (23/395) | 6.13% (28/457) | 0.852 |
| Term birth | 86.33% (341/395) | 87.96% (402/457) | 0.476 |
| Postterm delivery | 7.59% (30/395) | 5.25% (24/457) | 0.162 |
| Neonatal weight (g) | 3459.86 ± 468.92 | 3419.74 ± 519.33 | 0.240 |
| Newborn's sex | | | 0.441 |
| Male | 54.94% (217/395) | 52.30% (239/457) | |
| Female | 45.06% (178/395) | 47.70% (218/457) | |
| LBW | 2.78% (11/395) | 3.06% (14/457) | 0.810 |
| Macrosomia | 10.38% (41/395) | 10.72% (49/457) | 0.871 |
| SGA | 5.06% (20/395) | 5.69% (26/457) | 0.687 |
| LGA | 18.23% (72/395) | 17.72% (81/457) | 0.849 |
| GDM | 7.85% (31/395) | 8.32% (38/457) | 0.803 |
| HDP | 6.33% (25/395) | 4.60% (21/457) | 0.264 |
| PROM | 5.06% (20/395) | 3.58% (17/457) | 0.337 |
| Placenta previa | 0.25% (1/395) | 0.44% (2/457) | 0.555 |
| Others | 0.76% (3/395) | 1.09% (5/457) | 0.882 |
| Congenital malformation | 1.27% (5/395) | 0.22% (1/457) | 0.158 |

*Data are presented as the mean ± SD for continuous variables and % (n/N) for categorical variables. Student's t test was used for continuous variables, and the Pearson's chi-squared test was used for categorical variables with Fisher's exact test when necessary.*
*VPTD, very preterm delivery; PTD, preterm delivery; LBW, low birth weight; SGA, small for gestational age; LGA, large for gestational age; GDM, gestational diabetes mellitus; HDP, hypertensive disorders of pregnancy; PROM, premature rupture of membranes.*

**TABLE 7 |** Unadjusted and adjusted odds ratios of perinatal and neonatal outcomes of singleton live birth undergoing L-OI versus HRT FET cycles.

| | Unadjusted OR (95%CI) | Adjusted OR (95%CI) |
|---|---|---|
| Very preterm delivery (VPTD) | 0.384 (0.040-3.707) | 0.222 (0.021-2.368) |
| Preterm delivery (PTD) | 0.947 (0.536-1.637) | 1.054 (0.585-1.898) |
| Postterm delivery | 1.483 (0.852-2.582) | 1.147 (0.643-2.049) |
| LBW | 0.906 (0.407-2.020) | 0.875 (0.385-1.989) |
| Macrosomia | 0.964 (0.622-1.496) | 0.951 (0.607-1.491) |
| SGA | 0.687 (0.884-1.610) | 0.751 (0.404-1.398) |
| LGA | 0.849 (0.729-1.469) | 1.122 (0.780-1.614) |
| GDM | 0.925 (0.573-1.494) | 0.959 (0.584-1.575) |
| HDP | 1.384 (0.780-2.456) | 1.218 (0.678-2.187) |
| Placenta previa | 0.577 (0.052-6.392) | 0.881 (0.067-11.634) |
| Congenital malformation | 5.846 (0.680-50.253) | 5.370 (0.606-47.591) |

*The analysis was adjusted for female age, number of previous FET failures, BMI, AFC, endometrial thickness on the day of embryo transfer, thin endometrium and number of transferred embryos.*

*CI, confidence interval; FET, frozen embryo transfer; VPTD, very preterm delivery; PTD, preterm delivery; LBW, low birth weight; SGA, small for gestational age; LGA, large for gestational age; GDM, gestational diabetes mellitus; HDP, hypertensive disorders of pregnancy; PROM, premature rupture of membranes.*

factors instead of ex ante PSM. Although they could correct some confounding factors, the statistical effectiveness did not seem to be more convincing than PSM.

Several limitations associated with this study warrant mentioning. 1) The number of samples was lower after PSM than before, and the study was a retrospective study with some deviation; hence, additional prospective research is needed to verify our results. 2) Patients with diabetes and hypertension were not excluded, but blood pressure and blood glucose were controlled normally before FET, which might have led to some inaccuracy in the results. 3) Because maternal complications and offspring outcomes were obtained by telephone and reported by patients, incomplete and missing data were present. 3) Not all the patients included were undergoing their first FET cycle, though the number of previous transplantation failures between the two groups was compared, some bias in outcome may exist.

In conclusion, for women with abnormal ovulation undergoing FET, both HRT and L-OI protocols are safe and effective in the clinic. Although maternal and infant outcomes appear to be reassuring, they need to be confirmed by additional prospective research with large samples.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Third Affiliated Hospital of Zhengzhou University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

## AUTHOR CONTRIBUTIONS

WZ and YG designed the study and selected the population to be included and excluded. WZ and ZL were involved in the data extraction and analysis. JZ and BR reviewed the data. WZ and ZL were involved in drafting this article. ML, JL, and WZ modified the manuscript. All authors contributed to the article and approved the submitted version.

## ACKNOWLEDGMENTS

The authors are grateful to the physicians and coordinators who enrolled the patients and collected the data from all of the women who participated in this study. We acknowledge the patients who participated in the study. We also thank American Journal Experts for their professional manuscript editing service.

## REFERENCES

1. Zeilmaker GH, Alberda AT, Gent IV, Rijkmans C, Drogendijk A. Two Pregnancies Following Transfer of Intact Frozen-Thawed Embryos. *Fertil Steril* (1984) 42:293–6. doi: 10.1016/S0015-0282(16)48029-5
2. De Geyter C, Calhaz-Jorge C, Kupka MS, Wyns C, Mocanu E, Motrenko T, et al. ART in Europe, 2014 Results Generated From European Registries by ESHRE The European IVF-Monitoring Consortium (EIM) for the European Society of Human Reproduction and Embryology (ESHRE). *Hum Reprod* (2018) 33(9):1586–601. doi: 10.1093/humrep/dey242
3. Groenewoud ER, Cohlen BJ, Macklon NS. Programming the Endometrium for Deferred Transfer of Cryopreserved Embryos: Hormone Replacement *Versus* Modified Natural Cycles. *Fertil Steril* (2018) 109:768–74. doi: 10.1016/j.fertnstert.2018.02.135
4. Maheshwari A, Pandey S, Shetty A, Hamilton M, Bhattacharya S. Obstetric and Perinatal Outcomes in Singleton Pregnancies Resulting From the Transfer of Frozen Thawed Versus Fresh Embryos Generated Through *In Vitro* Fertilization Treatment: A Systematic Review and Meta-Analysis. *Fertil Steril* (2012) 98:368. doi: 10.1016/j.fertnstert.2012.05.019
5. Tiitinen H, Halttunen M, Härkki V, Vuoristo P, Hyden-Granskog C. Elective Single Embryo Transfer: The Value of Cryopreservation. *Hum Reprod* (2001) 16(6):1140–4. doi: 10.1093/humrep/16.6.1140
6. Evans J, Hannan NJ, Edgell TA, Vollenhoven BJ, Lutjen PJ, Osianlis T, et al. Fresh Versus Frozen Embryo Transfer: Backing Clinical Decisions

With Scientific and Clinical Evidence. *Hum Reprod Update* (2014) 20 (6):808–21. doi: 10.1093/humupd/dmu027
7. Groenewoud ER, Cohlen BJ, Al-Oraiby A, Brinkhuis EA, Broekmans FJ, de Bruin JP, et al. A Randomized Controlled, Non-Inferiority Trial of Modified Natural *Versus* Artificial Cycle for Cryo-Thawed Embryo Transfer. *Hum Reprod* (2016) 31(7):1483–92. doi: 10.1093/humrep/dew120
8. Belva H, Henriet S, Van den Abbeel C, Camus M, Devroey P, Van der Elst J, et al. Neonatal Outcome of 937 Children Born After Transfer of Cryopreserved Embryos Obtained by ICSI and IVF and Comparison With Outcome Data of Fresh ICSI and IVF Cycles. *Hum Reprod (Oxf Engl)* (2008) 23(10):2227–38. doi: 10.1093/humrep/den254
9. Palomba S, Homburg R, Santagni S, Sala GL, Orvieto R. Risk of Adverse Pregnancy and Perinatal Outcomes After High Technology Infertility Treatment: A Comprehensive Systematic Review. *Reprod Biol Endocrinol* (2016) 14:76. doi: 10.1186/s12958-016-0211-8
10. Sha T, Yin X, Cheng W, Massey IY. Pregnancy-Related Complications and Perinatal Outcomes Resulting From Transfer of Cryopreserved Versus Fresh Embryos *In Vitro* Fertilization: A Meta-Analysis. *Fertil Steril: Off J Am Fertil Soc Pac Coast Fertil Soc Can Fertil Androl Soc* (2018) 109(2):330–42. doi: 10.1016/j.fertnstert.2017.10.019
11. Sites CK, Wilson D, Barsky M, Bernson D, Bernstein IM, Boulet S, et al. Embryo Cryopreservation and Preeclampsia Risk. *Fertil Steril* (2017) 108 (5):784–90. doi: 10.1016/j.fertnstert.2017.08.035
12. Maheshwari A, Raja E, Bhattacharya S. Obstetric and Perinatal Outcomes After Either Fresh or Thawed Frozen Embryo Transfer: An Analysis of 112,432 Singleton Pregnancies Recorded in the Human Fertilisation and Embryology Authority Anonymized Dataset. *Fertil Steril* (2016) 106 (7):1703–8. doi: 10.1016/j.fertnstert.2016.08.047
13. Maheshwari A, Pandey S, Amalraj Raja E, Shetty A, Hamilton M, Bhattacharya S. Is Frozen Embryo Transfer Better for Mothers and Babies? Can Cumulative Meta-Analysis Provide a Definitive Answer? *Hum Reprod* (2018) 24(1):35–58. doi: 10.1093/humupd/dmx031
14. Hu KL, Zhang D, Li R. Endometrium Preparation and Perinatal Outcomes in Women Undergoing Single-Blastocyst Transfer in Frozen Cycles. *Fertil Steril* (2021) 115(6):1487–94. doi: 10.1016/j.fertnstert.2020.12.016
15. Li C, He YC, Xu JJ, Wang Y, Liu H, Duan CC, et al. Perinatal Outcomes of Neonates Born From Different Endometrial Preparation Protocols After Frozen Embryo Transfer: A Retrospective Cohort Study. *BMC Pregnancy Childbirth* (2021) 21(1):341. doi: 10.1186/s12884-021-03791-9
16. Zhang J, Liu H, Wang Y, Mao X, Kuang Y. Letrozole Use During Frozen Embryo Transfer Cycles in Women With Polycystic Ovary Syndrome. *Fertil Steril* (2019) 112:371–7. doi: 10.1016/j.fertnstert.2019.04.014
17. Aleyasin A, Aghahosseini M, Safdarian L, Noorzadeh M, Fallahi P. Can Letrozole Plus HMG Protocol Improve Pregnancy Outcomes in Frozen-Thawed Embryo Transfer? An RCT. *Int J Reprod Biomed* (2017) 15:83–6. doi: 10.29252/ijrm.15.2.83
18. Mitwally M, Casper RF. Use of an Aromatase Inhibitor for Induction of Ovulation in Patients With an Inadequate Response to Clomiphene Citrate. *Fertil Steril* (2001) 75:305–9. doi: 10.1016/S0015-0282(00)01705-2
19. Legro RS, Arslanian SA, Ehrmann DA, Hoeger KM, Welt CK. Diagnosis and Treatment of Polycystic Ovary Syndrome: An Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab* (2013) 98:4565–92. doi: 10.1210/jc.2013-2350
20. Miller PB, Parnell BA, Bushnell G, Tallman N, Forstein DA, Higdon HL 3rd, et al. Endometrial Receptivity Defects During IVF Cycles With and Without Letrozole. *Hum Reprod* (2012) 27(3):881–8. doi: 10.1093/humrep/der452
21. Garcia-Velasco JA. The Use of Aromatase Inhibitors in Invitro Fertilization. *Fertil Steril* (2012) 98(6):1356–8. doi: 10.1016/j.fertnstert.2012.09.042
22. Requena A, Herrero J, Landeras J, Navarro E, Neyro JL, Salvador C, et al. Use of Letrozole in Assisted Reproduction: A Systematic Review and Meta-Analysis. *Hum Reprod Update* (2008) 14(6):571–82. doi: 10.1093/humupd/dmn033
23. Peeraer K, Couck I, Debrock S, De Neubourg D, De Loecker P, Tomassetti C, et al. Frozen-Thawed Embryo Transfer in a Natural or Mildly Hormonally Stimulated Cycle in Women With Regular Ovulatory Cycles: A RCT. *Hum Reprod* (2015) 30(11):2552–62. doi: 10.1093/humrep/dev224
24. Llaa B, Als A, Akah A, Tdc C, Szd A, Rbj B, et al. Adverse Obstetric and Perinatal Outcomes in 1,136 Singleton Pregnancies Conceived After Programmed Frozen Embryo Transfer (FET) Compared With Natural Cycle FET - ScienceDirect. *Fertil Steril* (2021) 115:947–56. doi: 10.1016/j.fertnstert.2020.10.039
25. Dai L, Deng C, Li Y, Zhu J, Zhang Y. Birth Weight Reference Percentiles for Chinese. *PloS One* (2014) 9:e104779. doi: 10.1371/journal.pone.0104779
26. Healey S, Tan SL, Tulandi T, Biljan MM. Effects of Letrozole on Superovulation With Gonadotropins in Women Undergoing Intrauterine Insemination. *Fertil Steril* (2003) 80:1325–9. doi: 10.1016/j.fertnstert.2003.03.001
27. Li SJ, Zhang YJ, Chai XS, Nie MF, Zhou YY, Chen JL, et al. Letrozole Ovulation Induction: An Effective Option in Endometrial Preparation for Frozen-Thawed Embryo Transfer. *Arch Gynecol Obstet* (2014) 289:687–93. doi: 10.1007/s00404-013-3044-0
28. Hosseini-Najarkolaei A, Moini A, Kashani L, Farid Mojtahedi M, Hosseini-Najarkolaee H, Salehi E. The Effect of Letrozole *Versus* Artificial Hormonal Endometrial Preparation on Pregnancy Outcome After Frozen-Thawed Embryos Transfer Cycles: A Randomized Clinical Trial. *Reprod Biol Endocrinol* (2020) 18(1):115. doi: 10.1186/s12958-020-00675-z
29. Zhang J, Li Z, Sun L, Guan Y, Du M. Comparison of Pregnancy and Neonatal Outcomes of Single Frozen Blastocyst Transfer Between Letrozole-Induction and HRT Cycles in Patients With Abnormal Ovulation. *Front Endocrinol (Lausanne)* (2021) 12:664072. doi: 10.3389/fendo.2021.664072
30. Badawy A, Aal IA, Abulatta M. Clomiphene Citrate or Letrozole for Ovulation Induction in Women With Polycystic Ovarian Syndrome: A Prospective Randomized Trial. *Fertil Steril* (2009) 92:849–52. doi: 10.1016/j.fertnstert.2007.02.062
31. Tatsumi T, Jwa SC, Kuwahara A, Irahara M, Kubota T, Saito H. Pregnancy and Neonatal Outcomes Following Letrozole Use in Frozen-Thawed Single Embryo Transfer Cycles. *Hum Reprod* (2017) 32(6):1244–8. doi: 10.1093/humrep/dex066
32. Hu YJ, Chen YZ, Zhu YM, Huang HF. Letrozole Stimulation in Endometrial Preparation for Cryopreserved-Thawed Embryo Transfer in Women With Polycystic Ovarian Syndrome: A Pilot Study. *Clin Endocrinol* (2014) 80:283–9. doi: 10.1111/cen.12280
33. Zong L, Liu P, Zhou L, Wei D, Ding L, Qin Y. Increased Risk of Maternal and Neonatal Complications in Hormone Replacement Therapy Cycles in Frozen Embryo Transfer. *Reprod Biol Endocrinol* (2020) 18(1):36. doi: 10.1186/s12958-020-00601-3
34. Jing S, Li XF, Zhang S, Gong F, Lu G, Lin G. Increased Pregnancy Complications Following Frozen-Thawed Embryo Transfer During an Artificial Cycle. *J Assisted Reprod Genet* (2019) 36(5):925–33. doi: 10.1007/s10815-019-01420-1
35. Saito K, Kuwahara A, Ishikawa T, Morisaki N, Miyado M, Miyado K, et al. Endometrial Preparation Methods for Frozen-Thawed Embryo Transfer are Associated With Altered Risks of Hypertensive Disorders of Pregnancy, Placenta Accreta, and Gestational Diabetes Mellitus. *Hum Reprod* (2019) 34 (8):1567–75. doi: 10.1093/humrep/dez079
36. Horcajadas JA, Mínguez P, Dopazo J, Esteban FJ, Domínguez F, Giudice LC, et al. Controlled Ovarian Stimulation Induces a Functional Genomic Delay of the Endometrium With Potential Clinical Implications. *J Clin Endocrinol Metab* (2008) 93:4500–10. doi: 10.1210/jc.2008-0588
37. von Versen-Höynck F, Schaub AM, Chi YY, Chiu KH, Liu J, Lingis M, et al. Increased Preeclampsia Risk and Reduced Aortic Compliance With *In Vitro* Fertilization Cycles in the Absence of a Corpus Luteum. *Hypertension* (2019) 73(3):640–9. doi: 10.1161/HYPERTENSIONAHA.118.12043
38. von Versen-Hynck F, Narasimhan P, Selamet Tierney E, Martinez N, Conrad KP, Baker VL. Absent or Excessive Corpus Luteum Number Is Associated With Altered Maternal Vascular Health in Early Pregnancy. *Hypertension* (2019) 73(3):680–90. doi: 10.1161/HYPERTENSIONAHA.118.12046
39. Novak J, Danielson LA, Kerchner LJ, Sherwood OD, Conrad KP. Relaxin is Essential for Renal Vasodilation During Pregnancy in Conscious Rats. *J Clin Invest* (2001) 107:1469–75. doi: 10.1172/JCI11975
40. Singh B, Reschke L, Segars J, Baker VL. Frozen-Thawed Embryo Transfer: The Potential Importance of the Corpus Luteum in Preventing Obstetrical Complications. *Fertil Steril* (2020) 113(2):252–7. doi: 10.1016/j.fertnstert.2019.12.007
41. Zarei A, Sohail P, Parsanezhad ME, Alborzi S, Samsami A, Azizi M. Comparison of Four Protocols for Luteal Phase Support in Frozen-Thawed Embryo Transfer Cycles: A Randomized Clinical Trial. *Arch Gynecol Obstet* (2017) 295:1–8. doi: 10.1007/s00404-016-4217-4

42. Ebrahimi M, Asbagh FA, Darvish S. The Effect of Luteal Phase Support on Pregnancy Rates of the Stimulated Intrauterine Insemination Cycles in Couples With Unexplained Infertility. *Int J Fertil Steril* (2010) 4:51–6.
43. Miles RA, Paulson RJ, Lobo RA, Press MF, Dahmoush L, Sauer MV. Pharmacokinetics and Endometrial Tissue of Progesterone After Administration by Instramuscular and Vaginal Routes:a Comparative Study. *Fertil Steril* (1994) 62(3):485–90. doi: 10.1016/s0015-0282(16)56935-0
44. Yanushpolsky EH. Luteal Phase Support in *In Vitro* Fertilization. *Semin Reprod Med* (2015) 33(2):118–27. doi: 10.1055/s-0035-1545363

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Physiology

ORIGINAL RESEARCH
published: 04 April 2022
doi: 10.3389/fphys.2022.850180

# Concurrent Ovarian and Tubal Ectopic Pregnancy After IVF-ET: Case Report and Literature Review

*Yating Huang*[1,2†], *Qin Huang*[1,2†], *Jinglan Liu*[1,2†], *Mengxi Guo*[1,2], *Yuan Liu*[1,2] and *Dongmei Lai*[1,2*]

[1]School of Medicine, The International Peace Maternity and Child Health Hospital, Shanghai Jiaotong University, Shanghai, China, [2]Shanghai Key Laboratory of Embryo Original Disease, School of Medicine, Shanghai Jiaotong University, Shanghai, China

Ovarian pregnancy (OP) coupled with tubal ectopic pregnancy is rare. We present a case of coexistent ovarian and tubal ectopic pregnancies in the same adnexa resulting from *in vitro* fertilization and embryo transfer (IVF-ET) for tubal occlusion. The patient presented with mild vaginal bleeding without abdominal pain. OP was diagnosed *via* sonographic findings of an ectopic gestational sac (GS) and yolk sac that seemed to be inside her left ovary. Laparoscopic exploration confirmed this diagnosis, and ipsilateral tubal ectopic pregnancy was suspected during surgery. The patient underwent left salpingectomy and resection of the ovarian lesion. A subsequent histopathological examination verified the diagnosis of coexistent ovarian and tubal ectopic pregnancy. Though the mechanism underlying concurrent OP and tubal ectopic pregnancy is still unclear, clinicians should be cautious of potential combined ectopic pregnancy when dealing with patients who have received more than one embryo transfer.

**Keywords: ovarian pregnancy, tubal ectopic pregnancy, *in vitro* fertilization and embryo transfer, laparoscopy, mutiple embryo transfer**

OPEN ACCESS

***Edited by:***
*Yimin Zhu,*
*Zhejiang University, China*

***Reviewed by:***
*Yi Feng,*
*Fudan University, China*
*Ahmad Mustafa Metwalley,*
*Women's Health Fertility Clinic, Saudi Arabia*

***Correspondence:**
*Dongmei Lai*
*laidongmei@hotmail.com*

*†These authors have contributed equally to this work*

***Specialty section:***
*This article was submitted to Reproduction, a section of the journal Frontiers in Physiology*

***Received:*** *07 January 2022*
***Accepted:*** *08 March 2022*
***Published:*** *04 April 2022*

***Citation:***
*Huang Y, Huang Q, Liu J, Guo M, Liu Y and Lai D (2022) Concurrent Ovarian and Tubal Ectopic Pregnancy After IVF-ET: Case Report and Literature Review. Front. Physiol. 13:850180. doi: 10.3389/fphys.2022.850180*

## INTRODUCTION

Ovarian pregnancy (OP), a rare subgroup of ectopic pregnancy, comprised 0.15–3.2% of ectopic pregnancies (Bouyer et al., 2002; Raziel et al., 2004; Choi et al., 2011). It is even rarer for it to co-occur with tubal ectopic pregnancy (TP). To the best of our knowledge, only a few such cases have been reported (M Sueldo et al., 2014; Eom et al., 2018; Trindade et al., 2019).

Overall, the risk factors for OP are similar to those of TP, including a history of pelvic inflammatory disease, IVF, and previous abdominal surgery (Kamath et al., 2010; Weiss et al., 2016; Jennings and Krywko, 2020). In addition, polycystic ovarian syndrome, intra-uterine device usage, and endometriosis are also considered specific risk factors for OP patients (Wang et al., 2013; Parker and Srinivas, 2016; Alalade et al., 2017).

Most OP patients present with non-specific symptoms with lower abdominal pain and/or mild vaginal bleeding (Choi et al., 2011; Parker and Srinivas, 2016). If ultrasound fails to detect any signs of combined pregnancy, an integral preoperative diagnosis including OP can be difficult to determine. Most cases have been confirmed by operation and postoperative pathological analysis. Currently, the diagnosis of OP is still based on the original criteria reported by (Spiegelberg, 1878).

Here, we report a case of coexistent OP with unexpected TP after the transfer of two fresh embryos. Accordingly, we review several previous works for clinical features and advances in diagnosis and treatment.

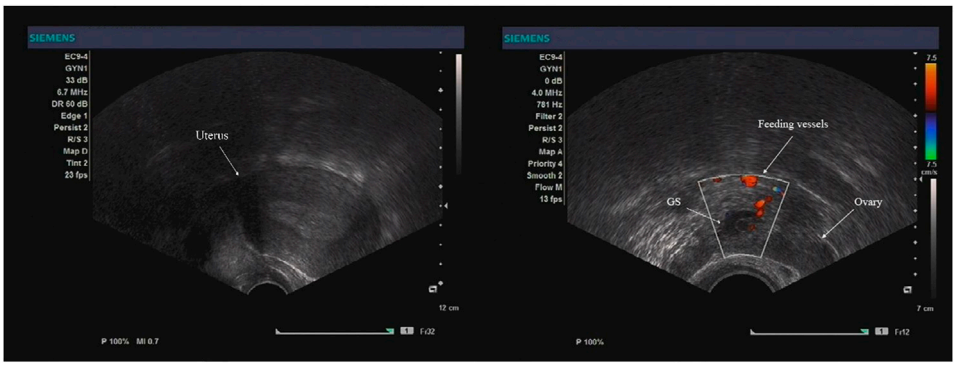



**FIGURE 1 |** Ultrasound image of the left ovarian ectopic pregnancy, showing the GS with a yolk sac inside and feeding vessels around.

## CASE REPORT

A 35-year-old nulligravid woman was hospitalized with a suspected OP 28 days after the transfer of two fresh embryos. Her previous menstrual cycles had been irregular, with a period occurring every one to 3 months that lasted three to 5 days, with average flow and mild dysmenorrhea. She had experienced a hysterography (HSG), which revealed a complete obstruction in the right fallopian tube and a partial obstruction in the left fallopian tube. She underwent two cycles of conventional IVF, both of which failed. A third IVF procedure was performed. Ovarian stimulation was performed with clomiphene citrate 100 mg (days 3–7), followed by daily injections of HMG 75 IU/150 IU based on follicular response. When the follicle was found to have reached a size of ≥16 mm, GnRH antagonist Cetrorelix 0.25 mg was administered. Then, five eggs were retrieved, and, under ultrasonographic guidance, two fresh embryos (one 9-celled embryo/grade II and one 12-celled embryo/grade II) were transferred to cleavage state (D3). Dydrogesterone (30 mg/day, orally; Duphaston®, Abbott Biologicals B.V., Netherlands) was prescribed for luteal support. Two weeks after transfer, the patient was confirmed to have conceived, and the human chorionic gonadotrophin and beta fraction (β-hCG) levels were 414.2 IU/L. About 3 weeks after transfer, she had slight vaginal bleeding for 1 day, but no other discomfort.

Routine viability ultrasonography was performed at 4-week gestation. Transvaginal ultrasonography revealed an empty uterus measuring 71 mm × 65 mm × 54 mm with an endometrial thickness of 12 mm. Her right ovary and tubal structures seemed to be normal, and a 30 × 25 × 20 mm heterogeneous mass was noted in the left adnexal area. A gestational sac (GS) with a beating fetal heart was seen inside, surrounded by ovary-like tissue, suggesting OP. Vascular proliferation was detected around the GS under power Doppler (**Figure 1**).

The patient was asymptomatic and hemodynamically stable when sent to the wards. On bimanual examination, no tenderness or masses were palpable on any side of her abdomen; no cervical pain was reported. A speculum examination showed no active bleeding at the cervix and only a trace of bloodstain on the vaginal wall. Furthermore, no abnormality was found in laboratory analysis of blood routine and blood biochemistry. The patient denied any history of endometriosis, pelvic inflammatory disease, or other relevant medical history.

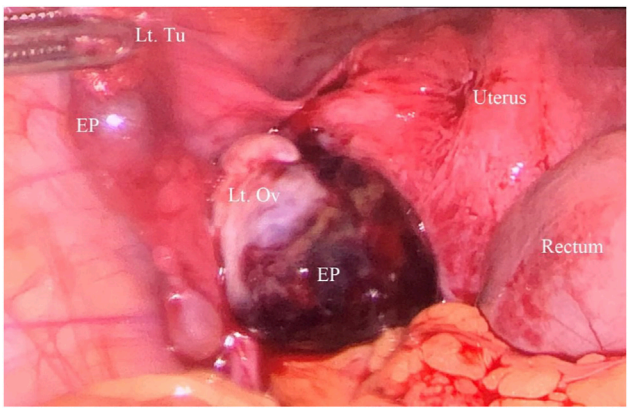



**FIGURE 2 |** Laparoscopic view of unruptured left ovarian pregnancy and ipsilateral tubal pregnancy (Lt. Tu = Left Fallopian tube, Lt. Ov = Left Ovary, EP = ectopic pregnancy).

A provisional diagnosis of left OP was made, and laparoscopic exploration was performed immediately. The surgeons explored the pelvic and abdominal cavities after aspirating about 200 ml of blood from the pelvis. The right fallopian tube and ovary were found to be normal, and the left ovary was enlarged and blueish, swelling to 6 cm in diameter. The left tube was exposed in a routine manner and found to be slightly distended and purple in appearance in the ampulla, which was dilated about 1.5 cm in diameter; both were intact (**Figure 2**). Considering the patient's recent embryo transfer, surgeons decided to perform the left salpingectomy and remove ectopic tissue while preserving the ovary. The trophoblastic tissue was removed from the left ovary with monopolar laparoscopic forceps, and the ovary was reconstructed with vicryl.

Pathological examination with hematoxylin and eosin staining of the surgical specimen showed a left OP (**Figure 3**) and ipsilateral tubal pregnancy (**Figure 4**) with the presence of trophoblastic tissues.

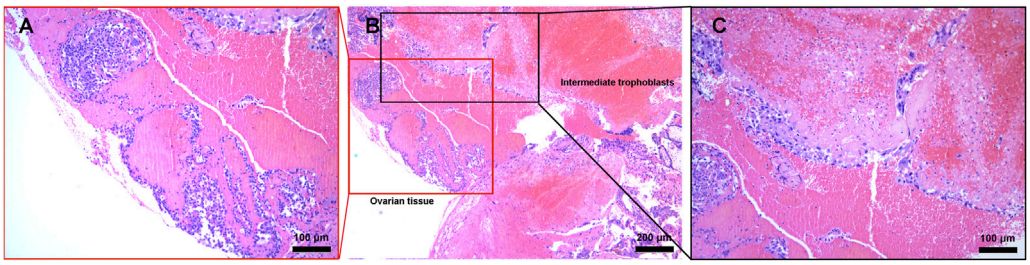



**FIGURE 3 |** Histopathological image showed ovarian tissue and intermediate trophoblasts were seen in the pathology slide of ovarian lesion. Scale bars, **(A)**, 100 μm **(B)**, 200 μm and **(C)**, 100 μm.

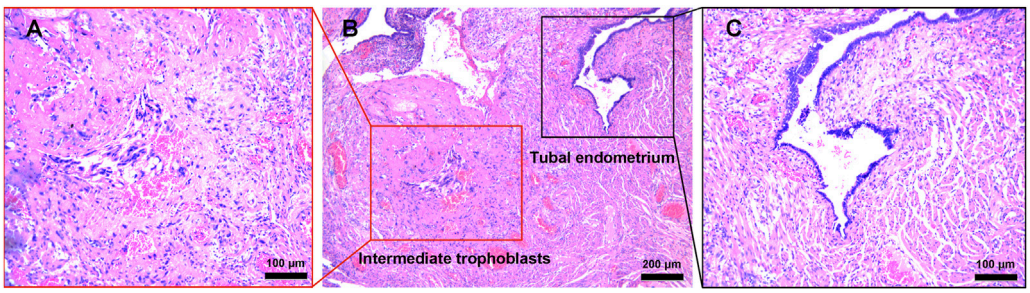



**FIGURE 4 |** Histopathological staining showed a small amount of intermediate trophoblasts infiltration into the fallopian tube tissue. Scale bars, **(A)**, 100 μm **(B)**, 200 μm and **(C)**, 100 μm.

## DISCUSSION

Combined pregnancy is rare and poses early diagnostic challenges. In existing reports, the clinical features of OP and TP patients have been unspecific, thus posing a dilemma for rupture and massive intra-abdominal bleeding with delayed diagnosis (Trindade et al., 2019). Particularly in cases of OP, pre-operative diagnosis is difficult to perform; however, this situation is improving owing to recent advances in ultrasound. Some authors state that the ultrasonic appearance suggestive of OP is a hypo-echoic, predominantly solid mass surrounded with blood flow signals (Comstock et al., 2005; Joseph and Irvine, 2012; Alalade et al., 2017), which is called the "ring of fire" structure. Moreover, an ectopic yolk sac and cardiac activity can facilitate provisional diagnosis of OP during ultrasonography (Comstock et al., 2005). It should be noted that advances in ultrasound technology can rectify the shortcomings of intra- and post-operative diagnosis involving the criteria established. MRI can also be an effective adjunct to ultrasound in the case of a patient with a hemodynamically stable status (Alalade et al., 2017; Ramanathan et al., 2018).

Here, we reported a case of concurrent OP and TP following IVF-ET to determine the causes thereof. ART was observed as a major risk factor in this case, as shown in **Supplementary Table S1**. This was consistent with three previous reports (M Sueldo et al., 2014; Eom et al., 2018; Trindade et al., 2019). Among these, M Sueldo et al. and Trindade et al. reported concurrent OP and TP after the transfer of two fresh embryos, and Eom et al. reported a patient who had undergone IUI treatment. Importantly, multiple embryo transfer was believed to be an important cause that significantly raised the rate of ectopic pregnancy over elective single transfer (Clayton et al., 2006; Bu et al., 2016). Several retrospective cohort studies have shown that more patients following IVF were found to be associated with fresh embryo transfer than frozen embryo transfer (FET) (Ishihara et al., 2011; Shapiro et al., 2011; Shapiro et al., 2012; Huang et al., 2014; Fang et al., 2015; Londra et al., 2015). In addition, receiving an embryo at the cleavage state (D3) was associated with a higher risk of ectopic pregnancy than a blastocyst on day 5 (Huang et al., 2014; Fang et al., 2015). Thus, fresh embryo transfer at the cleavage stage and multiple embryo transfer may be risk factors for multi-site ectopic pregnancy after ART. Other specific risk factors were also speculated; moreover, a high volume of culture medium was used when loading embryo or embryos, when there was an excessive ovarian response, in the transfer of an embryo in an abnormally high estrogen environment, and when there was a decreased transfer distance from the fundus (Pope et al., 2004; Chang and Suh, 2010; Wang et al., 2013; Jeon et al., 2016; Weiss et al., 2016; Lin et al., 2019).

Two hypotheses may explain the mechanism underlying concurrent ectopic pregnancy. First, the embryo or blastocyst may migrate in retrograde through the tube and implant in the ovary. Second, it may pass into one of the puncture sites created by the aspiration needle (Boronow et al., 1965). During the fresh

cycle, ovarian injury after oocyte retrieval may provide an opportunity for ectopic implantation (Ishihara et al., 2011). Elevation of the E2/P ratio with the administration of stimulating drugs or exogenous hormone supplementation may lead to uncoordinated movement of the uterus and fallopian tubes, causing the embryo to migrate in reverse into the abdominal cavity (Wang et al., 2013; Fang et al., 2015). Another mechanism is some manner of interference in the release of the ovum from the follicle, followed by fertilization *in situ* by the sperm (Dolinko et al., 2018).

As with tubal pregnancies, surgery remains the first choice treatment (Dolinko et al., 2018), especially for patients with significant hypoxia or hemodynamic instability (Odejinmi et al., 2011). Furthermore, minimal access surgery is now becoming a universal option (Joseph and Irvine, 2012). Although wedge resection of the ovary is still the most common procedure for OP (Choi et al., 2011), enucleation of the gestational product is receiving increasing acceptance from doctors, as it is considered the gentlest type of operation, able to preserve as much ovarian cortex as possible (Alkatout et al., 2011). Such a procedure includes enucleating the GS from the ovary, bluntly or with the help of monopolar or bipolar cautery (Einenkel et al., 2000; Nadarajah et al., 2002; Andrade et al., 2015), and subsequently hemostasis with electrocoagulation, thereby protecting the ovarian function to the greatest extent possible. However, for patients in life-threatening situations (e.g., excessive bleeding, difficult hemostasis), it may be appropriate to remove the entire ovary.

Furthermore, methotrexate therapy, including systemic application and local intra-GS injection (Shamma and Schwartz, 1992; Mittal et al., 2003; Dolinko et al., 2018), could be considered an alternative treatment with strict indications and monitoring (Andrade et al., 2015). However, it is not recommended as a first-line treatment by the American Society of Reproductive Medicine (ASRM).

Co-existing ectopic pregnancies may be misdiagnosed and treatment may be delayed, which may lead to life-threatening complications and necessitate additional surgery. Upon review of reported cases, we developed several specifications for the prevention of co-existing ectopic pregnancy after IVF-ET: 1) clinicians should be alert that more than one embryo was transferred in IVF-ET, or ovarian hyperstimulation was conducted in the pregnancy; 2) clinicians should be alert to abnormal changes in β-HCG after IVF-ET; 3) ultrasonography may show an empty uterus with GS occupying the position of the adnexa; 4) because either ipsilateral or contralateral ovarian and tubal pregnancy could occur, laparoscopic exploration of both lateral fallopian tubes and ovaries is needed, and clinicians should pay attention to laparoscopic images showing purple bulging of the tube or ovarian hemorrhage; and 5) pathologic evidence may include ovarian tissue in the wall of the GS and a GS in the fallopian tubal tissue.

## CONCLUSION

Concurrent OP and tubal pregnancy after ART have been reported in a few cases. In this report, we found that preoperative diagnosis involves considerable challenges. Risk factors include the transfer of multiple embryos in IVF-ET or ovarian hyperstimulation. As such, surgery remains the preferred treatment. Routine intra-operatory inspection of both fallopian tubes and ovaries is strongly recommended in any ectopic pregnancy, especially in high-risk patients.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committees of the International Peace Maternity and Child Health Hospital. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

## AUTHOR CONTRIBUTIONS

YH, JL, and MG participated in the operation, QH performed the ultrasonic diagnosis. YL made the pathology diagnosis. DL and YH conceived the study design, data collection, and manuscript preparation.

## FUNDING

This study was funded by the National Key Research and Developmental Program of China (2018YFC1004800 and 2018YFC1004802).

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.850180/full#supplementary-material

## REFERENCES

Alalade, A. O., Smith, F. J. E., Kendall, C. E., and Odejinmi, F. (2017). Evidence-based Management of Non-tubal Ectopic Pregnancies. *J. Obstet. Gynaecol.* 37, 982–991. doi:10.1080/01443615.2017.1323852

Alkatout, I., Stuhlmann-Laeisz, C., Mettler, L., Jonat, W., and Schollmeyer, T. (2011). Organ-preserving Management of Ovarian Pregnancies by Laparoscopic Approach. *Fertil. Sterility* 95, 2467–2470. doi:10.1016/j.fertnstert.2010.12.060

Andrade, A. G., Rocha, S., Marques, C. O., Simões, M., Martins, I., Biscaia, I., et al. (2015). Ovarian Ectopic Pregnancy in Adolescence. *Clin. Case Rep.* 3, 912–915. doi:10.1002/ccr3.336

Boronow, R. C., McElin, T. W., West, R. H., and Buckingham, J. C. (1965). Ovarian Pregnancy. *Am. J. Obstet. Gynecol.* 91, 1095–1106. doi:10.1016/0002-9378(65)90708-8

Bouyer, J., Coste, J., Fernandez, H., Pouly, J. L., and Job-Spira, N. (2002). Sites of Ectopic Pregnancy: a 10 Year Population-Based Study of 1800 Cases. *Hum. Reprod.* 17, 3224–3230. doi:10.1093/humrep/17.12.3224

Bu, Z., Xiong, Y., Wang, K., and Sun, Y. (2016). Risk Factors for Ectopic Pregnancy in Assisted Reproductive Technology: a 6-year, Single-center Study. *Fertil. Sterility* 106, 90–94. doi:10.1016/j.fertnstert.2016.02.035

Chang, H. J., and Suh, C. S. (2010). Ectopic Pregnancy after Assisted Reproductive Technology: what Are the Risk Factors? *Curr. Opin. Obstet. Gynecol.* 22, 202–207. doi:10.1097/gco.0b013e32833848fd

Choi, H.-J., Im, K.-S., Jung, H.-J., Lim, K.-T., Mok, J.-E., and Kwon, Y.-S. (2011). Clinical Analysis of Ovarian Pregnancy: a Report of 49 Cases. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 158, 87–89. doi:10.1016/j.ejogrb.2011.04.015

Clayton, H. B., Schieve, L. A., Peterson, H. B., Jamieson, D. J., Reynolds, M. A., and Wright, V. C. (2006). Ectopic Pregnancy Risk with Assisted Reproductive Technology Procedures. *Obstet. Gynecol.* 107, 595–604. doi:10.1097/01.aog.0000196503.78126.62

Comstock, C., Huston, K., and Lee, W. (2005). The Ultrasonographic Appearance of Ovarian Ectopic Pregnancies. *Obstet. Gynecol.* 105, 42–45. doi:10.1097/01.aog.0000148271.27446.30

Dolinko, A. V., Vrees, R. A., and Frishman, G. N. (2018). Non-tubal Ectopic Pregnancies: Overview and Treatment via Local Injection. *J. Minimally Invasive Gynecol.* 25, 287–296. doi:10.1016/j.jmig.2017.07.008

Einenkel, J., Baier, D., Horn, L.-C., and Alexander, H. (2000). Laparoscopic Therapy of an Intact Primary Ovarian Pregnancy with Ovarian Hyperstimulation Syndrome: Case Report. *Hum. Reprod.* 15, 2037–2040. doi:10.1093/humrep/15.9.2037

Eom, J. M., Choi, J. S., Bae, J., Lee, W. M., Lee, E., Lee, J., et al. (2018). Coexisting Ovarian and Tubal Pregnancies on Opposite Sides after Intrauterine Insemination: a Case Report. *BMC Pregnancy Childbirth* 18, 259. doi:10.1186/s12884-018-1801-6

Fang, C., Huang, R., Wei, L.-N., and Jia, L. (2015). Frozen-thawed Day 5 Blastocyst Transfer Is Associated with a Lower Risk of Ectopic Pregnancy Than Day 3 Transfer and Fresh Transfer. *Fertil. Sterility* 103, 655–661. doi:10.1016/j.fertnstert.2014.11.023

Huang, B., Hu, D., Qian, K., Ai, J., Li, Y., Jin, L., et al. (2014). Is Frozen Embryo Transfer Cycle Associated with a Significantly Lower Incidence of Ectopic Pregnancy? an Analysis of More Than 30,000 Cycles. *Fertil. Sterility* 102, 1345–1349. doi:10.1016/j.fertnstert.2014.07.1245

Ishihara, O., Kuwahara, A., and Saitoh, H. (2011). Frozen-thawed Blastocyst Transfer Reduces Ectopic Pregnancy Risk: an Analysis of Single Embryo Transfer Cycles in Japan. *Fertil. Sterility* 95, 1966–1969. doi:10.1016/j.fertnstert.2011.02.015

Jennings, L. K., and Krywko, D. M. (2020). "Pelvic Inflammatory Disease (PID)," in *StatPearls (Treasure Island (FL)* (Florida, USA : StatPearls Publishing StatPearls Publishing LLC.).

Jeon, J. H., Hwang, Y. I., Shin, I. H., Park, C. W., Yang, K. M., and Kim, H. O. (2016). The Risk Factors and Pregnancy Outcomes of 48 Cases of Heterotopic Pregnancy from a Single Center. *J. Korean Med. Sci.* 31, 1094–1099. doi:10.3346/jkms.2016.31.7.1094

Joseph, R. J., and Irvine, L. M. (2012). Ovarian Ectopic Pregnancy: Aetiology, Diagnosis, and Challenges in Surgical Management. *J. Obstet. Gynaecol.* 32, 472–474. doi:10.3109/01443615.2012.673039

Kamath, M. S., Aleyamma, T. K., Muthukumar, K., Kumar, R. M., and George, K. (2010). A Rare Case Report: Ovarian Heterotopic Pregnancy after *In Vitro* Fertilization. *Fertil. Steril* 94, 1910–1911. doi:10.1016/j.fertnstert.2010.02.045

Lin, J., Wang, N., Huang, J., Cai, R., Fan, Y., Kuang, Y., et al. (2019). Pregnancy and Neonatal Outcomes of hMG Stimulation with or without Letrozole in Endometrial Preparation for Frozen-Thawed Embryo Transfer in Ovulatory Women: A Large Retrospective Cohort Study. *Ddt* Vol. 13, 3867–3877. doi:10.2147/dddt.s212235

Londra, L., Moreau, C., Strobino, D., Garcia, J., Zacur, H., and Zhao, Y. (2015). Ectopic Pregnancy after *In Vitro* Fertilization: Differences between Fresh and Frozen-Thawed Cycles. *Fertil. Sterility* 104, 110–118. doi:10.1016/j.fertnstert.2015.04.009

Mittal, S., Dadhwal, V., and Baurasi, P. (2003). Successful Medical Management of Ovarian Pregnancy. *Int. J. Gynecol. Obstet.* 80, 309–310. doi:10.1016/s0020-7292(02)00304-1

Nadarajah, S., Sim, L. N., and Loh, S. F. (2002). Laparoscopic Management of an Ovarian Pregnancy. *Singapore Med. J.* 43, 095–096.

Odejinmi, F., Sangrithi, M., and Olowu, O. (2011). Operative Laparoscopy as the Mainstay Method in Management of Hemodynamically Unstable Patients with Ectopic Pregnancy. *J. Minimally Invasive Gynecol.* 18, 179–183. doi:10.1016/j.jmig.2010.11.005

Parker, V. L., and Srinivas, M. (2016). Non-tubal Ectopic Pregnancy. *Arch. Gynecol. Obstet.* 294, 19–27. doi:10.1007/s00404-016-4069-y

Pope, C., Cook, E. K., Arny, M., Novak, A., and Grow, D. R. (2004). Influence of Embryo Transfer Depth on *In Vitro* Fertilization and Embryo Transfer Outcomes. *Fertil. Sterility* 81, 51–58. doi:10.1016/j.fertnstert.2003.05.030

Ramanathan, S., Raghu, V., Ladumor, S. B., Nagadi, A. N., Palaniappan, Y., Dogra, V., et al. (2018). Magnetic Resonance Imaging of Common, Uncommon, and Rare Implantation Sites in Ectopic Pregnancy. *Abdom. Radiol.* 43, 3425–3435. doi:10.1007/s00261-018-1604-2

Raziel, A., Schachter, M., Mordechai, E., Friedler, S., Panski, M., and Ron-El, R. (2004). Ovarian Pregnancy-A 12-year Experience of 19 Cases in One Institution. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 114, 92–96. doi:10.1016/j.ejogrb.2003.09.038

Shamma, F. N., and Schwartz, L. B. (1992). Primary Ovarian Pregnancy Successfully Treated with Methotrexate. *Am. J. Obstet. Gynecol.* 167, 1307–1308. doi:10.1016/s0002-9378(11)91706-2

Shapiro, B. S., Daneshmand, S. T., De Leon, L., Garner, F. C., Aguirre, M., and Hudson, C. (2012). Frozen-thawed Embryo Transfer Is Associated with a Significantly Reduced Incidence of Ectopic Pregnancy. *Fertil. Sterility* 98, 1490–1494. doi:10.1016/j.fertnstert.2012.07.1136

Shapiro, B. S., Daneshmand, S. T., Garner, F. C., Aguirre, M., Hudson, C., and Thomas, S. (2011). Evidence of Impaired Endometrial Receptivity after Ovarian Stimulation for *In Vitro* Fertilization: a Prospective Randomized Trial Comparing Fresh and Frozen-Thawed Embryo Transfers in High Responders. *Fertil. Sterility* 96, 516–518. doi:10.1016/j.fertnstert.2011.02.059

Spiegelberg, O. (1878). Zur Casuistik der Ovarialschwangerschaft. *Arch. Gynak.* 13, 73–79. doi:10.1007/bf01991416

Sueldo, C. M., Budinetz, T., Maslow, B.-S., Nulsen, J., Engmann, L., and Benadiva, C. (2014). Concurrent Ovarian and Ipsilateral Tubal Ectopic Pregnancies after a Double Embryo Transfer: a Case Report. *J. Assist. Reprod. Genet.* 31, 1643–1646. doi:10.1007/s10815-014-0338-5

Trindade, V. D., Burmann, L., Viégas, D., Hentschke, M. R., Azambuja, R., Okada, L., et al. (2019). Ectopic Pregnancy in Left Ovary and Contralateral Uterine Tube Diagnosed One Week Apart in *In Vitro* Fertilization with Donor Eggs: Case Report. *JBRA Assist. Reprod.* 23, 439–441. doi:10.5935/1518-0557.20190030

Wang, J., Wei, Y., Diao, F., Cui, Y., Mao, Y., Wang, W., et al. (2013). The Association between Polycystic Ovary Syndrome and Ectopic Pregnancy after *In Vitro* Fertilization and Embryo Transfer. *Am. J. Obstet. Gynecol.* 209, 139–9. doi:10.1016/j.ajog.2013.05.007

Weiss, A., Beck-Fruchter, R., Golan, J., Lavee, M., Geslevich, Y., and Shalev, E. (2016). Ectopic Pregnancy Risk Factors for ART Patients Undergoing the GnRH Antagonist Protocol: a Retrospective Study. *Reprod. Biol. Endocrinol.* 14, 12. doi:10.1186/s12958-016-0146-0

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Endocrinology

ORIGINAL RESEARCH
published: 14 April 2022
doi: 10.3389/fendo.2022.853473

# Insulin Resistance is a Risk Factor for Early Miscarriage and Macrosomia in Patients With Polycystic Ovary Syndrome From the First Embryo Transfer Cycle: A Retrospective Cohort Study

*Yuanhui Chen*[1,2], *Jiayu Guo*[1,2], *Qingwen Zhang*[2] *and Cuilian Zhang*[2]*

[1] *Reproductive Medical Center, People's Hospital of Zhengzhou University, Zhengzhou, China,* [2] *Reproductive Medical Center, Henan Provincial People's Hospital, Zhengzhou, China*

OPEN ACCESS

**Edited by:**
*Yiping Shen,*
*Harvard Medical School, United States*

**Reviewed by:**
*Lianghui Diao,*
*Shenzhen Zhongshan Urology Hospital, China*
*Krzysztof Cezary Lewandowski,*
*Medical University of Lodz, Poland*

***Correspondence:**
*Cuilian Zhang*
*lluckyzcl@qq.com*

**Specialty section:**
*This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology*

**Received:** *12 January 2022*
**Accepted:** *08 March 2022*
**Published:** *14 April 2022*

**Citation:**
*Chen Y, Guo J, Zhang Q and Zhang C (2022) Insulin Resistance is a Risk Factor for Early Miscarriage and Macrosomia in Patients With Polycystic Ovary Syndrome From the First Embryo Transfer Cycle: A Retrospective Cohort Study. Front. Endocrinol. 13:853473. doi: 10.3389/fendo.2022.853473*

**Objective:** The objective of the study was to explore the effect of insulin resistance on pregnancy outcomes in patients with polycystic ovary syndrome (PCOS) from the first embryo transfer cycle.

**Design:** This was a single-center, retrospective, observational cohort study.

**Patients:** Included in the study were women with PCOS for the first embryo transfer.

**Main Outcome Measures:** Early miscarriage rate and macrosomia rate were the main outcome measures.

**Results:** With increased HOMA-IR, the early miscarriage rate (7.14, 13.21, and 16.22%, respectively; $P = 0.039$), macrosomia rate (5.78, 11.79, and 17.58%, respectively; $P = 0.026$) and the incidence of gestational diabetes (GDM) (10.00, 14.50, and 25.67% respectively; $P = 0.002$) significantly increased, while the live birth rate markedly decreased (63.03, 55.27, and 47.88%, respectively; $P = 0.004$). No significant difference was found in clinical pregnancy rate, late miscarriage rate, low birthweight rate and baby gender ratio (all $P > 0.05$). After adjusting for confounding factors, HOMA-IR was an independent risk factor of early miscarriage rate and macrosomia rate.

**Conclusion:** Insulin resistance is an independent risk factor for early miscarriage and macrosomia in PCOS patients during the first embryo transfer cycle. It is essential to give more attention before and after pregnancy for PCOS women with high HOMA-IR.

**Keywords: insulin resistance, polycystic ovary syndrome, early miscarriage, macrosomia, *in vitro* fertilization**

## INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common endocrine disorder that affects about 5–10% reproductive women (1, 2). Common clinical features for PCOS patients include ovulatory disturbances, obesity, hyperandrogenism, hyperinsulinemia, and insulin resistance (IR). IR plays an important role in regulating energy metabolism and follicular growth and development, thus is considered as an important factor in the pathogenesis of PCOS. The incidence of IR in the PCOS population varies from 50 to 70% by different races and regions (3, 4). IR is defined as reduced insulin sensitivity and an increased amount of insulin is needed to perform its normal function. It is generally believed that IR is closely related to obesity. Women with PCOS combined with IR are more prone to metabolic syndrome and cardiovascular disease.

At present, the hyperinsulinemic–euglycemic clamp technique is considered as the gold standard for assessing insulin sensitivity (5), but the complexity and high expense of the method limit its large-scale clinical application. Clinically, the homeostasis model assessment of insulin resistance (HOMA-IR) provides an efficient formula for evaluating B-cell function and insulin sensitivity. It is now a widely used method for assessing IR in many studies (6, 7). For women undergoing assisted reproduction technology, IR is easy to be ignored when fasting blood glucose is normal. A deep understanding about the influence of IR on PCOS may help to explore the pathophysiology of PCOS (8). Moreover, bringing awareness of IR in the reproductive health is crucial for disease management among PCOS women. It was found that although the clinical pregnancy rate with assisted reproduction technology of PCOS was similar to that of non-PCOS patients, the adverse maternal and fetal complications such as the risk of miscarriage, premature delivery, macrosomia, gestational diabetes (GDM) and hypertension were significantly higher (9). Therefore, this study aims to examine the association between IR and clinical pregnancy outcomes by comparing the outcomes of PCOS women with different insulin resistance levels, and discussing the influence of IR on clinical outcomes after the first embryo transfer treatment.

## MATERIALS AND METHODS

### Study Design and Population

This was a single-center retrospective cohort study approved by the Ethics Committee of the People's Hospital of Zhengzhou University. Enrolled as subjects of the study were PCOS patients who underwent *in vitro* fertilization (IVF) or intracytoplasmic sperm microinjection (ICSI) procedures for the first time between January 2017 and June 2020 at the Reproductive Medicine Center of People's Hospital of Zhengzhou University. The diagnosis of PCOS was based on the Rotterdam criteria established in the 2003 Rotterdam consensus workshop, which required that at least two of the following three criteria were met: oligomenorrhea and/or anovulation, clinical and/or biochemical signs of hyperandrogenism, and polycystic ovaries on ultrasound scanning (10).

The exclusion criteria included: 1): cycles with incomplete data; 2) no embryo transfer cycles; 3) with endometrium factors such as intrauterine adhesion and uterine malformation; 4) with recurrent spontaneous abortion and autoimmune disease; 5) with chromosome abnormalities screened by preimplantation genetic screening of preimplantation genetic diagnosis; and 6) other endocrine disorders such as thyroid diseases, diabetes mellitus, impaired fasting glucose and hyperprolactinemia (**Figure 1**). All couples in the study had been given informed consent and signed informed consent for assisted reproduction therapy. This study complied with the basic principles of the Declaration of Helsinki.

Fasting blood glucose and fasting insulin were included in the routine examination of IVF treatment in our center. The two tests were performed six months before the start of ovarian stimulation, and in the same laboratory. As there is no consensus about the cutoff value of IR at present, the patients was divided into three groups according to 25th and 75th quartile of the HOAM-IR in this study: Group 1: HOMA-IR ≤1.87 (n = 238); group 2: 1.87 <HOMA-IR <4.28 (n = 474); and group 3: HOMA-IR ≥4.28 (n = 236). The insulin resistance index was calculated using the HOMA-IR according to the following formula: HOMA-IR = fasting blood glucose × fasting insulin/22.5. The unit of fasting blood glucose was mmol/L and the unit of fasting insulin was μ U/ml.

### Ovarian Stimulation Protocols

In this study, the controlled ovulation induction protocol was conducted by the same team according to the condition of the patients. All the women underwent either GnRH agonist or flexible GnRH antagonist protocol.

### GnRH Agonist Protocol

For the GnRH agonist protocol, 30 to 35 days after a single injection of 3.75 mg of long-acting GnRH agonist (Diphereline, Ipsen, Tianjin) on the second or third day of menstrual cycle, or injection of the short-acting GnRH agonist (Decapeptyl, 0.1 mg/d, Germany ferring) for 14 to 18 days began in the middle luteal phase of the previous menstrual cycle. Once the condition reached the downregulation standard, a dose of 75–300 IU gonadotropin (Gn) was administered based on the age, ovarian reserve, body mass index (BMI), and anti-Mullerian hormone (AMH) level of the patient. Gonadotropin doses were adjusted according to ovarian response and hormone levels after 4 to 5 days. Urinary human chorionic gonadotropin (hCG) was administered subcutaneously for triggering when at least two follicles measured ≥18 mm or three follicles measured ≥17 mm. A dose of 4,000 to 10,000 IU of hCG (Lizhu Pharmaceutical Trading, China) was given to induce ovulation depending on peak estradiol level and age. Oocyte retrieval guided by vaginal ultrasound was performed 36–37 h later.

### GnRH Antagonist Protocol

Gn was injected from the second or third day of menstruation, and the starting dose of Gn was the same as above. Follicular size and hormone levels were monitored after four or five days of Gn

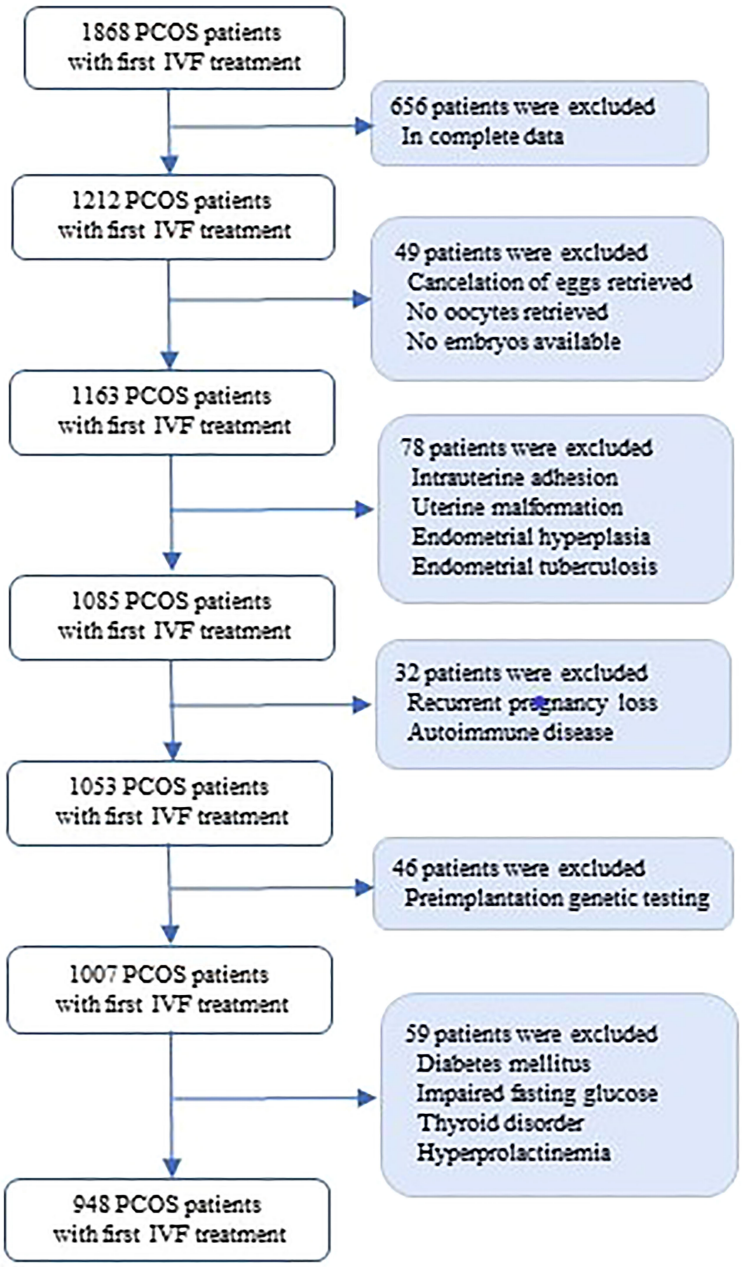



**FIGURE 1** | Flow chart of patients' selection and exclusions.

treatment. A daily dose of 0.25 mg GnRH antagonist was initiated when a dominant follicle reached a mean diameter of 12 mm or estrogen level ≥200 ng/L or when blood luteinizing hormone (LH) levels began to show a notable upward trend. The dose was administered until the day of hCG administration. When at least two follicles measured ≥18 mm or three follicles measured ≥17 mm, a dose of 4,000 to 10,000 IU hCG was administered subcutaneously for triggering. Oocyte retrieval guided by vaginal ultrasound was performed 35–36 h later.

## Embryo Transfer and Luteal Support

IVF/ICSI fertilization was performed depending on male semen parameters. On the 3rd to 5th day after oocyte retrieval, 1–2 high-quality cleavage embryos or blastocysts were selected for embryo transfer. During the frozen embryo transfer cycle, the endometrial preparation protocol was selected individually according to the condition of the patient, and 1–2 cleavage embryos or blastocysts should be transferred timely after the endometrial transformation. The hCG level in peripheral blood

was measured on the 14th day after embryo transfer. Clinical pregnancy was defined as the presence of at least one intrauterine gestational sac on the 4–5 weeks after transfer. Luteal support drugs were discontinued in non-pregnant patients, and luteal support drugs were continued in pregnant patients until 8–10 weeks of pregnancy.

## Outcomes

The primary outcomes of this study were the early miscarriage rate, macrosomia birth rate, and live birth rate. Live birth was defined as the complete removal or delivery of the fertilized product from the mother after more than 28 weeks of gestation with the presence of respiration or any signs of life (heartbeat, umbilical cord pulsation, voluntary muscle movement) after separation from the mother. Early miscarriage was defined as embryo loss before 12 weeks of pregnancy. Low birth weight was defined as fetal birth weight <2,500 g. Macrosomia was defined as birth weight ≥4,000 g.

## Statistical Analysis

All measurement data were expressed by mean ± standard deviation (mean ± SD). One-way ANOVA was used for comparison between groups. All counting data were expressed by percentage (%), and chi-squared test was used to compare the count data between groups. Logistic regression model was used for multivariate analysis.

All statistical management and analyses were performed using SPSS software, version 24.0. A two-sided *P*-value <0.05 was considered statistically significant.

# RESULTS

## Study Population

A total of 948 PCOS women who underwent first embryo transfer cycle and met the study inclusion and exclusion criteria were enrolled (**Figure 1**). According to the 25th and 75th of HOMA-IR, all the patients were divided into three groups: group 1 with HOMA-IR ≤1.87 (n = 238), group 2 with HOMA-IR between 1.87 and 4.28 (n = 474), and group 3 with HOMA-IR ≥4.28 (n = 236).

## Patient Demographic and Characteristics

**Table 1** showed the demographic and clinical characteristics among the three groups. The mean HOMA-IR was significant different in the three groups (1.43 ± 0.35, 2.92 ± 0.68, 6.72 ± 2.78, respectively; $P$ <0.001). The BMI (22.1 ± 2.9, 24.9 ± 3.4, 28.3 ± 3.4, respectively; $P$ <0.001) and basal testosterone (T) (0.41 ± 0.2, 0.42 ± 0.19, 0.46 ± 0.22; $P$ = 0.020) significantly increased with HOMA-IR. In group 3, obese patients (BMI ≥28 kg/m$^2$) accounted for as high as 52.1%. The level of AMH, basal follicular stimulation hormone (FSH) and basal luteinizing hormone (LH) decreased significantly among the three groups. There were no significant differences in age, duration of infertility, type of infertility and fertilization method ($P$ >0.05).

## Ovarian Stimulation and First Embryo Transfer Results

As shown in **Table 2**, with increased HOMA-IR, the starting dosage of Gn, the total dosage of Gn and the duration of Gn became higher, while the number of oocytes retrieved, number of mature oocytes, number of normal fertilization oocytes, number of available embryos and number of good embryos became significantly lower ($P$ <0.05). No statistically significant difference was observed in type of protocol.

After the first embryo transfer, the type of transfer (fresh cycle or frozen cycle), number of embryos transferred and the thickness of endometrium were comparable among the groups ($P$ >0.05). With increased HOMA-IR, the early miscarriage rate (7.14, 13.21, and 16.22%, respectively; $P$ = 0.039), macrosomia rate (5.78, 11.79, and 17.58%, respectively; $P$ = 0.026) and the incidence of GDM (10.00, 14.50, and 25.67% respectively; $P$ = 0.002) significantly increased, while the live birth rate markedly decreased (63.03, 55.27, and 47.88%, respectively; $P$ = 0.004). No macrosomia baby

**TABLE 1** | Comparison of demographic and clinical characteristics of the three groups.

| Item | Group 1 | Group 2 | Group 3 | *P* |
|---|---|---|---|---|
| No. of cases | 238 | 474 | 236 | |
| Age (year) | 29.1 ± 3.9 | 28.9 ± 3.6 | 28.5 ± 4.2 | 0.208 |
| HOMA-IR | 1.43 ± 0.35 | 2.92 ± 0.68 | 6.72 ± 2.78 | <0.001 |
| BMI (kg/m$^2$) | 22.1 ± 2.9 | 24.9 ± 3.4 | 28.3 ± 3.4 | <0.001 |
| <24 | 76.5 (182/238) | 43.0 (204/474) | 9.3 (22/236) | |
| 24–27.9 | 19.7 (47/238) | 38.9 (184/474) | 38.6 (91/236) | |
| ≥28 | 3.8 (9/238) | 18.1 (86/474) | 52.1 (123/236) | |
| AMH (ng/ml) | 9.2 ± 4.9 | 8.3 ± 4.5 | 7.2 ± 4.1 | <0.001 |
| FSH (IU/L) | 6.1 ± 1.5 | 5.7 ± 1.3 | 5.5 ± 1.4 | <0.001 |
| LH (IU/L) | 10.5 ± 5.8 | 8.7 ± 4.7 | 7.6 ± 4.2 | <0.001 |
| T (ng/ml) | 0.41 ± 0.2 | 0.42 ± 0.19 | 0.46 ± 0.22 | 0.020 |
| Duration of infertility (year) | 2.5 ± 0.1 | 2.5 ± 0.1 | 2.5 ± 0.2 | 0.068 |
| Type of infertility (%) | | | | 0.103 |
| Primary | 63.9 (152/238) | 61.8 (293/474) | 69.9 (165/236) | |
| Secondary | 36.1 (86/238) | 38.2 (181/474) | 30.1 (71/236) | |
| Methods of ART (%) | | | | 0.314 |
| IVF | 84.5 (201/238) | 87.6 (415/474) | 89.0 (210/236) | |
| ICSI | 15.5 (37/238) | 12.4 (59/474) | 11.0 (26/236) | |

**TABLE 2 |** Ovarian stimulation characteristics among the three groups.

| Item | Group 1 | Group 2 | Group 3 | *P* |
|---|---|---|---|---|
| No. of cases | 238 | 474 | 236 | |
| Protocol (%) | | | | 0.078 |
| GnRH agonist protocol | 83.2 (198/236) | 86.5 (410/474) | 90.3 (213/236) | |
| GnRH antagonist protocol | 16.8 (40/236) | 13.5 (64/474) | 9.7 (13/236) | |
| Starting dosage of Gn (IU) | 122.1 ± 27.7 | 131.9 ± 29.2 | 142.9 ± 31.7 | <0.001 |
| Total dosage of Gn (IU) | 1,741.4 ± 87.6 | 2,200.8 ± 1,202.6 | 2,880.0 ± 1,254.2 | <0.001 |
| Duration of Gn (d) | 11.2 ± 3.2 | 12.2 ± 3.7 | 13.7 ± 3.7 | <0.001 |
| No. of oocytes retrieved | 16.0 ± 7.7 | 14.8 ± 7.9 | 13.8 ± 7.8 | 0.011 |
| No. of mature oocytes | 13.6 ± 7.0 | 12.7 ± 7.1 | 11.8 ± 6.8 | 0.017 |
| No. of normal fertilization oocytes | 9.6 ± 5.6 | 9.0 ± 5.5 | 8.1 ± 5.2 | 0.009 |
| No. of available embryos | 7.7 ± 4.8 | 7.6 ± 5.0 | 6.7 ± 4.5 | 0.048 |
| No. of good embryos | 4.1 ± 2.5 | 3.8 ± 2.4 | 3.6 ± 2.1 | 0.036 |

was born in twin pregnancy patients. Furthermore, the live birth rate of single baby was prominently lower, while the rate of twin live birth was comparable. No significant difference was found in clinical pregnancy rate, late miscarriage rate, low birth weight rate, and baby gender ratio (**Table 3**).

Multivariate logistic regression analysis was performed to explore the risk factors of early miscarriage rate and macrosomia rate. The regression model included the following factors: age, HOMA-IR, BMI, AMH, number of available embryos, number of embryos transferred, type of transfer embryo and endometrial thickness. The results showed that HOMA-IR was an independent risk factor of early miscarriage rate and macrosomia rate. Compared with group 1, the group 2 and group 3 had significantly higher early miscarriage rate (group 2, a*OR* = 1.640, 95% CI: 1.101–2.443, $P = 0.015$; group 3, a*OR* = 1.685, 95% CI: 1.049, 2.708, $P = 0.031$) and macrosomia rate (group 2, a*OR* = 1.983, 95% CI: 1.089–3.611, $P = 0.025$; group 3, a*OR* = 2.218, 95% CI: 1.149–4.281, $P = 0.018$). The details are shown in **Table 4**.

## DISCUSSION

In the present study, we found that HOMA-IR was associated with early miscarriage, macrosomia, live birth rate, and the incidence of GDM. With increasing of HOMA-IR, the early miscarriage rate, the macrosomia rate and the prevalence of GDM elevated remarkedly, and the live birth rate decreased significantly in their first embryo transfer. The influences still remained after adjusting for the following factors: age, BMI, AMH, number of available embryos, number of embryos transferred, type of transfer embryo and endometrial thickness.

**TABLE 3 |** Outcomes of first embryo transfer cycle.

| Item | Group 1 | Group 2 | Group 3 | *P* |
|---|---|---|---|---|
| No. of cases | 238 | 474 | 236 | |
| Type of transfer (%) | | | | 0.128 |
| Fresh cycle | 43.3 (103/238) | 47.3 (224/474) | 52.5 (124/236) | |
| Frozen cycle | 56.6 (135/238) | 52.7 (250/474) | 47.4 (112/236) | |
| No of embryo transferred | 1.47 ± 0.50 | 1.47 ± 0.50 | 1.50 ± 0.50 | 0.631 |
| Type of transfer embryos (%) | | | | 0.021 |
| cleavage | 57.1 (136/238) | 55.9 (265/474) | 66.5 (157/236) | |
| blastocyst | 42.9 (102/238) | 44.1 (209/474) | 33.5 (79/236) | |
| Endometrium (mm) | 9.8 ± 1.9 | 10.0 ± 2.0 | 10.1 ± 2.2 | 0.337 |
| Clinical pregnancy rate (%) | 70.59 (168/238) | 67.09 (318/474) | 62.71 (148/236) | 0.188 |
| Early miscarriage rate (%) | 7.14 (12/168) | 13.21 (42/318) | 16.22 (24/148) | 0.039 |
| Late miscarriage rate (%) | 2.38 (4/168) | 2.83 (9/318) | 5.41 (8/148) | 0.258 |
| Live birth rate (%) | 63.03 (150/238) | 55.27 (262/474) | 47.88 (113/236) | 0.004 |
| Single live birth rate (%) | 50.84 (121/238) | 44.73 (212/474) | 38.56 (91/236) | 0.027 |
| Low birth weight rate (%) | 5.78 (7/121) | 6.60 (14/212) | 9.89 (9/91) | 0.478 |
| Macrosomia rate (%) | 5.78 (7/121) | 11.79 (25/212) | 17.58 (16/91) | 0.026 |
| Twin live birth rate (%) | 12.18 (29/238) | 10.55 (50/474) | 9.32 (22/236) | 0.597 |
| Low birthweight rate (%) | 31.03 (18/58) | 44 (44/100) | 50 (22/44) | 0.125 |
| Macrosomia rate (%) | 0 | 0 | 0 | |
| GDM (%) | 10.00 (15/150) | 14.50 (38/262) | 25.67 (29/113) | 0.002 |
| Baby gender ratio (male/female) | 1.11 | 1.17 | 1.11 | 0.948 |
| Total Male | 94 | 168 | 71 | |
| Total Female | 85 | 144 | 64 | |

**TABLE 4** | Logistic regression analysis to account for confounding variables of early miscarriage and macrosomia.

| | Early miscarriage rate | | | Macrosomia rate | | |
|---|---|---|---|---|---|---|
| | **B** | ***aOR* (95% confidence interval)** | ***P*** | **B** | ***aOR* (95% confidence interval)** | ***P*** |
| HOMA-IR | | | 0.039 | | | 0.035 |
| Group 1 | | Ref (1) | | | Ref (1) | |
| Group 2 | 0.495 | 1.640 (1.101, 2.443) | 0.015 | 0.685 | 1.983 (1.089, 3.611) | 0.025 |
| Group 3 | 0.522 | 1.685 (1.049, 2.708) | 0.031 | 0.779 | 2.218 (1.149, 4.281) | 0.018 |

Further, we found that with increased HOMA-IR, there were significant decreasing in number of oocytes retrieved, number of available embryos, and number of good embryos. We suspect that with fewer available embryos and good embryos, reduced chance for embryo selection in the first embryo transfer cycle might lead to adverse pregnancy outcomes, and high HOMA-IR may be detrimental to the oocyte and embryo quality.

## IR and Early Miscarriage

Several previous studies have shown that PCOS patients had a higher miscarriage rate than non-PCOS patients in IVF treatment. Su et al. found that women with PCOS had an increased risk miscarriage (aOR 1.629, 95% CI 1.240–2.141) for the first IVF treatment (11). A meta-analysis including twenty-nine studies also demonstrated that PCOS women had higher risks of miscarriage (OR 1.41, 95% CI 1.04–1.91) than control group (9). Due to the complexity of endocrine disorders in PCOS population, no clear indicators exist concerning the exact risk factors for adverse pregnancy outcomes. A meta-analysis found that high BMI (OR 1.48, 95% CI [1.32, 1.67], MD = 1.35, 95% CI [0.58,2.12]) and insulin resistance (MD = 0.32, 95% CI [0.15, 0.49]) were associated with an increased risk of miscarriage in PCOS patients undergoing ART (12). Li et al. found that IR was an independent risk factor for spontaneous abortion (13), which was consistent with our study. However, the definition of IR used in our study was different compared with previous studies. In the study of Li et al. (13), patients with HOMA-IR greater than 4.5 were classified as IR, while in our study HOMA-IR was grouped by the percentile. At present time, there is no consensus on the definition of IR, as previous studies have variously defined IR with the level of HOMA-IR. In this study, we grouped the patients by 25th quantile and 75th quantile of HOMA-IR and explore the relationship between HOMA-IR and adverse pregnancy outcomes.

IR might affect early miscarriage through downstream physiological changes. IR or hyperinsulinemia may affect the secretion of androgen, and excess androgen can aggravate endocrine disorders and follicular dysplasia, which may further result in poor quality eggs and embryos. Besides, from an *in vivo* study, hyperandrogenism and insulin resistance could induce mitochondria-mediated damage and result in an imbalance between oxidative and antioxidative stress responses in the gravid uterus, which correlates with high abortion risk (14). An experiment in pregnant rats suggested that deleterious effects of hyperandrogenism and insulin resistance on fetal survival were related to placental mitochondrial abnormalities and elevated reactive oxygen species production (15). Additionally, gut microbiota dysbiosis can promote metabolism, immune response through interaction with the external environment, which may closely relate with IR in PCOS patients and cause adverse pregnancy outcomes (16). Other factors, such as serum testosterone and serum chemerin level, might also contribute to the early abortion in PCOS women (17, 18).

## IR Affecting Macrosomia

In this study, we found that macrosomia rate and the incidence of GDM significantly increased with HOMA-IR elevation, and the influence was still remained after adjusting for the possible confounding factors. A meta-analysis including fifty-nine studies of Chinese PCOS women suggested that the estimates of GDM and macrosomia among women with PCOS were significantly higher than those in women without PCOS (all $P$ <0.05). Further subgroup analysis found that PCOS women with pre-pregnancy insulin resistance were at an increased risk for GDM and macrosomia (all $P$ <0.05) (19). A retrospective cohort study including 1,357 pregnant women with PCOS and 6,940 without PCOS suggested that PCOS women had a higher rate of macrosomia (9.14% vs 6.64%, $P$ = 0.008), and the difference was prominent among obese PCOS women with no significant difference (18.92% vs 8.00%, $P$ = 0.15) (20).

At present, a large number of studies have found that maternal weight was a high-risk factor of macrosomia (21–24). Additionally, a study found that insulin resistance was a link between maternal overweight and fetal macrosomia in nondiabetic pregnancies (25). Study has shown that there was a significant positive correlation between maternal weight and HOMA-IR (r = 0.248, $P$ <0.05) (26). In our study, the BMI increased significantly in accordance with HOMA-IR ($P$ <0.001), and more than half women (52.1%) were obese (BMI ≥28) when HOMA-IR was more than 4.28. PCOS is commonly characterized by endocrine disorder such as insulin resistance, hyperandrogenism, and obesity. Obesity and insulin resistance are closely interrelated.

Macrosomia has short-term and long-term adverse health effects and is thus an important public health concern. A murine model suggested that neonatal macrosomia was an independent risk factor of adult metabolic syndrome (27). Another research including 1,767 infants explored the risk of childhood under 3 years, and found that obesity for macrosomic babies was 3.74 (1.96–7.14) and 1.64 (0.89–3.00) times higher based on weight-for-age and BMI-for-age, respectively (28). It is essential to explore the risk factors and possible mechanisms of macrosomia. The higher rate of macrosomia maybe associated with the greater risk of GDM in PCOS patients. PCOS patients had a high incidence of GDM and prevalence of GDM diagnosis in the first trimester, especially in patients with obesity and insulin resistance (29–31). In our study, the incidence of GDM significantly increased with HOMA-IR, which was in accordance

with the occurrence of macrosomia. However, even with no GDM during pregnancy, there still was an increased risk of macrosomia with insulin resistance (aOR:1.71; 95% CI: 1.12–1.97) (32). In addition, during pregnancy, maternal tissues become increasingly insensitive to insulin in order to liberate nutritional supply to the growing fetus. Thus, IR might be an important risk factor for macrosomia among PCOS patients.

### Strengths and Limitations

To our best knowledge, this is the first study to explore the effects of insulin resistance both on early miscarriage and macrosomia in PCOS patients during their first embryo transfer cycles. Most of the previous studies have compared the influence of IR on PCOS patients and non-PCOS patients. It provides valuable data support for clinical consultation and new ideas for future clinical and basic research. This study also has certain limitations that should be noticed. First, this study was designed as a retrospective cohort study, and thus limited its scope to explore the relevant biological mechanism by which insulin resistance affects pregnancy outcomes. Additionally, the assessment of HOMA-IR has some limitations (33). HOMA-IR reflects predominantly hepatic insulin resistance, while peripheral insulin resistance is better described by oral glucose tolerance test-derived (OGTT) insulin resistance indices. However, the data of OGTT and other maternal complications during pregnancy were not studied in this study since the data were collected retrospectively.

## CONCLUSION

In summary, this study showed that insulin resistance was an independent risk factor for early miscarriage and macrosomia in PCOS patients during the first embryo transfer cycle. The early miscarriage rate and macrosomia rate were significantly higher with the increasing of HOMA-IR. Therefore, for PCOS patients with high insulin level, it is essential to give effective treatment before pregnancy, and the perinatal period may require more attention from obstetricians and pediatricians.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Zhengzhou University and the Henan Provincial People's Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

## AUTHOR CONTRIBUTIONS

YC designed the study. JG and QZ were involved in the data extraction and analysis. CZ was responsible for providing data and guiding research. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

## FUNDING

This work was supported by the National Natural Science Foundation of China (NSFC) (project number: U2004130).

## ACKNOWLEDGMENTS

The authors thank all participants involved in this study, and wish to express their thanks to all clinicians and clinical embryologists in the reproductive center of People's Hospital of Zhengzhou University.

## REFERENCES

1. Bozdag G, Mumusoglu S, Zengin D, Karabulut E, Yildiz OB. The Prevalence and Phenotypic Features of Polycystic Ovary Syndrome: A Systematic Review and Meta-Analysis. *Hum Reprod* (2016) 31(12):2841–55. doi: 10.1093/humrep/dew218
2. Li R, Zhang Q, Yang D, Li S, Lu S, Wu X, et al. Prevalence of Polycystic Ovary Syndrome in Women in China: A Large Community-Based Study. *Hum Reprod* (2013) 28(9):2562–9. doi: 10.1093/humrep/det262
3. Hassani F, Oryan S, Eftekhari-Yazdi P, Bazrgar M, Moini A, Nasiri N, et al. Association Between The Number of Retrieved Mature Oocytes and Insulin Resistance or Sensitivity in Infertile Women With Polycystic Ovary Syndrome. *Int J Fertil Steril* (2019) 12(4):310–5. doi: 10.22074/ijfs.2019.5422
4. Diamanti-Kandarakis E, Dunaif A. Insulin Resistance and the Polycystic Ovary Syndrome Revisited: An Update on Mechanisms and Implications. *Endocr Rev* (2012) 33(6):981–1030. doi: 10.1210/er.2011-1034
5. DeFronzo RA, Tobin JD, Andres R. Glucose Clamp Technique: A Method for Quantifying Insulin Secretion and Resistance. *Am J Physiol-Endocrinol Metab* (1979) 237(3):E214. doi: 10.1152/ajpendo.1979.237.3
6. Alebić MŠimun, Bulum T, Stojanović Nataša, Duvnjak L. Definition of Insulin Resistance Using the Homeostasis Model Assessment (HOMA-IR) in IVF Patients Diagnosed With Polycystic Ovary Syndrome (PCOS) According to the Rotterdam Criteria. *Endocrine* (2014) 47(2):625–30. doi: 10.1007/s12020-014-0182-5
7. Gao J, Zhou L, Hong J, Chen C. Assessment of Insulin Resistance in Chinese PCOS Patients With Normal Glucose Tolerance. *Gynecol Endocrinol* (2017) 33(11):888–91. doi: 10.1080/09513590.2017.1342238
8. Ding H, Zhang J, Zhang F, Zhang S, Chen X, Liang W, et al. Resistance to the Insulin and Elevated Level of Androgen: A Major Cause of Polycystic Ovary Syndrome. *Front Endocrinol (Lausanne)* (2021) 12:741764. doi: 10.3389/fendo.2021.741764
9. Sha T, Wang X, Cheng W, Yan Y. A Meta-Analysis of Pregnancy-Related Outcomes and Complications in Women With Polycystic Ovary Syndrome Undergoing IVF. *Reprod BioMed Online* (2019) 39(2):281–93. doi: 10.1016/j.rbmo.2019.03.203
10. Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group. Revised 2003 Consensus on Diagnostic Criteria and Long-Term Health Risks Related to Polycystic Ovary Syndrome. *Fertil Steril* (2004) 81:19–25. doi: 10.1016/j.fertnstert.2003.10.004

11. Liu S, Mo M, Xiao S, Li L, Hu X, Hong L, et al. Pregnancy Outcomes of Women With Polycystic Ovary Syndrome for the First In Vitro Fertilization Treatment: A Retrospective Cohort Study With 7678 Patients. *Front Endocrinol (Lausanne)* (2020) 11:575337. doi: 10.3389/fendo.2020.575337
12. Sun Y-F, Zhang J, Xu Y-M, Cao Z-Y, Wang Y-Z, Hao G-M, et al. High BMI and Insulin Resistance Are Risk Factors for Spontaneous Abortion in Patients With Polycystic Ovary Syndrome Undergoing Assisted Reproductive Treatment: A Systematic Review and Meta-Analysis. *Front Endocrinol (Lausanne)* (2020) 11:592495. doi: 10.3389/fendo.2020.592495
13. Tian L, Shen H, Lu Q, Norman RJ, Wang J. Insulin Resistance Increases the Risk of Spontaneous Abortion After Assisted Reproduction Technology Treatment. *J Clin Endocrinol Metab* (2007) 92(4):1430–3. doi: 10.1210/jc.2006-1123
14. Hu M, Zhang Y, Guo X, Jia W, Liu G, Zhang J, et al. Hyperandrogenism and Insulin Resistance Induce Gravid Uterine Defects in Association With Mitochondrial Dysfunction and Aberrant Reactive Oxygen Species Production. *Am J Physiol Endocrinol Metab* (2019) 316(5):E794–809. doi: 10.1152/ajpendo.00359.2018
15. Zhang Y, Zhao W, Xu H, Hu M, Guo X, Jia W, et al. Hyperandrogenism and Insulin Resistance-Induced Fetal Loss: Evidence for Placental Mitochondrial Abnormalities and Elevated Reactive Oxygen Species Production in Pregnant Rats That Mimic the Clinical Features of Polycystic Ovary Syndrome. *J Physiol* (2019) 597(15):3927–50. doi: 10.1113/JP277879
16. He F-F, Li Y-M. Role of Gut Microbiota in the Development of Insulin Resistance and the Mechanism Underlying Polycystic Ovary Syndrome: A Review. *J Ovarian Res* (2020) 13(1):73. doi: 10.1186/s13048-020-00670-3
17. Lathi RB, Dahan MH, Reynolds-May MF, Milki AA, Behr B, Westphal LM. The Role of Serum Testosterone in Early Pregnancy Outcome: A Comparison in Women With and Without Polycystic Ovary Syndrome. *J Obstet Gynaecol Can* (2014) 36(9):811–6. doi: 10.1016/S1701-2163(15)30483-7
18. Yang X, Quan X, Lan Y, Wei Q, Ye J, Yin X, et al. Serum Chemerin Level in Women With PCOS and Its Relation With the Risk of Spontaneous Abortion. *Gynecol Endocrinol* (2018) 34(10):864–7. doi: 10.1080/09513590.2018.1462316
19. Wang T, Fu H, Chen L, Xu Y. Pregnancy Complications Among Women With Polycystic Ovary Syndrome in China: A Meta-Analysis. *Zhong Nan Da Xue Xue Bao Yi Xue Ban* (2017) 42(11):1300–10. doi: 10.11817/j.issn.1672-7347.2017.11.010
20. Liu Q, Wang J, Xu Q, Kong L, Wang J. A Retrospective Cohort Study of Obstetric Complications and Birth Outcomes in Women With Polycystic Ovarian Syndrome. *J Obstet Gynaecol* (2021) 08:1–6. doi: 10.1080/01443615.2021.1931066
21. Pereda J, Bove I, Pineyro MM. Excessive Maternal Weight and Diabetes Are Risk Factors for Macrosomia: A Cross-Sectional Study of 42,663 Pregnancies in Uruguay. *Front Endocrinol (Lausanne)* (2020) 11:588443. doi: 10.3389/fendo.2020.588443
22. Lewandowska Małgorzata. The Role of Maternal Weight in the Hierarchy of Macrosomia Predictors; Overall Effect of Analysis of Three Prediction Indicators. *Nutrients* (2021) 13(3):801. doi: 10.3390/nu13030801
23. Triunfo S, Lanzone A. Impact of Overweight and Obesity on Obstetric Outcomes. *J Endocrinol Invest* (2014) 37(4):323–9. doi: 10.1007/s40618-014-0058-9
24. Zhang YF, Luo HN, Shi R, Zhang YJ, Tai XM, Hu XY, et al. Effect of Body Mass Index on the Assisted Reproductive Outcome of Frozen-Thawed Embryo Transfer in Patients With Polycystic Ovary Syndrome. *Zhonghua Fu Chan Ke Za Zhi* (2021) 56(4):257–63. doi: 10.3760/cma.j.cn112141-20201223-00906
25. Ahlsson F, Diderholm B, Jonsson Björn, Nordén-Lindberg S, Olsson R, Ewald U, et al. Insulin Resistance, a Link Between Maternal Overweight and Fetal Macrosomia in Nondiabetic Pregnancies. *Horm Res Paediatr* (2010) 74(4):267–74. doi: 10.1159/000295710
26. Imoh LC, Ocheke AN. Correlation Between Maternal Weight and Insulin Resistance in Second Half of Pregnancy. *Niger Med Jf* (2014) 55(6):465–8. doi: 10.4103/0300-1652.144697
27. Hermann GM, Dallas LM , Haskell SE, Roghair RD. Neonatal Macrosomia Is an Independent Risk Factor for Adult Metabolic Syndrome. *Neonatology* (2010) 98(3):238–44. doi: 10.1159/000285629
28. Pan X-F, Tang L, Lee AH, Binns C, Yang C-X, Xu Z-P, et al. Association Between Fetal Macrosomia and Risk of Obesity in Children Under 3 Years in Western China: A Cohort Study. *Comp Study World J Pediatr* (2019) 15(2):153–60. doi: 10.1007/s12519-018-0218-7
29. Sawada M, Masuyama H, Hayata K, Kamada Y, Nakamura K, Hiramatsu Y. Pregnancy Complications and Glucose Intolerance in Women With Polycystic Ovary Syndrome. *Endocr J* (2015) 62(11):1017–23. doi: 10.1507/endocrj
30. de Wilde MA, Goverde AJ, Veltman-Verhulst SM, Eijkemans MJC, Franx A, Fauser BCJM. Insulin Action in Women With Polycystic Ovary Syndrome and Its Relation to Gestational Diabetes. *Hum Reprod* (2015) 30(6):1447–53. doi: 10.1093/humrep/dev072
31. Lewandowski K, Głuchowska M, Garnysz K, Horzelski W, Grzesiak M, Lewiński A. High Prevalence of Early (1st Trimester) Gestational Diabetes Mellitus in Polish Women Is Accompanied by Insulin Resistance Similar to Women With Polycystic Ovary Syndrome (PCOS). *Endokrynol Pol* (2021). doi: 10.5603/EP.a2021.0095
32. Temming LA, Tuuli MG, Stout MJ, Macones GA, Cahill AG. Maternal and Perinatal Outcomes in Women With Insulin Resistance. *Am J Perinatol* (2016) 33(8):776–80. doi: 10.1055/s-0036-1572434
33. Lewandowski KC, Płusajska J, Horzelski W, Bieniek E, Lewiński A. Limitations of Insulin Resistance Assessment in Polycystic Ovary Syndrome. *Endocr Connect* (2018) 7(3):403–12. doi: 10.1530/EC-18-0021


**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Endocrinology

ORIGINAL RESEARCH
published: 23 May 2022
doi: 10.3389/fendo.2022.851213

# Pregnancy and Perinatal Outcomes of Patients With Prior Cesarean Section After a Single Embryo Transfer in IVF/ICSI: A Retrospective Cohort Study

*Lin Wang, Jing Wang, Nan Lu, Jiayin Liu and Feiyang Diao* *

*State Key Laboratory of Reproductive Medicine, Clinical Center of Reproductive Medicine, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China*

OPEN ACCESS

**Edited by:**
*Yiping Shen,*
*Harvard Medical School, United States*

**Reviewed by:**
*Lang Qin,*
*Sichuan University, China*
*Yichun Guan,*
*Third Affiliated Hospital of Zhengzhou University, China*
*Linli Hu,*
*Zhengzhou University, China*

***Correspondence:**
*Feiyang Diao*
*phenix_y@163.com*

**Specialty section:**
*This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology*

**Received:** *09 January 2022*
**Accepted:** *07 March 2022*
**Published:** *23 May 2022*

**Citation:**
*Wang L, Wang J, Lu N, Liu J and Diao F (2022) Pregnancy and Perinatal Outcomes of Patients With Prior Cesarean Section After a Single Embryo Transfer in IVF/ICSI: A Retrospective Cohort Study. Front. Endocrinol. 13:851213. doi: 10.3389/fendo.2022.851213*

**Objective:** To study the influence of the previous cesarean section on the pregnancy outcomes and perinatal outcomes in single embryo transfer (SET) cycles in an *in vitro* fertilization/intracytoplasmic sperm injection-embryo transfer (IVF/ICSI-ET) setting compared to those with previous vaginal delivery (VD). In addition, the association between fertility outcomes and different cesarean scar defect (CSD) sizes was studied.

**Method:** This was a retrospective cohort study conducted in the Reproductive Center of the First Affiliated Hospital of Nanjing Medical University. A total of 4,879 patients with previous delivery history undergoing SET were included between January 2015 and April 2019. Patients were divided into the VD group and cesarean delivery (CD) group according to different modes of previous delivery. The primary outcome was live birth rate. The pregnancy outcomes of CD were analyzed as a subgroup and the relationship between pregnancy outcomes as well as the different sizes of CSD were explored by logistic regression analysis.

**Results:** There were no significant differences in live birth rate, clinical pregnancy rate, and miscarriage rate between the CD group and VD group. The incidence rates of pregnancy complications such as pregnancy hypertension, gestational diabetes mellitus, placenta abnormalities, premature rupture of membrane, and postpartum hemorrhage were similar in the two groups. Live birth rate was significantly lower in the CSD group (23.77% vs 37.01%, aOR: 0.609, 95% CI: 0.476-0.778) comparing to patients without CSD. There were also significant differences in clinical pregnancy rate (37.52% vs 47.64%, aOR: 0.779, 95%CI: 0.623-0.973) and miscarriage rate (34.55% vs 20.59%, aOR: 1.407, 95% CI:1.03-1.923). Large size CSD significantly decreased live birth rate (13.33% vs 26.29%, aOR: 0.422, 95%CI: 0.197-0.902) and clinical pregnancy rate (25.33% vs 40.09%, aOR: 0.503, 95%CI: 0.272-0.930) compared with small size CSD.

**Conclusion:** For women with previous cesarean sections, the pregnancy outcomes were similar to those with previous VD without increased perinatal complications following SET. The presence of CSD was associated with a marked reduction in live birth rate, especially in patients with large size CSD.

**Keywords: live birth, single embryo transfer, *in vitro* fertilization, Cesarean delivery, Cesarean section defect**

# 1 INTRODUCTION

In recent decades, the prevalence of cesarean section (CS) in the global scope grew two-fold, increasing from 12% in 2000 to 21% in 2015 in all deliveries (1). In China, the percentage of CS delivery increased from 28.8% to 36.7% from 2008 to 2018 (2), which was much higher than the reasonable range of 10-15% recommended by the World Health Organization (WHO). There are rising concerns regarding the short-term complications and long-term risks of CS, including placental implantation and uterine rupture in the next pregnancy (3). It is well established that pregnancy risks dramatically increase with twin pregnancies than singleton pregnancies (4), especially in patients with scarred uterus (5). There is an urgent need to reduce the multiple pregnancies rate in patients with the previous cesarean delivery (CD) (6). However, the incidence of multiple pregnancies increases in patients undergoing IVF by multiple embryos transfer to achieve a higher pregnancy rate (7). Single embryo transfer (SET) is an effective strategy to avoid multiple pregnancies without compromising the cumulative live birth rates compared with double embryos transfer (8). Previous studies showed SET not only decreased multiple pregnancies risk but also improved the perinatal outcomes compared with singletons resulting from double-embryo transfers (9). Hence, SET is recommended for patients with a scarred uterus. Keeping that in mind, the pregnancy and perinatal outcomes after SET in patients with previous CS are still unknown.

A significantly lower rate of natural conception after CD was reported (10). Several studies investigated the association of prior CD and pregnancy outcomes in IVF cycles. The conclusions have been controversial as these studies lack homogeneity and different studies evaluated different numbers (one or more) of embryos transfer including a mix of cleavage-stage and blastocyst-stage embryos transfer. It is important to further explore the effect of previous CD on pregnancy outcomes in a SET setting.

Cesarean scar defect (CSD) is also called niche or diverticulum, which refers to poor healing of uterine scar after CS (11). Its prevalence varies from 6.9-69% depending on the study population and methodology used (12). Some reports suggested that CSD impaired embryo implantation and subsequent fertility (13, 14). Residual myometrial thickness (RMT) measured less than 3mm is defined as large CSD (15) with a high risk of spontaneous uterine rupture (16, 17). No published studies have investigated the relationship between pregnancy outcomes with different sizes of CSD.

Therefore, this study aimed to investigate the impact of previous CD compared with previous vaginal delivery (VD) on the reproductive outcomes and perinatal outcomes in patients undergoing SET. We also explored the relationship between the pregnancy outcomes and different CSD sizes in patients undergoing IVF treatment.

# 2 MATERIAL AND METHOD

## 2.1 Study Population

This retrospective study was conducted at the Department of Assisted Reproduction Center of the First Affiliated Hospital of Nanjing Medical University from January 2015 to April 2019. Patients included into this study had at least one previous delivery (including CS and VD) and SET was performed. Only the first embryo transfer was included in the analysis. According to the previous modes of delivery, patients were divided into two groups: the previous CD group and the previous VD group. Exclusion criteria was: advanced maternal age (>43years); recurrent pregnant loss: two or more pregnancy loss before 24 weeks of gestation; untreated mild to severe hydrosalpinx, endometriosis, uterine adhesion; Preimplantation Genetic Testing (PGT) cycles; and oocytes donation cycles.

## 2.2 CSD Evaluation

All patients were assessed by Voluson E8 ultrasound system (General Electric Voluson, 2014, USA) equipped with a 5-9 MHz three-dimensional transvaginal probe. The three-dimensional-transvaginal ultrasound (3D-TVS) was taken 3-7 days after menstruation. CSD is defined as a wedge-shaped anechoic area with an indentation of the myometrium larger than 2 mm at the site of CS. The depth, width of CSD, and RMT were measured in the sagittal plane (18). The large CSD was estimated as RMT less than 3mm, middle size CSD was RMT in a range of 3-6 mm, small size CSD was defined as RMT more than 6mm.

## 2.3 Treatment Protocol

### 2.3.1 Ovarian Stimulation

Conventional gonadotropin releasing hormone (GnRH) agonist (GnRHa) (midluteal GnRHa suppression) and GnRH antagonist (antagonist administration when the leading follicle diameter reaches 13mm) regimens were performed for ovarian stimulation. The initial dose of recombinant follicle-stimulating hormone (FSH) was 100–300 IU/day depending on age, body mass index (BMI), ovarian reserve, and possible response to stimulation.

### 2.3.2 Ovulation Trigger and Luteal Phase Support

When at least the diameter of two follicles reached 18 mm or three follicles greater than 17mm, a single bonus of 6500 IU

recombinant human chorionic gonadotropin (hCG) injection was administered subcutaneously and oocyte retrieval was performed 36 hours later. Only one embryo was transferred 3–5 days after oocyte retrieval. The luteal phase was daily supported by progesterone from the day of oocyte retrieval and continued for 14 days after the embryo transfer. In the cases of potential severe ovarian hyperstimulation syndrome, all embryos were frozen.

#### 2.3.3 Frozen-Thawed Embryo Transfer (FET) Protocol

Endometrial preparation for FET was performed by four regimens, including natural cycle, induced ovulation cycle, hormone replacement therapy (HRT), and GnRHa combined HRT (GnRHa+HRT) regimen. The natural cycle was performed in women with regular menstruation with or without hCG trigger. An induced ovulation cycle was conducted among anovulatory women with letrozole in combination with human menopausal gonadotropin (hMG). Luteal phase support was administered on the day of ovulation. For the HRT cycle, exogenous estrogen was administered until the endometrium reached optimal thickness, then the supplement of exogenous progesterone was performed. The GnRHa+HRT was mainly for women with endometriosis or adenomyosis. Pituitary down-regulation was achieved by a full dose of GnRHa 3.75mg at day 1 or day 2 of the menstrual cycle and HRT was performed 25-28 days later. A cleavage-stage or a blastocyst-stage embryo was transferred 3-5 days after endometrial development with progesterone.

### 2.4 Outcome Measures

The primary outcome was live birth rate, defined as live births after 28 gestational weeks. The secondary outcome parameters included biochemical pregnancy, clinical pregnancy, miscarriage, ectopic pregnancy, twin pregnancies, neonatal outcomes, and maternal pregnancy complications. Biochemical pregnancy was detected as positive serum hCG 14 days after embryo transfer. Clinical pregnancy was defined as the presence of a gestational sac with or without fetal heart detected by the ultrasound examination at the eighth gestational week. Miscarriage referred to pregnancy loss before 28 gestational weeks. Ectopic pregnancy referred to the gestational sac detected out of the uterine cavity. Twin pregnancies was defined as two fetal heartbeats detected by ultrasound. The interested maternal complications included gestational hypertension, gestational diabetes, placental abnormalities such as placenta previa and placental abruption, premature rupture of the membrane, and postpartum hemorrhage. Neonatal outcomes comprised preterm birth (<37 weeks of gestation), stillbirth (fetal death after 28 gestational weeks), low birth weight (< 2500g), and very low birth weight (<1500g).

### 2.5 Statistical Analysis

Data were analyzed by SPSS statistics (version 26; IBM, Armonk, NY). Continuous variables were described as mean values with standard deviation and categorical variables were described as numbers with percentages. Propensity score matching (PSM) was applied to balance the distributions of observed baseline characteristics between the CD groups and the VD groups with a 1:1 nearest-neighbor matching strategy and caliper was set as 0.2. Age, BMI, infertility diagnosis, fertilization methods, fresh or frozen-thawed cycle, the protocol of fresh and frozen embryo transfer, and endometrial thickness on the day of embryo transfer were selected as the matching factors. After PSM, Student's t-test or Mann-Whitney U test was used for continuous variables, depending on the normality of the data distribution. Fisher's exact test and Pearson's $\chi 2$ were used for categorical data. Univariate and multivariate logistic regression analysis was performed to test the relationship between the presence of CSD and reproductive outcomes. The adjusted covariates of logistic regression included age, BMI, fresh or frozen-thawed cycle, the stage of embryo transferred, and endometrial thickness on the day of transfer. The association of the different sizes of CSD and the reproductive outcomes were performed by the logistic regression by the adjusted factors described above. The crude and adjusted results were expressed as odds ratio (OR) with 95% confidence intervals (CIs). A two-sided P value of less than 0.05 was considered statistically significant.

## 3 RESULTS

### 3.1 General Information of Patients With Different Delivery Modes Following SET

As shown in **Figure 1**, 3,135 women who underwent single cleavage-stage embryo transfer and 1,744 women who underwent single blastocyst-stage embryo transfer were included. Before matching, the baseline characteristics such as age, BMI, and the thickness of the endometrium were not balanced in VD and CD groups. After subsequent propensity score matching, 1,350 patients were assigned to the VD and CD groups, respectively, in patients with single cleavage-stage embryo transfer and 729 patients were included in each group with single blastocyst-stage embryo transfer. The baseline variables such as age, BMI, infertility factors, endometrial thickness, the protocol of fresh and frozen embryo transfer were all comparable between the VD and CD groups in both cleavage-stage and blastocyst-stage embryo transfer populations (all $P>0.05$) (**Table 1**).

### 3.2 The Pregnancy and Perinatal Outcomes of VD and CD Groups

The pregnancy outcomes of the VD and CD groups are presented in **Table 2**. The biochemical pregnancy rate (cleavage-stage: 40.67% vs 39.18%, $P=0.432$; blastocyst-stage: 71.60% vs 69.41%, $P=0.358$), clinical pregnancy rate (cleavage-stage: 36.22% vs 34.29%, $P=0.295$; blastocyst-stage: 66.67% vs 65.29%, $P=0.543$), and live birth rate (cleavage-stage: 26.59% vs 23.70%%, $P=0.084$; blastocyst-stage: 57.20% vs 52.40%, $P=0.066$) were higher in the VD groups but the differences failed to reach significant difference. In addition, no significant differences were observed in miscarriage rate, ectopic pregnancy rate, or twin pregnancies rate in different groups. **Table 3** shows the perinatal outcomes including maternal complications and neonatal outcomes. The prevalence rate of preterm birth, low birth, very low birth, and obstetric complications did not differ in VD and

**TABLE 1** | Demographics and cycle characteristics of patients with different previous delivery modes.

| | Cleavage-stage embryo | | | | | | Blastocyst -stage embryo | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | before PSM | | | after PSM | | | before PSM | | | after PSM | | |
| | VD (n=1707) | CD (n=1428) | *P* value | VD (n=1350) | CD (n=1350) | *P* value | VD (n=926) | CD (n=818) | *P* value | VD (n=729) | CD (n=729) | *P* value |
| Age (year) | 35.52 ±4.71 | 36.81 ±5.00 | <0.001* | 36.52 ±4.96 | 36.3±4.64 | 0.237 | 33.48 ±4.76 | 33.39 ±4.25 | 0.707 | 33.43 ±4.77 | 33.30 ±4.41 | 0.58 |
| BMI (kg/m2) | 23.13 ±2.78 | 23.63 ±2.79 | 0.004* | 23.13 ±2.82 | 23.25 ±2.96 | 0.283 | 22.38 ±2.73 | 23.68 ±2.81 | 0.028* | 22.38 ±2.74 | 22.63 ±2.7 | 0.079 |
| Infertility diagnosis, n (%) | | | | | | | | | | | | |
| Tubal factors | 597 (34.97) | 469 (32.84) | 0.294 | 438 (32.44) | 446 (33.04) | 0.613 | 460 (49.68) | 402 (49.14) | 0.976 | 376 (51.58) | 363 (49.19) | 0.898 |
| Decreased ovarian reservation | 551 (32.28) | 448 (31.37) | | 402 (29.78) | 426 (31.56) | | 195 (21.06) | 169 (20.66) | | 149 (20.44) | 154 (20.87) | |
| Unexplained infertility | 157 (9.20) | 135 (9.45) | | 125 (9.26) | 118 (8.74) | | 95 (10.26) | 85 (10.39) | | 61 (8.37) | 60 (8.13) | |
| Combined factors | 402 (23.55) | 376 (26.33) | | 385 (28.52) | 360 (26.67) | | 176 (19.01) | 162 (19.8) | | 143 (19.62) | 152 (20.6) | |
| Fertilization method, n (%) | | | | | | | | | | | | |
| IVF | 1371 (80.32) | 1139 (79.76) | 0.699 | 1090 (80.74) | 1065 (78.89) | 0.231 | 745 (80.45) | 687 (83.99) | 0.055 | 599 (82.17) | 602 (82.58) | 0.837 |
| ICSI | 336 (19.68) | 289 (20.24) | | 260 (19.26) | 285 (21.11) | | 181 (19.55) | 131 (16.01) | | 130 (17.83) | 127 (17.42) | |
| transfer cycle, n (%) | | | | | | | | | | | | |
| Fresh | 720 (42.18) | 602 (42.16) | 0.990 | 526 (38.96) | 575 (42.59) | 0.055 | 40 (4.32) | 45 (5.50) | 0.253 | 24 (3.29) | 28 (3.84) | 0.572 |
| Frozen | 987 (57.82) | 826 (57.84) | | 824 (61.04) | 775 (57.41) | | 886 (95.68) | 773 (94.50) | | 705 (96.71) | 701 (96.16) | |
| Stimulation protocol, n (%) | | | | | | | | | | | | |
| Agonist | 371 (51.53) | 319 (52.99) | 0.596 | 277 (52.66) | 305 (53.04) | 0.899 | 32 (80) | 35 (77.78) | 0.802 | 22 (91.67) | 20 (71.43) | 0.065 |
| Antagonist | 349 (48.47) | 283 (47.01) | | 249 (47.34) | 270 (46.96) | | 8 (20) | 10 (22.22) | | 2 (8.33) | 8 (28.57) | |
| Endometrial preparation method, n (%) | | | | | | | | | | | | |
| Natural cycle | 435 (44.07) | 375 (45.40) | 0.314 | 384 (46.6) | 352 (45.42) | 0.272 | 357 (40.29) | 305 (39.46) | 0.292 | 270 (38.30) | 280 (39.94) | 0.641 |
| Induced ovulation | 302 (30.60) | 272 (32.93) | | 246 (29.85) | 261 (33.69) | | 350 (39.50) | 299 (38.68) | | 275 (39.01) | 277 (39.51) | |
| Hormone replacement treatment (HRT) | 169 (17.12) | 119 (14.41) | | 135 (16.38) | 106 (13.68) | | 108 (12.19) | 87 (11.25) | | 93 (13.19) | 77 (10.98) | |
| GnRHa+HRT | 81 (8.21) | 60 (7.26) | | 59 (7.16) | 56 (7.23) | | 71 (8.01) | 82 (10.61) | | 67 (9.50) | 67 (9.56) | |
| Endometrial thickness (mm) | 9.62±1.89 | 9.50±1.70 | 0.040* | 9.56±1.85 | 9.49±1.79 | 0.296 | 9.76±1.74 | 9.59±1.64 | 0.040* | 9.70±1.68 | 9.65±1.67 | 0.505 |

*PSM, propensity score matching; VD, vaginal delivery; CD, cesarean delivery; BMI, body mass index; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; GnRHa, gonadotropin-releasing hormone agonist; Values are described as mean ± standard deviation or number (percentage); *P<0.05.*

CD groups (all *P*>0.05). However, we observed a statistically significant decrease in gestational weeks of delivery in patients with previous CS (cleavage-stage: 38.39 ± 1.89 weeks vs 38.08 ± 1.55 weeks, *P*=0.02; blastocyst-stage: 38.36 ± 1.63 weeks vs 37.95 ±1.49 weeks, *P*<0.001).

## 3.3 The Baseline Characteristics and Logistic Regression Analysis of Reproductive Outcomes Between Previous CD Patients With and Without CSD

As shown in **Table 4**, the number of patients with previous CD without visible scars was 1,570 and the number of patients with CSD was 509. The baseline characteristics such as age, BMI, endometrial thickness, and the proportion of blastocyst-stage transfer were comparable between the patients with and without CSD (all *P*>0.05), while the proportion of fresh embryo transfer was significantly different between the two groups (34.77% vs 27.13%, *P*=0.001). We investigated the pregnancy outcomes by logistic regression to overcome the imbalance and the results are shown in **Table 5**. After adjusting for age, BMI, fresh or frozen-thawed cycle, the stage of embryo transferred, endometrial thickness, the live birth rate was significantly lower in patients with CSD than those without CSD (23.77% vs 37.01%, aOR: 0.609, 95%CI: 0.476-0.778). The probability of clinical pregnancy rate (37.52% vs 47.64%, aOR: 0.779, 95%CI: 0.623-0.973) also decreased in patients with CSD. A significantly increased risk of

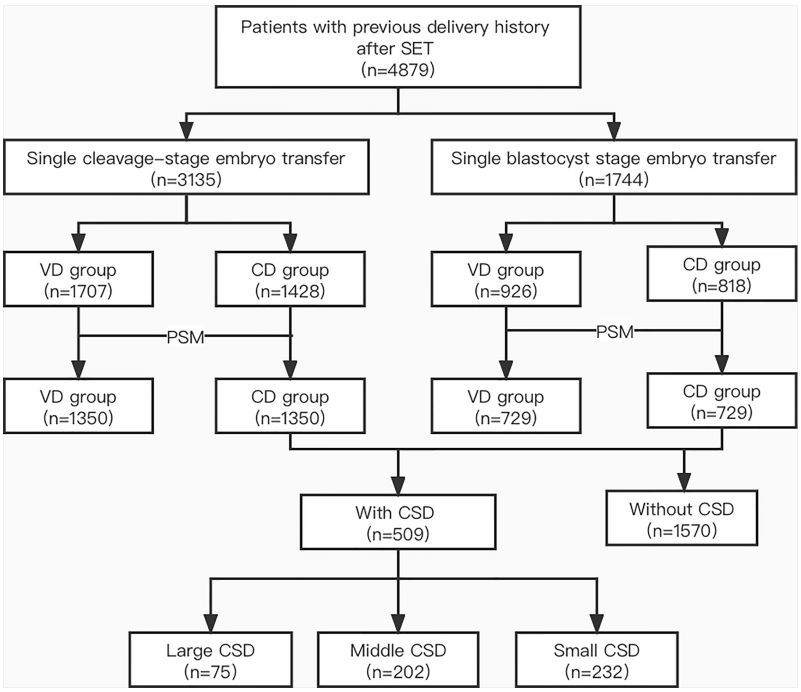



**FIGURE 1** | Flow chart of the study. SET, single embryo transfer; VD, vaginal delivery; CD, cesarean delivery; PSM, propensity score matching; CSD, cesarean scar defect.

**TABLE 2** | Pregnancy outcomes of patients with different previous delivery modes.

| Parameter | Cleavage-stage embryo | | | Blastocyst-stage embryo | | |
|---|---|---|---|---|---|---|
| | VD group (n=1350) | CD group (n=1350) | *P* value | VD (n=729) | CD (n=729) | *P* value |
| Biochemical pregnancy rate, %(n/N) | 40.67 (549/1350) | 39.18 (529/1350) | 0.432 | 71.60 (522/729) | 69.41 (506/729) | 0.358 |
| Clinical pregnancy rate,%(n/N) | 36.22 (489/1350) | 34.29 (463/1350) | 0.295 | 66.67 (487/729) | 65.29 (476/729) | 0.543 |
| Miscarriage rate, %(n/N) | 24.13 (118/489) | 28.94 (134/463) | 0.093 | 13.78 (67/487) | 18.07 (86/476) | 0.067 |
| Ectopic pregnancy rate, %(n/N) | 2.86 (14/489) | 1.94 (9/463) | 0.356 | 0.62 (3/487) | 1.68 (8/476) | 0.120 |
| Twin pregnancies rate, %(n/N) | 1.02 (5/489) | 0.86 (4/463) | 0.179 | 1.64 (8/487) | 0.63 (3/476) | 0.139 |
| Live birth rate, %(n/N) | 26.59 (359/1350) | 23.70 (320/1350) | 0.084 | 57.20 (417/729) | 52.40 (382/729) | 0.066 |

*VD, vaginal delivery; CD, cesarean delivery. Values are described as percentage (number/total number),*P<0.05.*

**TABLE 3** | Perinatal outcomes of patients with different modes of previous delivery.

| Parameter | Cleavage-stage embryo | | | Blastocyst -stage embryo | | |
|---|---|---|---|---|---|---|
| | VD group (n=359) | CD group (n=320) | *P* value | VD (n=417) | CD (n=382) | *P* value |
| Maternal complications, n (%) | 43 (11.98) | 47 (14.69) | 0.299 | 41 (9.83) | 52 (13.61) | 0.096 |
| Gestational diabetes mellitus, n (%) | 18 (5.01) | 24 (7.50) | 0.179 | 26 (6.24) | 28 (7.33) | 0.538 |
| Gestational hypertension, n (%) | 11 (3.06) | 8 (2.50) | 0.656 | 3 (0.72) | 5 (1.31) | 0.403 |
| Placenta previa, n (%) | 8 (2.23) | 8 (2.50) | 0.816 | 4 (0.96) | 9 (2.36) | 0.119 |
| Placental abruption, n (%) | 1 (0.28) | 0 (0.00) | 0.345 | 1 (0.24) | 0 (0.00) | 0.338 |
| Premature rupture of membrane, n (%) | 4 (1.11) | 6 (1.88) | 0.411 | 6 (1.44) | 7 (1.83) | 0.660 |
| Postpartum hemorrhage, n (%) | 1 (0.28) | 1 (0.31) | 0.935 | 1 (0.24) | 3 (0.79) | 0.275 |
| Neonatal outcomes | | | | | | |
| Gestational age of delivery (weeks) | 38.39±1.89 | 38.08±1.55 | 0.020* | 38.36±1.63 | 37.95±1.49 | <0.001* |
| Birth weight (g) | 3497.78±614.20 | 3418.38±539.95 | 0.646 | 3481.08±513.40 | 3408.96±536.82 | |
| Preterm birth, n (%) | 35 (9.75) | 34 (10.63) | 0.379 | 34 (8.15) | 41 (10.73) | 0.212 |
| Stillbirth, n (%) | 2 (0.55) | 0 (0.00) | 0.182 | 0 (0.00) | 1 (0.26) | 0.317 |
| Low birth weight (<2500g), n (%) | 10 (5.29) | 13 (4.06) | 0.400 | 9 (2.16) | 13 (3.40) | 0.283 |
| Very low birth weight (<1500g), n (%) | 1 (0.28) | 2 (0.62) | 0.462 | 1 (0.24) | 1 (0.26) | 0.950 |

*VD, vaginal delivery; CD, cesarean delivery. Values are described as mean±standard deviation or number (percentage) *P<0.05.*

**TABLE 4 |** Baseline characteristics of patients with and without CSD.

| Item | Without CSD group (n=1570) | CSD group (n=509) | *P* value |
|---|---|---|---|
| Ages (years) | 35.04±4.71 | 35.84±4.99 | 0.339 |
| BMI (kg/m2) | 22.85±2.86 | 23.56 ±2.87 | 0.240 |
| Endometrial thickness(mm) | 9.59 ±1.73 | 9.49 ±1.79 | 0.522 |
| Blastocyst-stage embryo transfer rate, %(n/N) | 36.18% (568/1570) | 31.63% (161/509) | 0.062 |
| Fresh embryo transfer rate, %(n/N) | 27.13% (426/1570) | 34.77% (177/509) | 0.001* |

*CSD, cesarean section defect; BMI, body mass index; Values are described as mean±standard deviation or percentage (number/total number); *P<0.05.*

**TABLE 5 |** Logistic regression analysis of reproductive outcomes of patients with and without CSD.

| Parameter | Without CSD group (n=1570) | CSD group (n=509) | Crude OR | *P* value | Adjusted OR | *P* value |
|---|---|---|---|---|---|---|
| Biochemical pregnancy rate, % (n/N) | 51.78 (813/1570) | 43.61 (222/509) | 0.720 (0.589-0.881) | 0.001* | 0.865 (0.696-1.076) | 0.194 |
| Clinical pregnancy rate, % (n/N) | 47.64 (748/1570) | 37.52 (191/509) | 0.660 (0.538-0.810) | 0.001* | 0.779 (0.623-0.973) | 0.027* |
| Miscarriage rate, % (n/N) | 20.59 (154/748) | 34.55 (66/191) | 1.370 (1.007-1.863) | 0.045* | 1.407 (1.030-1.923) | 0.032* |
| Ectopic pregnancy rate, % (n/N) | 1.74 (13/748) | 2.09 (4/191) | 0.949 (0.308-2.923) | 0.927 | 1.088 (0.349-3.389) | 0.885 |
| Live birth rate, % (n/N) | 37.01 (581/1570) | 23.77 (121/509) | 0.531 (0.422-0.667) | <0.001* | 0.609 (0.476-0.778) | <0.001* |

*CSD, cesarean section defect. Values are described as percentage (number/total number); Adjusted for age, BMI, fresh or frozen-thawed cycle, the stage of embryo at transfer, endometrial thickness; *P<0.05.*

miscarriage was observed in the CSD group (34.55% vs 20.59%, aOR: 1.407, 95%CI: 1.03-1.923). There were no significant differences in biochemical pregnancy rate or ectopic pregnancy rate.

## 3.4 The Relationship Between the Reproductive Outcomes and Different CSD Size

After adjusting for important confounders (age, BMI, fresh or frozen-thawed cycle, the stage of embryo transferred, endometrial thickness), patients with large CSD were associated with a significantly lower live birth rate (13.33% vs 26.29%, aOR: 0.422, 95%CI: 0.197-0.902) compared with patients with small CSD. Similarly, biochemical pregnancy rate (32.00% vs 45.69%,aOR: 0.546, 95%CI: 0.305-0.978) and clinical pregnancy rate(25.33% vs 40.09%, aOR: 0.503, 95%CI: 0.272-0.93) were significantly lower in the large CSD group. However, there were no significant differences observed in miscarriage rate among patients with different sizes of CSD (**Table 6**).

# 4 DISCUSSION

CS rate is increasing worldwide and continues to grow. CS leads to an anatomic change of the uterus and contributed to a lower rate of childbearing (9, 19). Recent studies attempt to demonstrate the relation between CD and subsequent pregnancy outcomes in IVF, but the results have been controversial. The underlying cause of the difference was considered to be the heterogeneity of these studies. One of the factors was the imbalanced baseline characteristics of the patients, including maternal age, endometrial thickness at the day of transfer, and BMI. Zhang et al. (20) observed no difference in live birth rate (40.59% vs 45.38%, *P*=0.466) between the CD and VD groups, however, there was imbalanced maternal age. Diao (21) et al. also revealed no significant difference in live birth rate (33.1% vs36.4%, OR: 0.86, 95%CI: 0.64~1.16, *P*>0.05) with thinner endometrial thickness in the CD group. In another study, Friedenthal J et al. (22) reported nearly a 10% reduction in the live birth rate of the CD group with imbalanced BMI. Our preliminary data also showed some imbalanced characteristics including higher age, larger BMI, and thinner endometrium in the CD group. To overcome this imbalance, we utilized PSM and reported a lower live birth rate, clinical pregnancy rate, and higher miscarriage rate without a statistically significant difference in women with previous CD compared with women with previous VD following SET. Furthermore, previous studies included patients with a mix of cleavage-stage and blastocyst-stage embryo transfers in different proportions, which could lead to a biased interpretation of the results. In a prospective study performed by Patounakis et al. (23), the live birth rate (39% vs 32%, *P*=0.366) was similar between different modes of the previous delivery with 35-39% blastocyst-stage embyro transfer rate. When the blastocyst transfer rate was only 7.9-9% [Huang et al. (24)], an obviously lower live birth rate (27.5% vs 33.4%, *P*=0.03) in patients with previous CD was discovered. Previous work demonstrates that blastocyst-stage embryo transfer was associated with an increased pregnancy rate than cleavage-stage embryo transfer (25, 26), so we further stratified patients with different stages of embryo development, respectively, to avoid bias. The results showed the same trend of lower live birth rate regardless of cleavage-stage embryo or blastocyst-stage embryo transfer.

Patients with previous CD history had an increased risk of life-threatening pregnancy complications with the subsequent twin gestation than singleton pregnancy (27). SET was defined as a multiple birth minimization strategy (28). Our data shows comparable perinatal outcomes following SET between patients with different previous delivery modes. The incidences of adverse obstetric and neonatal outcomes did not show significant differences between the CD and VD groups. The twin pregnancies rates were 0.63-1.64% in patients with CD history

**TABLE 6** | Logistic regression analysis of patients with different sizes of CSD.

| Parameter | (%) (n/N) | Crude OR (95%CI) | *P* value | Adjusted OR (95%CI) | *P* value |
|---|---|---|---|---|---|
| **Biochemical pregnancy rate** | | | | | |
| Small CSD | 45.69 (106/232) | reference | | reference | |
| Middle CSD | 45.54 (92/202) | 0.994 (0.681-1.452) | 0.976 | 0.884 (0.586-1.334) | 0.557 |
| Large CSD | 32.00 (24/75) | 0.559 (0.323-0.969) | 0.038* | 0.546 (0.305-0.978) | 0.042* |
| **Clinical pregnancy rate** | | | | | |
| Small CSD | 40.09 (93/232) | reference | | reference | |
| Middle CSD | 39.11 (79/202) | 0.960 (0.653-1.412) | 0.836 | 0.862 (0.567-1.310) | 0.488 |
| Large CSD | 25.33 (19/75) | 0.507 (0.283-0.908) | 0.022* | 0.503 (0.272-0.930)* | 0.028* |
| **miscarriage rate** | | | | | |
| Small CSD | 31.18 (29/93) | reference | | reference | |
| Middle CSD | 35.44 (28/79) | 1.126 (0.645-1.967) | 0.675 | 1.105 (0.620-1.967) | 0.735 |
| Large CSD | 47.37 (9/19) | 0.955 (0.430-2.120) | 0.909 | 1.012 (0.450-2.278) | 0.976 |
| **Live birth rate** | | | | | |
| Small CSD | 26.29 (61/232) | reference | | reference | |
| Middle CSD | 24.75 (50/202) | 0.922 (0.598-1.422) | 0.714 | 0.832 (0.522-1.326) | 0.439 |
| Large CSD | 13.33 (10/75) | 0.431 (0.208-0.892) | 0.023* | 0.422 (0.197-0.902) | 0.026* |

*CSD, cesarean section defect; Values are described as percentage ( number/total number ); Adjusted for age, BMI, fresh or frozen-thawed cycle, stage of embryo transferred, endometrial thickness;*P<0.05.*

following SET. In contrast to our study, some studies (29, 30) transferred one or more embryos in patients with previous CS, and the twin birth rate approximately reached 30% with significantly higher preterm birth rate than singleton births. Some patients even received selective fetal reduction to decrease the risk of adverse events in twin birth. Selective fetal reduction was an invasive procedure complicated with infection and miscarriage (31) and SET was more likely to be the first option to achieve a healthy live birth. Moreover, the CD group showed a significantly lower gestational age than VD group (cleavage-stage: 38.39 ± 1.89 weeks vs 38.08 ± 1.55 weeks, $P$=0.02, blastocyst-stage: 38.36 ± 1.63 weeks vs 37.95 ± 1.49 weeks, $P$<0.001). This might be associated with the timing of elective repeat CS without labor. Most repeat cesarean deliveries were performed around 37-39 weeks of gestation (32) in patients with previous history of CS concerning maternal and neonatal safety (33).

The presence of CSD had a negative effect on subsequent fertility (34). In this study, the presence of CSD shows a detrimental effect on subsequent pregnancy. The results remained robust after adjusting for the possible confounders and effect-modifying factors. Patients with CSD were associated with a significantly lower rate of subsequent live birth (aOR: 0.609, 95%CI: 0.476~0.778, $P$<0.001) and clinical pregnancy (aOR: 0.779, 95%CI: 0.623~0.973, $P$=0.027), as well as a higher likelihood of miscarriage (aOR:1.407, 95%CI: 1.03~1.923, $P$=0.032) compared with those without defect at the site of the cesarean incision. The results were in agreement with previous studies (21, 35). The existence of CSD could lead to poor pregnancy outcomes in patients undergoing IVF.

In literature, large CSD (RMT<3mm) in non-pregnant women is regarded as a high risk of uterine dehiscence or rupture in subsequent pregnancies (36). However, there is no definitive classification of CSD to predict the pregnancy outcomes in IVF. This study explored the relationship between different sizes of scar defects and pregnancy outcomes with a logistic regression model adjusted for potential confounding factors. Live birth rate (13.33% vs 26.29%, aOR: 0.422, 95%CI: 0.197-0.902) and clinical pregnancy rate (25.33% vs 40.09%, aOR: 0.503, 95%CI: 0.272-0.930) sharply decreased in patients with large CSD compared with those with small CSD. The underlying mechanisms appear to be associated with reduced scar contractility around the fibrotic scar (37). The impaired ability of myometrium cannot expel the blood completely in the niche with degradation of hemoglobin (38). The fluid accumulated at the CS site may hamper the embryo implantation like in patients with hydrosalpinx (39). The toxic environment with excess iron might disturb the endometrial receptivity and uterine microbiota (40). Another explanation is that CSD may compromise the process of decidualization (41). The delayed endometrial maturation has a negative effect on steroid receptor expression and impairs embryo implantation (42). Furthermore, the altered immune microenvironment in the scar can lead to a decline in fertility with less vascularization and leukocytes (13).

The major weakness of our study was its retrospective design. We were unable to get more detailed previous information about the CS, such as previous pregnancy complications, emergent or elective CS, single or double-layer suture of the uterus and the ability to assess the role of related information on pregnancy outcomes was not available. Another limitation was the sensitivity of 3D-TVS examination for CSD. Saline contrast sonography, hysteroscopy, or magnetic resonance imaging (MRI) might provide more accuracy but were also more invasive and expensive (43, 44). A better diagnosis tool and classification for CSD needs to be explored.

## 5 CONCLUSION

This study demonstrated no significant differences in pregnancy outcomes and no higher incidences of perinatal complications in patients with different modes of previous delivery in SET cycles. Further subgroup analyses suggested the presence of CSD was

associated with a lower live birth rate, and large CSD was identified as the main deleterious factor for live birth. Our findings suggest clinicians should assess the healing of uterus scars and inform patients of the adverse impacts of CSD in the subsequent pregnancy.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Reproductive Medical Ethics Committee of the first affiliated hospital of Nanjing Medical University. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

LW and FD conceived and designed the study. JW collected data and performed the statistical analysis. LW wrote the first draft which was revised by NL and JW. The study was supervised by JL and FD. All the authors contributed to the study and approved the submitted version.

## FUNDING

This work was supported by National Key Research and Development Program of China (2018YFC100258, 2017YFC1001303) and National Natural and Science Foundation of China (81730041).

## ACKNOWLEDGMENTS

The authors thank all participants involved in this study.

## REFERENCES

1. Boerma T, Ronsmans C, Melesse DY, Barros AJD, Barros FC, Juan L, et al. Global Epidemiology of Use of and Disparities in Caesarean Sections. *Lancet* (2018) 392:1341–8. doi: 10.1016/S0140-6736(18)31928-7
2. Li HT, Hellerstein S, Zhou YB, Liu JM, Blustein J. Trends in Cesarean Delivery Rates in China, 2008-2018. *JAMA* (2020) 323(1):89–91. doi: 10.1001/jama.2019.17595
3. Sandall J, Tribe RM, Avery L, Mola G, Visser GH, Homer CS, et al. Short-Term and Long-Term Effects of Caesarean Section on the Health of Women and Children. *Lancet* (2018) 392(10155):1349–57. doi: 10.1016/S0140-6736(18)31930-5
4. Santana DS, Surita FG, Cecatti JG. Multiple Pregnancy: Epidemiology and Association With Maternal and Perinatal Morbidity. *Rev Bras Ginecol Obstet* (2018) 40(9):554–62. doi: 10.1055/s-0038-1668117
5. Ngene NC, Naidoo TD, Titus M, Moodley J, Craib S, Stutterheim J. Antepartum Uterine Rupture in a Twin Gestation Presenting as an Advanced Extrauterine Pregnancy in a Patient With a Previous Lower Segment Caesarean Section. *Trop Doct* (2014) 44(3):176–8. doi: 10.1177/0049475514524954
6. Adamson GD, Norman RJ. Why are Multiple Pregnancy Rates and Single Embryo Transfer Rates So Different Globally, and What do We do About it? *Fertil Steril* (2020) 114(4):680–9. doi: 10.1016/j.fertnstert.2020.09.003
7. Gleicher N, Kushnir VA, Barad D. Fertility Treatments and Multiple Births in the United States. *N Engl J Med* (2014) 370(11):1069. doi: 10.1056/NEJMc1400242
8. Thurin A, Hausken J, Hillensjö T, Jablonowska B, Pinborg A, Strandell A, et al. Elective Single-Embryo Transfer Versus Double-Embryo Transfer in *In Vitro* Fertilization. *N Engl J Med* (2004) 351(23):2392–402. doi: 10.1056/NEJMoa041032
9. Martin AS, Chang J, Zhang Y, Kawwass JF, Boulet SL, McKane P, et al. Perinatal Outcomes Among Singletons After Assisted Reproductive Technology With Single-Embryo or Double-Embryo Transfer Versus No Assisted Reproductive Technology. *Fertil Steril* (2017) 107(4):954–60. doi: 10.1016/j.fertnstert.2017.01.024
10. Gurol-Urganci I, Bou-Antoun S, Lim CP, Cromwell DA, Mahmood TA, Templeton A, et al. Impact of Caesarean Section on Subsequent Fertility: A Systematic Review and Meta-Analysis. *Hum Reprod* (2013) 28(7):1943–52. doi: 10.1093/humrep/det130
11. Tulandi T, Cohen A. Emerging Manifestations of Cesarean Scar Defect in Reproductive-Aged Women. *J Minim Invasive Gynecol* (2016) 23(6):893–902. doi: 10.1016/j.jmig.2016.06.020
12. Antila-Långsjö RM, Mäenpää JU, Huhtala HS, Tomás EI, Staff SM. Cesarean Scar Defect: A Prospective Study on Risk Factors. *Am J Obstet Gynecol* (2018) 219(5):458.e1–8. doi: 10.1016/j.ajog.2018.09.004
13. Naji O, Wynants L, Smith A, Abdallah Y, Saso S, Stalder C, et al. Does the Presence of a Caesarean Section Scar Affect Implantation Site and Early Pregnancy Outcome in Women Attending an Early Pregnancy Assessment Unit? *Hum Reprod* (2013) 28(6):1489–96. doi: 10.1093/humrep/det110
14. Gubbini G, Centini G, Nascetti D, Marra E, Moncini I, Bruni L, et al. Surgical Hysteroscopic Treatment of Cesarean-Induced Isthmocele in Restoring Fertility: Prospective Study. *J Minim Invasive Gynecol* (2011) 18(2):234–7. doi: 10.1016/j.jmig.2010.10.011
15. Donnez O, Donnez J, Orellana R, Dolmans MM. Gynecological and Obstetrical Outcomes After Laparoscopic Repair of a Cesarean Scar Defect in a Series of 38 Women. *Fertil Steril* (2017) 107(1):289–96.e2. doi: 10.1016/j.fertnstert.2016.09.033
16. Risager JK, Uldbjerg N, Glavind J. Cesarean Scar Thickness in non-Pregnant Women as a Risk Factor for Uterine Rupture. *J Matern Fetal Neonatal Med* (2022) 35(2):389–94. doi: 10.1080/14767058.2020.1719065
17. Kok N, Wiersma IC, Opmeer BC, de Graaf IM, Mol BW, Pajkrt E. Sonographic Measurement of Lower Uterine Segment Thickness to Predict Uterine Rupture During a Trial of Labor in Women With Previous Cesarean Section: A Meta-Analysis. *Ultrasound Obstet Gynecol* (2013) 42(2):132–9. doi: 10.1002/uog.12479
18. Wang CB, Chiu WW, Lee CY, Sun YL, Lin YH, Tseng CJ. Cesarean Scar Defect: Correlation Between Cesarean Section Number, Defect Size, Clinical Symptoms and Uterine Position. *Ultrasound Obstet Gynecol* (2009) 34(1):85–9. doi: 10.1002/uog.6405.5
19. Kjerulff KH, Paul IM, Weisman CS, Hillemeier MM, Wang M, Legro RS, et al. Association Between Mode of First Delivery and Subsequent Fecundity and Fertility. *JAMA Netw Open* (2020) 3(4):e203076. doi: 10.1001/jamanetworkopen.2020.3076
20. Zhang N, Chen H, Xu Z, Wang B, Sun H, Hu Y. Pregnancy, Delivery, and Neonatal Outcomes of *In Vitro* Fertilization-Embryo Transfer in Patient With Previous Cesarean Scar. *Med Sci Monit* (2016) 22:3288–95. doi: 10.12659/MSM.900581
21. Diao J, Gao G, Zhang Y, Wang X, Zhang Y, Han Y, et al. Caesarean Section Defects may Affect Pregnancy Outcomes After *In Vitro* Fertilization-Embryo Transfer: A Retrospective Study. *BMC Pregnancy Childbirth* (2021) 21(1):487. doi: 10.1186/s12884-021-03955-7
22. Friedenthal J, Alkon-Meadows T, Hernandez-Nieto C, Gounko D, Lee JA, Copperman A, et al. The Association Between Prior Cesarean Delivery and Subsequent *In Vitro* Fertilization Outcomes in Women Undergoing

Autologous, Frozen-Thawed Single Euploid Embryo Transfer. *Am J Obstet Gynecol* (2021) 225(3):287.e1–8. doi: 10.1016/j.ajog.2021.03.026
23. Patounakis G, Ozcan MC, Chason RJ, Norian JM, Payson M, DeCherney AH, et al. Impact of a Prior Cesarean Delivery on Embryo Transfer: A Prospective Study. *Fertil Steril* (2016) 106(2):311–6. doi: 10.1016/j.fertnstert.2016.03.045
24. Huang J, Lin J, Cai R, Lu X, Song N, Gao H, et al. Effect of a Prior Cesarean Delivery on Pregnancy Outcomes of Frozen-Thawed Embryo Transfer: A Retrospective Cohort Study in a Freeze-All Setting. *Acta Obstet Gynecol Scand* (2020) 99(10):1303–10. doi: 10.1111/aogs.13863
25. Glujovsky D, Farquhar C, Quinteiro Retamar AM, Alvarez Sedo CR, Blake D. Cleavage Stage Versus Blastocyst Stage Embryo Transfer in Assisted Reproductive Technology. *Cochrane Database Syst Rev* (2016) (6): CD002118. doi: 10.1002/14651858.CD002118.pub5
26. Chen H, Lv JQ, Wu XM, Xiao Y, Xi HT, Zhu CF, et al. Blastocyst-Stage Versus Cleavage-Stage Embryo Transfer in the First Frozen Cycles of OHSS-Risk Patients Who Deferred From Fresh Embryo Transfer. *Gynecol Endocrinol* (2015) 31(9):698–701. doi: 10.3109/09513590.2015.1062858
27. Kabiri D, Masarwy R, Schachter-Safrai N, Masarwa R, Hirsh Raccah B, Ezra Y, et al. Trial of Labor After Cesarean Delivery in Twin Gestations: Systematic Review and Meta-Analysis. *Am J Obstet Gynecol* (2019) 220(4):336–47. doi: 10.1016/j.ajog.2018.11.125
28. Meldrum DR. *In Vitro* Fertilization Twins: Acceptable When Desired, or Iatrogenic Complication Preventable Through Elective Single Embryo Transfer? *Fertil Steril* (2018) 109(2):221–2. doi: 10.1016/j.fertnstert.2017.12.006
29. Wang YQ, Yin TL, Xu WM, Qi QR, Wang XC, Yang J. Reproductive Outcomes in Women With Prior Cesarean Section Undergoing *In Vitro* Fertilization: A Retrospective Case-Control Study. *J Huazhong Univ Sci Technolog Med Sci* (2017) 37(6):922–7. doi: 10.1007/s11596-017-1828-3
30. Chen T, Li B, Shi H, Bu ZQ, Zhang FQ, Su YC. Reproductive Outcomes of Single Embryo Transfer in Women With Previous Cesarean Section. *Reprod Sci* (2021) 28(4):1049–59. doi: 10.1007/s43032-020-00345-w
31. Evans MI, Ciorica D, Britt DW, Fletcher JC. Update on Selective Reduction. *Prenat Diagn* (2005) 25(9):807–13. doi: 10.1002/pd.1261
32. Tita AT, Landon MB, Spong CY, Lai Y, Leveno KJ, Varner MW, et al. Timing of Elective Repeat Cesarean Delivery at Term and Neonatal Outcomes. *N Engl J Med* (2009) 360(2):111–20. doi: 10.1056/NEJMoa0803267
33. Landon MB, Hauth JC, Leveno KJ, Spong CY, Leindecker S, Varner MW, et al. National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network. Maternal and Perinatal Outcomes Associated With a Trial of Labor After Prior Cesarean Delivery. *N Engl J Med* (2004) 351 (25):2581–9. doi: 10.1056/NEJMoa040405
34. Vissers J, Hehenkamp W, Lambalk CB, Huirne JA. Post-Caesarean Section Niche-Related Impaired Fertility: Hypothetical Mechanisms. *Hum Reprod* (2020) 35(7):1484–94. doi: 10.1093/humrep/deaa094
35. Huang L, Huang S, Yuan Y, Li Y, Chen M, Zhou C. Reduced Pregnancy and Live Birth Rates After *In Vitro* Fertilization in Women With Cesarean Section Scar Diverticulum: A Retrospective Cohort Study. *J Obstet Gynaecol Res* (2022) 48(1):146–54. doi: 10.1111/jog.15061
36. Api M, Boza A, Gorgen H, Api O. Should Cesarean Scar Defect Be Treated Laparoscopically? A Case Report and Review of the Literature. *J Minim Invasive Gynecol* (2015) 22(7):1145–52. doi: 10.1016/j.jmig.2015.06.013
37. Donnez O. Cesarean Scar Defects: Management of an Iatrogenic Pathology Whose Prevalence has Dramatically Increased. *Fertil Steril* (2020) 113(4):704–16. doi: 10.1016/j.fertnstert.2020.01.037
38. Van Langendonckt A, Casanas-Roux F, Donnez J. Iron Overload in the Peritoneal Cavity of Women With Pelvic Endometriosis. *Fertil Steril* (2002) 78 (4):712–8. doi: 10.1016/s0015-0282(02)03346-0
39. Lawrenz B, Melado L, Garrido N, Coughlan C, Markova D, Fatemi H. Isthmocele and Ovarian Stimulation for IVF: Considerations for a Reproductive Medicine Specialist. *Hum Reprod* (2020) 35(1):89–99. doi: 10.1093/humrep/dez241
40. Moreno I, Simon C. Relevance of Assessing the Uterine Microbiota in Infertility. *Fertil Steril* (2018) 110(3):337–43. doi: 10.1016/j.fertnstert.2018.04.041
41. Brosens JJ, Gellersen B. Something New About Early Pregnancy: Decidual Biosensoring and Natural Embryo Selection. *Ultrasound Obstet Gynecol* (2010) 36(1):1–5. doi: 10.1002/uog.7714
42. Ben-Nagi J, Walker A, Jurkovic D, Yazbek J, Aplin JD. Effect of Cesarean Delivery on the Endometrium. *Int J Gynaecol Obstet* (2009) 106(1):30–4. doi: 10.1016/j.ijgo.2009.02.019
43. Roberge S, Boutin A, Chaillet N, Moore L, Jastrow N, Demers S, et al. Systematic Review of Cesarean Scar Assessment in the Nonpregnant State: Imaging Techniques and Uterine Scar Defect. *Am J Perinatol* (2012) 29 (6):465–71. doi: 10.1055/s-0032-1304829
44. Antila-Långsjö R, Mäenpää JU, Huhtala H, Tomás E, Staff S. Comparison of Transvaginal Ultrasound and Saline Contrast Sonohysterography in Evaluation of Cesarean Scar Defect: A Prospective Cohort Study. *Acta Obstet Gynecol Scand* (2018) 97(9):1130–6. doi: 10.1111/aogs.13367

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers | Frontiers in Endocrinology

ORIGINAL RESEARCH
published: 24 May 2022
doi: 10.3389/fendo.2022.868335

# Birthweight After Frozen Embryos Formed on the Fifth Day Versus the Sixth Day: A Retrospective Analysis Including 17,127 Singleton Newborns

*Junlan Yang[1†], Ze Wang[1†], Hairu Cao[1], Lu Liu[1], Qiaona Yuan[1], Haiyan Xu[1] and Rong Tang[1,2*]*

*[1] Center for Reproductive Medicine, Cheeloo College of Medicine, Shandong University, Jinan, China, [2] Shandong Provincial Hospital Affiliated to Shandong First Medical University, Shandong First Medical University, Jinan, China*

**Background:** Transferring blastocysts frozen on day 6 (D6) may adversely affect the pregnancy rate compared with day 5 (D5). Moreover, it remains unclear whether delayed embryo transfer affects neonatal birth weight.

**Methods:** A retrospective cohort study consisting of 17,127 singleton births from single frozen embryo transfer (FET) cycles, between January 2011 and January 2020, was performed including 14,166 blastocysts frozen on D5 and 2,961 on D6. The primary outcomes of this study were neonatal birth weight and incidence of small for gestational age (SGA), large for gestational age (LGA), low birth weight (LBW), and macrosomia.

**Results:** The mean neonatal birth weight in the D5 group (3.47 ± 0.49 kg) was significantly higher compared with the D6 group (3.45 ± 0.50 kg), although the discrepancy was only 0.02 kg. Multiple linear regression analysis for birth weight between the two groups showed no statistically significant difference (β= -0.01 t= -1.218; P>0.05). Logistic regression analysis revealed that the risks of SGA (OR 1.166; 95%CI, 0.911-1.491; P>0.05), LGA (OR 0.917; 95%CI, 0.831-1.012; P>0.05), LBW (OR 1.192; 95%CI, 0.926-1.533; P>0.05), and macrosomia (OR 0.975; 95%CI, 0.864-1.100; P>0.05) were similar in the two groups after adjusting for confounders.

**Conclusions:** In the FET cycle, the neonatal birth weight and incidence of LGA, SGA, LBW, or macrosomia were similar between the D5 and D6 groups, suggesting that delayed blastocyst transfer would not affect the neonatal birth weight.

**Keywords: frozen embryo transfer, blastocyst, birth weight, SGA, LGA**

OPEN ACCESS

***Edited by:***
*Yimin Zhu,*
*Zhejiang University, China*

***Reviewed by:***
*Luigi Carbone,*
*University of Naples Federico II, Italy*
*Yingpu Sun,*
*First Affiliated Hospital of Zhengzhou University, China*

***Correspondence:**
*Rong Tang*
*r.tang.sduivf@hotmail.com*

*[†]These authors have contributed equally to this work*

***Specialty section:***
*This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology*

***Received:*** *02 February 2022*
***Accepted:*** *24 March 2022*
***Published:*** *24 May 2022*

***Citation:***
*Yang J, Wang Z, Cao H, Liu L, Yuan Q, Xu H and Tang R (2022) Birthweight After Frozen Embryos Formed on the Fifth Day Versus the Sixth Day: A Retrospective Analysis Including 17,127 Singleton Newborns. Front. Endocrinol. 13:868335. doi: 10.3389/fendo.2022.868335*

## INTRODUCTION

Embryo transfer (ET) at the blastocyst stage has been widely recommended in assisted reproductive technology (ART), especially in the single ET program. Theoretically, prolonged in-vitro culture to blastocyst from the cleavage stage allows for better selection of the implantation potential of the embryo, thereby improving the pregnancy rate. Moreover, single ET reduces the incidence of

multiple pregnancy rates, which is associated with a higher risk of maternal and neonatal complications (1). Blastocysts are usually formed on the fifth day (D5) of in-vitro culture, while blastulation of some embryos can be delayed to the sixth day (D6) or even later. The embryonic development rate is suggested as an essential indicator of reproductive outcomes. It has been reported that D6 blastocysts generally have a higher rate of aneuploidy than D5 blastocysts (2). Irani et al. have observed that the transfer of D5 euploid blastocysts results in higher rates of clinical pregnancy and live birth compared with those at D6 in preimplantation genetic testing (PGT) cycles (3). A proposed explanation is that the superior implantation potential of D5 blastocysts can be attributed to metabolic or epigenetic factors that may differ in the embryos at different development stages.

The birth weight of neonates has long been regarded as an indicator of the offspring's health. Chiavaroli et al. have reported that infants born with small-for-gestational-age (SGA) or large-for-gestational-age (LGA) show adverse cardio-metabolic profiles during childhood and adolescence, leading to an increased risk of cardiovascular diseases later in life (4). In addition, LGA is associated with a high risk of offspring obesity and depression (5, 6). Previous findings have indicated that frozen embryo transfer (FET) is associated with a higher birthweight and an increased risk of delivering LGA babies as compared to fresh embryo transfer (7, 8), implying that the process of cryopreservation can adversely affect the embryo quality and developmental potential. Moreover, FET at the blastocyst stage is associated with higher birthweight and an increased risk of LGA compared with the cleavage stage (9, 10). However, limited studies have compared the perinatal outcomes after the transfer of frozen-thawed blastocysts formed on D5 and D6, and the results remain controversial (11, 12). In the present study, we aimed to explore the effect of frozen-thawed blastocyst, formed at different developmental stages, on perinatal outcomes.

# MATERIALS AND METHODS

## Study Design and Participants

We performed a retrospective cohort study including 17,127 singleton live births after the transfer of frozen-thawed blastocysts from January 2011 to January 2020. Live birth was defined as a birth exhibiting life signs with ≥24 gestational weeks (13). This study was approved by the Institutional Review Board of the Centre for Reproductive Medicine affiliated with Shandong University. Inclusion criteria were as follows: (1) age ≤ 40 years; (2) body mass index (BMI) ≤35 kg/m2; and (3) the transfer of frozen-thawed blastocysts formed on D5 or D6. The exclusion criteria were as follows: (1) women with uterine malformations or intrauterine adhesions; (2) women diagnosed hypertensive disorders, chronic diabetes, and gestational diabetes mellitus (GDM). Hypertensive disorders included gestational hypertension (blood pressure ≥140/90 mm Hg after 20 weeks of gestation), preeclampsia, and eclampsia; and (3) frozen embryos that had undergone the re-cryopreservation process or preimplantation genetic testing.

## Procedures

*In vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) was performed after oocyte collection by the routine procedure. On D3 after insemination, embryos were observed and graded by morphological criteria based on the number and size of the blastomere.

The extended culture of embryos was determined according to the quantity and quality of cleavage-stage embryos, in combination with the request of patients and the evaluation of clinicians. On D3, embryos were removed from the cleavage medium and placed in the blastocyst medium, followed by incubation to D5 or D6, and even D7 until the blastocyst formed. The blastocyst quality was assessed based on the Gardner score system (14), which was frozen if it developed up to 4BC by vitrification.

Endometrial preparation was carried out under natural cycles, stimulation cycles, and artificial cycles, depending on the clinician's discretion. In a natural ovulation regimen, detection of spontaneous or triggered ovulation was the indication of timing for FET. Oral dydrogesterone was administered for luteal phase support after ovulation (Duphaston, 20-30 mg, or Utrogestan, 200-300mg, once a day). The single frozen-thawed blastocyst was transferred on the 5th day after ovulation. If pregnancy was achieved, dydrogesterone was continued until the 10th week of gestation. In the artificial cycle, oral estradiol (Progynova, 4-8 mg) was taken once a day from day 1-3 of the menstrual cycle. Oral (dydrogesterone, 20 mg, once a day) and transvaginal progesterone (Crinone 8% vaginal gel, 90 mg, once a day) or (dydrogesterone, 40 mg, once a day) and transvaginal progesterone (Utrogestan, 200mg, once a day) was added when the endometrium reached 7 mm or more. Single FET was performed on the sixth day of the progesterone exposure. If pregnancy occurred, estrogen supplement was stopped at the 8th week of gestation and progesterone support was continued until the 10th week of gestation. For stimulation cycles, letrozole or human menopausal gonadotropin (HMG), either alone or in combination, was given from day 3-5 of the menstrual cycle. Ultrasound monitoring was repeated every 1-3 days according to follicle growth until ovulation triggering. When the dominant follicle reached a diameter of 17–20 mm, human chorionic gonadotropin (hCG) at a dosage of 5,000-10,000 IU was administered to trigger ovulation. Other procedures were the same as the natural cycle.

## Outcome Measures and Definitions

The primary outcomes included birth weight (including absolute birth weight), SGA [defined as weighing less than the 10th percentile of birth weight (15)], LGA (defined as weighing more than the 90th percentile of birth weight), LBW (defined as birth weight less than 2,500 g), and macrosomia (defined as infant birth weight more than 4,000 g). The secondary outcomes were cesarean delivery, gestational age [defined as pregnancy weeks from the 19th day before FET to delivery (16)], and preterm birth (defined as a baby born at less than 37 weeks of gestation).

## Statistical Analysis

Baseline characteristics and neonatal outcomes were compared between the study groups by t-test (for continuous variables) or chi-square test (for categoric variables). A multiple linear regression analysis was performed to assess the relationship between the blastocyst development rate and birth weight with adjustment for potential confounding factors, including maternal age (continuous variable), parity (binary variable), basal follicle-stimulating hormone (FSH, continuous variable), FET regimens for endometrial preparation (binary variable), fertilization method (binary variable), embryo quality (binary variable), and developmental stage (binary variable). Logistic regression analysis was performed to evaluate the effect on the incidence of LGA/SGA after adjustment for the potential confounding factors. Odds ratios (OR) and 95% confidence intervals (CI) were used. P<0.05 was considered statistically significant. All statistical analyses were performed in SPSS version 22 software.

# RESULTS

## Baseline Characteristics

A total of 17,127 women who met the study inclusion criteria were enrolled, including 14,166 cases in the D5 group and 2,961 cases in the D6 group. **Table 1** presents the baseline characteristics. No difference was observed in maternal BMI, gravidity, infertility cause, and endometrial thickness between the two groups. Maternal age, parity, basal FSH level, endometrial preparation protocols, and fertilization method showed significant differences between the two groups (P<0.05). These parameters were then adjusted as potential confounders in the logistic regression (**Table 3**) and multiple linear regression analyses (**Table 4**).

**TABLE 1** | Demographics and cycle characteristics.

| Characteristics | D5 (n=14166) | D6 (n=2961) | P |
|---|---|---|---|
| **Baseline demographics** | | | |
| Maternal age, (year) | 30.14 (±3.79) | 30.82 (±3.98) | <0.05 |
| Maternal BMI, (kg/m$^2$) | 23.11 (±3.39) | 23.11 (±3.46) | 0.99 |
| Nulligravida, n (%) | 8038 (56.7) | 1643 (55.5) | 0.21 |
| Nulliparity, n (%) | 11756 (83.0) | 2298 (77.6) | <0.05 |
| Causes of infertility, n (%) | | | 0.07 |
| Tubal factors | 8654 (61.1) | 1750 (59.1) | |
| Anovulatory factors | 385 (2.7) | 74 (2.5) | |
| Unexplained factors | 390 (2.8) | 95 (3.2) | |
| Male factors | 2368 (16.7) | 557 (18.8) | |
| Combined factors[a] | 2229 (15.7) | 456 (15.4) | |
| Others[b] | 140 (1.0) | 29 (1.0) | |
| Basal FSH level (IU/L) | 6.28 (±1.72) | 6.81 (±2.26) | <0.05 |
| Fasting blood glucose level (mmol/L) | 5.21 (±0.39) | 5.23 (±0.36) | 0.09 |
| Fresh **cycle characteristics** | | | |
| Fertilization method, n (%) | | | <0.05 |
| IVF | 10290 (72.6) | 1807 (61.0) | |
| ICSI | 3876 (27.4) | 1154 (39.0) | |
| Embryo quality, n(%) | | | <0.05 |
| Good | 12422 (87.7) | 1926 (65.0) | |
| Poor | 1744 (12.3) | 1035 (35.0) | |
| **FET cycle characteristics** | | | |
| endometrial preparation protocols, n (%) | | | <0.05 |
| Natural cycle | 7898 (55.8) | 1739 (58.7) | |
| Stimulated cycle | 1344 (9.5) | 301 (10.2) | |
| Artificial cycle | 4924 (34.8) | 921 (31.1) | |
| Endometrial thickness, (cm) | 0.98 (±0.16) | 0.98 (±0.16) | 0.17 |

*Data are presented as mean ± SD, median (IQR) or n (%).*
*[a]Combined factors, both with Male and female factors.*
*[b]Others, advanced age and/or diminished ovarian function.*

## Neonatal Outcomes

**Table 2** presents the neonatal outcomes based on blastocyst development rate. The mean birth weight was 3.47 ± 0.49 kg and 3.45 ± 0.50 kg in the D5 group and D6 group, respectively, showing a significant difference between the two groups, although the discrepancy was only 0.02 kg. Regarding the other neonatal outcomes, no differences were observed between the two groups in terms of the incidence of SGA (2.6% vs. 3.0%; P>0.05), LGA (23.9% vs. 22.7%; P>0.05), LBW (2.5% vs. 2.9%; P>0.05), macrosomia (14.0% vs. 13.6%; P>0.05), and preterm birth (5.0% vs. 5.4%; P>0.05). Notably, the rate of cesarean section was higher following the transfer of blastocysts vitrified on D6 compared with D5 (68.4% vs. 74.3%; P<0.05). Logistic regression was performed after adjustment for the effects of potential confounding factors on the risks of SGA, LGA, LBW, and macrosomia (**Table 3**). There were also no statistically significant differences in the risks of SGA (OR 1.166; 95%CI, 0.911-1.491; P>0.05), LGA (OR 0.917; 95%CI, 0.831-1.012; P>0.05), LBW (OR 1.192; 95%CI, 0.926-1.533; P>0.05), macrosomia (OR 0.975; 95%CI, 0.864-1.100; P>0.05), and preterm birth (OR 1.104; 95%CI, 0.917-1.329; P>0.05) between the two groups. Regression analysis showed that women who received D6 embryos were more likely to undergo cesarean section (OR 1.262; 95%CI, 1.148-1.387; P<0.05) compared with those who received D5 embryos. Following multiple linear regression, no difference was observed between the D5 and D6 groups in terms of birth weight (β= -0.01 t= -1.218; P>0.05), as shown in **Table 4**.

**TABLE 2** | Neonatal outcomes transferred from blastocysts frozen at day 5 vs day 6.

| Outcomes | D5 | D6 | P |
|---|---|---|---|
| Cesarean section, n (%) | 9683 (68.4) | 2199 (74.3) | <0.05 |
| Gestational age (wk) | 39.23 (±1.57) | 39.19 (±1.58) | 0.14 |
| Gestational age, n (%) | | | 0.612 |
| 24-28w | 10 (0.1) | 2 (0.1) | |
| 28-37w | 704 (5.0) | 156 (5.3) | |
| 37-42w | 13415 (94.7) | 2792 (94.3) | |
| ≥42w | 37 (0.3) | 11 (0.4) | |
| Birth weight (kg) | 3.47 (±0.49) | 3.45 (±0.50) | <0.05 |
| Z-scores | 0.62 (±1.07) | 0.58 (±1.07) | 0.067 |
| Male gender, n (%) | 7635 (53.9) | 1600 (54.0) | 0.89 |
| Preterm birth (<37 weeks), n (%) | 708 (5.0) | 160 (5.4) | 0.36 |
| Low birthweight, n (%) | 352 (2.5) | 85 (2.9) | 0.23 |
| SGA[c], n (%) | 363 (2.6) | 90 (3.0) | 0.14 |
| LGA[d], n (%) | 3392 (23.9) | 673 (22.7) | 0.16 |
| Macrosomia, n (%) | 1985 (14.0) | 403 (13.6) | 0.57 |

*Data are expressed as mean ± SD, or number (percent).*
*[c]SGA, small for gestational age.*
*[d]LGA, large for gestational age.*

**TABLE 3 |** Logistic regression analysis of potential confouders[e] for neonatal outcomes from frozen blastocyst at day 5 or day 6.

| Outcomes | OR (95% CI) | P | Adjust OR (95% CI) | P |
|---|---|---|---|---|
| Cesarean section | 1.336 (1.221-1.461) | <0.05 | 1.262 (1.148-1.387) | <0.05 |
| Preterm birth (<37 weeks) | 1.086 (0.910-1.295) | 0.360 | 1.104 (0.917-1.329) | 0.297 |
| Low birthweight | 1.160 (0.912-1.475) | 0.226 | 1.192 (0.926-1.533) | 0.173 |
| SGA | 1.192 (0.943-1.507) | 0.141 | 1.166 (0.911-1.491) | 0.222 |
| LGA | 0.943 (0.850-1.027) | 0.157 | 0.917 (0.831-1.012) | 0.083 |
| Macrosomia | 0.967 (0.862-1.085) | 0.566 | 0.975 (0.864-1.100) | 0.678 |

[e]*potential confouders including maternal age (continuous variable), parity (binary variable), basal follicle-stimulating hormone (continuous variable), FET regimens for endometrial preparation (binary variable), fertilization method (binary variable), embryo quality(binary variable), developmental stage (binary variable).*

**TABLE 4 |** Multiple regression analysis of potential confounders associating with neonatal birth weight.

| | Unstandardized coefficients | | Standardized coefficients | | |
|---|---|---|---|---|---|
| | B | Std. error | β | t | P |
| Model | | | | | |
| (constant) | 3.547 | 0.037 | | 97.167 | <0.05 |
| Maternal age, (year) | -0.001 | 0.001 | -0.009 | -1.016 | 0.309 |
| Nulliparity | 0.041 | 0.01 | 0.035 | 4.143 | <0.05 |
| Basal FSH level (IU/L) | -0.006 | 0.002 | -0.026 | -3.349 | <0.05 |
| Fertilization method | -0.007 | 0.008 | -0.007 | -0.864 | 0.388 |
| Embryo quality | -0.017 | 0.01 | -0.014 | -1.749 | 0.08 |
| Endometrial preparation protocols | 0.02 | 0.004 | 0.041 | 5.304 | <0.05 |
| Blastocyst (Day5/6) | -0.012 | -0.009 | -0.01 | -1.218 | 0.223 |

## DISCUSSION

To the best of our knowledge, our current study was, to date, the largest investigation on the effect of embryonic development duration before freezing at the blastocyst stage on neonatal birth weight after FET. The results of this retrospective cohort study indicate that embryonic development duration before freezing at the blastocyst stage did not affect neonatal birth weight, confirming the findings of previous small-scale retrospective studies (11, 17). Meanwhile, in our present study, no association was found between delayed blastocysts and increased risks of SGA, LGA, LBW, and macrosomia, suggesting that delayed blastulation did not adversely affect the birth weight of offspring in FET cycles. Hiraoka et al. have reported that there are no significant differences in gestational age, preterm delivery rate, and birth weight when the embryonic development duration is different. However, only 71 deliveries are included in their study (17). Wang et al. have also reported that the gestational age and birth weight show no significant difference between the D5 and D6 groups (11), with 515 cases in their study. Furthermore, both the above-mentioned studies are undermined by the presence of twins and the limited population size and none of them have reported the outcomes with adjustment for gestational age and gender. Our results show that there was no difference in the risks of LGA and SGA in singletons born from the blastocysts frozen on D6 compared with D5 after adjustment for gender and gestational age, which was consistent with previous findings (11). Regarding birth weight, there was no significant difference after multiple linear regression.

It remains unclear whether the blastocyst development rate affects the perinatal outcomes. Several studies have found that slow-growing blastocysts with delayed expansion on D6 have no impact on clinical results after transferring good-quality embryos, suggesting that delayed blastulation is not related to viability (18, 19). In Yang's study, the blastocyst quality is a crucial factor that affects pregnancy outcomes (18). Meanwhile, some other studies have indicated that implantation rate, clinical pregnancy rate, and live birth rate derived from the D5 group are higher compared with the D6 group (20–22). Ferreux et al. have speculated that the difference in clinical outcomes is ascribed to chromosomal abnormalities (20). Previous studies have reported a higher aneuploidy rate in D6 blastocysts (2, 23). These findings indicate that there are significant differences in reproductive potential between women undergoing D5 and D6 blastocyst transfers. The differences are partly ascribed to a higher aneuploidy rate among D6 blastocysts. Some studies have reported that D5 blastocysts exhibit significantly higher mitochondrial DNA (mtDNA) levels compared with the D6 group. Moreover, aneuploid blastocysts have higher amounts of mtDNA than euploid blastocysts (24, 25). Animal studies have reported a higher incidence rate of apoptosis in delayed blastocysts compared with blastocysts transferred early. Moreover, the gene expression profile and diameter of blastocysts depend on the developmental stage of the blastocysts (26). In addition, Hashimoto et al. have shown that the incidence of spindle abnormalities is higher in growth-retarded embryos. However, no significant differences are found in the birth weight and gestational age between the groups receiving embryos vitrified on D5 and D6. They conclude that most blastomeres with abnormal spindles are eliminated before implantation (27).

Wang et al. have reported a higher proportion of LBW in the D5 group compared with the D6 group. They consider that the increased birth weight is associated with the extended culture (11). Inconsistent with Cai's study, they have reported a significantly higher risk of LGA in singletons born after delayed blastocyst transfer on D6 (12). Ferreux's study has also found that the birth weight of neonates derived from the D5 group is less compared with the D6 group. They have proposed that extended *in vitro* culture contributes to the heavier birth weight. Nevertheless, they have not reported the outcomes with adjustment for gestational age and gender (20). Existing data on the effect of culture duration on neonatal birth weight are conflicting. Some studies have reported that compared with

cleavage embryos, blastocyst transfer tends to have a higher mean birth weight and an increased proportion of LGA (9, 10, 28). An animal study has demonstrated that the effects of delayed blastulation and extended culture on blastocysts can be cumulative (26). However, others have shown that culture duration is not correlated with neonatal birth weight (29, 30). Du et al. have reported retarded embryos do not result in a high risk of LBW, congenital malformations, and early neonatal death (31). Our data further indicated that there was no difference in neonatal weight with the increased duration to 6 days compared with 5 days for blastulation.

Previous studies have shown that the neonatal birth weight may be affected by circumstances of embryonic development, such as endometrial receptivity (32, 33), different culture medium (16, 34), vitrification (35), endometrium preparation (36), and endometrial thickness (37). After logistic regression analysis, we found that obstetrical history (parity), basal FSH level, and endometrial preparation regimens were independent risk factors for neonatal birth weight.

In our present study, we found that delayed blastocyst transfer did not pose adverse effects on neonatal birth weight. Therefore, it was reasonable to speculate that the growth potential of blastocysts could affect blastocysts at the early preimplantation period. The implantation process might eliminate blastocysts with lower adaptation capacity, thus theoretically selecting blastocysts with better growth potential. Several studies have illustrated that embryos with comparable developmental potential of cell division may eliminate a genetically abnormal cell line (38, 39).

In the present study, we focused on the effects of FET on singleton births based on a large sample, which ruled out the possibility of adverse fetal growth caused by some other factors, including twins and related pregnancy complications, and the possibility of adverse fetal growth caused by high estrogen levels in fresh cycles. Indeed, we acknowledged certain limitations in the present study. First, the present study was limited by the bias inherent in its retrospective nature. Despite adequate control for confounding factors, unavailable or unknown confounders may generate bias because of the retrospective design. Although anti-Mullerian hormone levels (AMH), thyroid-stimulating hormone (TSH), and polycystic ovary syndrome (PCOS) might be related to neonatal birth and endometrial thickness of HCG day (the day of HCG administration day) these variables were not available or had extensive missing data (>50%), and could not be included in this study. Second, in some cycles, top-quality embryos on D3 were transferred as a priority, while the morphologically poorer embryos were placed in extended culture until the blastocyst stage. The blastocyst quality was graded before being frozen, which might bias the difference. Third, most of the neonatal outcomes were accessed by telephone interview, which might result in underestimated birth defect rates. Finally, we showed that the rate of cesarean section was higher in the D6 group compared with the D5 group, while no statistically significant differences were found in the analysis of gestational age between the two groups. Moreover, there might be other factors that were not included in this study since the database was not fully completed. Further studies should investigate the mechanisms underlying the effect of delayed blastulation on neonatal outcomes.

## CONCLUSIONS

In summary, neonatal birth weight and the proportion of LGA, SGA, macrosomia, or LBW were similar between the D5 and D6 groups. We concluded that frozen and delayed blastocysts would not affect neonatal birth weight. However, further large prospective studies should be carried out to confirm these results and the underlying mechanisms should also be investigated.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hospital's Ethics Committee for Reproductive Medicine. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

## AUTHOR CONTRIBUTIONS

JY contributed significantly to analysis and wrote the manuscript. ZW performed the data analyses and manuscript preparation. HC, LL, QY and HX helped perform the analysis with constructive discussions. RT contributed to the conception of the study. All authors contributed to the article and approved the submitted version.

## FUNDING

This study was supported by the Shandong Province Medical and Health Technology Development Project (2016WS0442) and the National Key Research and Development Program of China (2017YFC10010004).

## ACKNOWLEDGMENTS

We are very grateful to all participants for their data analysis efforts.

## REFERENCES


1. Freeman MR, Hinds MS, Howard KG, Howard JM, Hill GA. Guidance for Elective Single-Embryo 245 Transfer Should Be Applied to Frozen Embryo Transfer Cycles. *J Assist Reprod Genet* (2019) 36(5):939–46. doi: 10.1007/s10815-019-01433-w
2. Taylor TH, Patrick JL, Gitlin SA, Wilson JM, Crain JL, Griffin DK. Comparison of Aneuploidy, Pregnancy and Live Birth Rates Between Day 5 and Day 6 Blastocysts. *Reprod BioMed Online* (2014) 29(3):305–10. doi: 10.1016/j.rbmo.2014.06.001
3. Irani M, O'Neill C, Palermo GD, Xu K, Zhang C, Qin X, et al. Blastocyst Development Rate Influences Implantation and Live Birth Rates of Similarly Graded Euploid Blastocysts. *Fertil Steril* (2018) 110(1):95–102 e1. doi: 10.1016/j.fertnstert.2018.03.032
4. Chiavaroli V, Marcovecchio ML, de Giorgis T, Diesse L, Chiarelli F, Mohn A. Progression of Cardio-Metabolic Risk Factors in Subjects Born Small and Large for Gestational Age. *PloS One* (2014) 9(8):e104278. doi: 10.1371/journal.pone.0104278
5. Cnattingius S, Villamor E, Lagerros YT, Wikstrom AK, Granath F. High Birth Weight and Obesity–a Vicious Circle Across Generations. *Int J Obes (Lond)* (2012) 36(10):1320–4. doi: 10.1038/ijo.2011.248
6. Colman I, Ataullahjan A, Naicker K, Van Lieshout RJ. Birth Weight, Stress, and Symptoms of Depression in Adolescence: Evidence of Fetal Programming in a National Canadian Cohort. *Can J Psychiatry* (2012) 57(7):422–8. doi: 10.1177/070674371205700705
7. Litzky JF, Boulet SL, Esfandiari N, Zhang Y, Kissin DM, Theiler RN, et al. Effect of Frozen/Thawed Embryo Transfer on Birthweight, Macrosomia, and Low Birthweight Rates in US Singleton Infants. *Am J Obstet Gynecol* (2018) 218(4):433 e1– e10. doi: 10.1016/j.ajog.2017.12.223
8. Conforti A, Picarelli S, Carbone L, La Marca A, Venturella R, Vaiarelli A, et al. Perinatal and Obstetric Outcomes in Singleton Pregnancies Following Fresh Versus Cryopreserved Blastocyst Transfer: A Meta-Analysis. *Reprod BioMed Online* (2021) 42(2):401–12. doi: 10.1016/j.rbmo.2020.09.029
9. Zhang J, Wang Y, Liu H, Mao X, Chen Q, Fan Y, et al. Effect of *In Vitro* Culture Period on Birth Weight After Vitrified-Warmed Transfer Cycles: Analysis of 4,201 Singleton Newborns. *Fertil Steril* (2019) 111(1):97–104. doi: 10.1016/j.fertnstert.2018.10.006
10. Wang N, Zhao X, Ma M, Zhu Q, Wang Y. Effect of Day 3 and Day 5/6 Embryo Quality on the Reproductive Outcomes in the Single Vitrified Embryo Transfer Cycles. *Front Endocrinol (Lausanne)* (2021) 12:641623. doi: 10.3389/fendo.2021.641623
11. Wang X, Zhen J, Sun Z, Yu Q, Deng C, Zhou Y, et al. Effects of Fifth Day (D5) or Sixth Day (D6) Frozen-Thawed Blastocysts on Neonatal Outcomes. *Zygote* (2016) 24(5):684–91. doi: 10.1017/S0967199415000696
12. Cai J, Liu L, Xu Y, Liu Z, Jiang X, Li P, et al. Day 6 Blastocyst is Associated With Increased Birth Weight in Full-Term Singleton Newborns After Frozen-Thawed Transfer. *Arch Gynecol Obstet* (2018) 298(2):397–403. doi: 10.1007/s00404-018-4808-3
13. Smith ADAC, Tilling K, Nelson SM, Lawlor DA. Live-Birth Rate Associated With RepeatIn Vitro Fertilization Treatment Cycles. *JAMA* (2015) 314 (24):2654–62. doi: 10.1001/jama.2015.17296
14. Gardner DK, Lane M, Stevens J, Schlenker T, Schoolcraft WB. Blastocyst Score Affects Implantation and Pregnancy Outcome: Towards a Single Blastocyst Transfer. *Fertil Steril* (2000) 73(6):1155–8. doi: 10.1016/s0015-0282(00)00518-5
15. Dai L, Deng C, Li Y, Zhu J, Mu Y, Deng Y, et al. Birth Weight Reference Percentiles for Chinese. *PloS One* (2014) 9(8):e104779. doi: 10.1371/journal.pone.0104779
16. Nelissen EC, Van Montfoort AP, Coonen E, Derhaag JG, Geraedts JP, Smits LJ, et al. Further Evidence That Culture Media Affect Perinatal Outcome: Findings After Transfer of Fresh and Cryopreserved Embryos. *Hum Reprod* (2012) 27(7):1966–76. doi: 10.1093/humrep/des145
17. Hiraoka K, Hiraoka K, Miyazaki M, Fukunaga E, Horiuchi T, Kusuda T, et al. Perinatal Outcomes Following Transfer of Human Blastocysts Vitrified at Day 5, 6 and 7. *J Exp Clin Assisted Reprod* (2009) 6:4.
18. Yang H, Yang Q, Dai S, Li G, Jin H, Yao G, et al. Comparison of Differences in Development Potentials Between Frozen-Thawed D5 and D6 Blastocysts and Their Relationship With Pregnancy Outcomes. *J Assist Reprod Genet* (2016) 33(7):865–72. doi: 10.1007/s10815-016-0712-6
19. Capalbo A, Rienzi L, Cimadomo D, Maggiulli R, Elliott T, Wright G, et al. Correlation Between Standard Blastocyst Morphology, Euploidy and Implantation: An Observational Study in Two Centers Involving 956 Screened Blastocysts. *Hum Reprod* (2014) 29(6):1173–81. doi: 10.1093/humrep/deu033
20. Ferreux L, Bourdon M, Sallem A, Santulli P, Barraud-Lange V, Le Foll N, et al. Live Birth Rate Following Frozen-Thawed Blastocyst Transfer is Higher With Blastocysts Expanded on Day 5 Than on Day 6. *Hum Reprod* (2018) 33 (3):390–8. doi: 10.1093/humrep/dey004
21. Sciorio R, Thong KJ, Pickering SJ. Increased Pregnancy Outcome After Day 5 Versus Day 6 Transfers of Human Vitrified-Warmed Blastocysts. *Zygote* (2019) 27(5):279–84. doi: 10.1017/S0967199419000273
22. Kato K, Ueno S, Yabuuchi A, Uchiyama K, Okuno T, Kobayashi T, et al. Women's Age and Embryo Developmental Speed Accurately Predict Clinical Pregnancy After Single Vitrified-Warmed Blastocyst Transfer. *Reprod BioMed Online* (2014) 29(4):411–6. doi: 10.1016/j.rbmo.2014.06.007
23. Alfarawati S, Fragouli E, Colls P, Stevens J, Gutierrez-Mateo C, Schoolcraft WB, et al. The Relationship Between Blastocyst Morphology, Chromosomal Abnormality, and Embryo Gender. *Fertil Steril* (2011) 95(2):520–4. doi: 10.1016/j.fertnstert.2010.04.003
24. Wu FS, Weng SP, Shen MS, Ma PC, Wu PK, Lee NC. Suboptimal Trophectoderm Mitochondrial DNA Level is Associated With Delayed Blastocyst Development. *J Assist Reprod Genet* (2021) 38(3):587–94. doi: 10.1007/s10815-020-02045-5
25. Klimczak AM, Pacheco LE, Lewis KE, Massahi N, Richards JP, Kearns WG, et al. Embryonal Mitochondrial DNA: Relationship to Embryo Quality and Transfer Outcomes. *J Assist Reprod Genet* (2018) 35(5):871–7. doi: 10.1007/s10815-018-1147-z
26. Lin T, Lee JE, Oqani RK, Kim SY, Cho ES, Jeong YD, et al. Delayed Blastocyst Formation or an Extra Day Culture Increases Apoptosis in Pig Blastocysts. *Anim Reprod Sci* (2017) 185:128–39. doi: 10.1016/j.anireprosci.2017.08.012
27. Hashimoto S, Amo A, Hama S, Ito K, Nakaoka Y, Morimoto Y. Growth Retardation in Human Blastocysts Increases the Incidence of Abnormal Spindles and Decreases Implantation Potential After Vitrification. *Hum Reprod* (2013) 28(6):1528–35. doi: 10.1093/humrep/det059
28. Wang X, Du M, Guan Y, Wang B, Zhang J, Liu Z. Comparative Neonatal Outcomes in Singleton Births From Blastocyst Transfers or Cleavage-Stage Embryo Transfers: A Systematic Review and Meta-Analysis. *Reprod Biol Endocrinol* (2017) 15(1):36. doi: 10.1186/s12958-017-0255-4
29. Marconi N, Raja EA, Bhattacharya S, Maheshwari A. Perinatal Outcomes in Singleton Live Births After Fresh Blastocyst-Stage Embryo Transfer: A Retrospective Analysis of 67 147 IVF/ICSI Cycles. *Hum Reprod* (2019) 34 (9):1716–25. doi: 10.1093/humrep/dez133
30. Orion G, Sokal-Arnon T, Son WY, Demirtas E, Buckette W, Zeadna A, et al. Extended Embryo Culture is Not Associated With Increased Adverse Obstetric or Perinatal Outcome. *Amm J Obstet Gynecol* (2014) 211(2):1097–6868. doi: 10.1016/j.ajog.2014.03.018
31. Du T, Wang Y, Fan Y, Zhang S, Yan Z, Yu W, et al. Fertility and Neonatal Outcomes of Embryos Achieving Blastulation on Day 7: Are They of Clinical Value? *Hum Reprod* (2018) 33(6):1038–51. doi: 10.1093/humrep/dey092
32. Shapiro BS, Daneshmand ST, Garner FC, Aguirre M, Ross R. Contrasting Patterns in *In Vitro* Fertilization Pregnancy Rates Among Fresh Autologous, Fresh Oocyte Donor, and Cryopreserved Cycles With the Use of Day 5 or Day 6 Blastocysts may Reflect Differences in Embryo-Endometrium Synchrony. *Fertil Steril* (2008) 89(1):20–6. doi: 10.1016/j.fertnstert.2006.08.092
33. Van Voorhis BJ, Dokras A. Delayed Blastocyst Transfer: Is the Window Shutting? *Fertil Steril* (2008) 89(1):31–2. doi: 10.1016/j.fertnstert.2007.01.172
34. Vergouw CG, Kostelijk EH, Doejaaren E, Hompes PG, Lambalk CB, Schats R. The Influence of the Type of Embryo Culture Medium on Neonatal Birthweight After Single Embryo Transfer in IVF. *Hum Reprod* (2012) 27 (9):2619–26. doi: 10.1093/humrep/des252

35. Liu SY, Teng B, Fu J, Li X, Zheng Y, Sun XX. Obstetric and Neonatal Outcomes After Transfer of Vitrified Early Cleavage Embryos. *Hum Reprod* (2013) 28(8):2093–100. doi: 10.1093/humrep/det104
36. Hu KL, Zhang D, Li R. Endometrium Preparation and Perinatal Outcomes in Women Undergoing Single-Blastocyst Transfer in Frozen Cycles. *Fertil Steril* (2021) 115(6):1487–94. doi: 10.1016/j.fertnstert.2020.12.016
37. Guo Z, Xu X, Zhang L, Zhang L, Yan L, Ma J. Endometrial Thickness is Associated With Incidence of Small-for-Gestational-Age Infants in Fresh *In Vitro* Fertilization-Intracytoplasmic Sperm Injection and Embryo Transfer Cycles. *Fertil Steril* (2020) 113(4):745–52. doi: 10.1016/j.fertnstert.2019.12.014
38. Singla S, Iwamoto-Stohl LK, Zhu M, Zernicka-Goetz M. Autophagy-Mediated Apoptosis Eliminates Aneuploid Cells in a Mouse Model of Chromosome Mosaicism. *Nat Commun* (2020) 11(1):2958. doi: 10.1038/s41467-020-16796-3
39. Bolton H, Graham SJL, van der Aa N, Kumar P, Theunis K, Fernandez Gallardo E, et al. Mouse Model of Chromosome Mosaicism Reveals Lineage-Specific Depletion of Aneuploid Cells and Normal Developmental Potential. *Nat Commun* (2016) 7:11165. doi: 10.1038/ncomms11165

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers | Frontiers in Endocrinology

ORIGINAL RESEARCH
published: 03 June 2022
doi: 10.3389/fendo.2022.889029

# Association of Polycystic Ovary Syndrome Phenotypes With Adverse Pregnancy Outcomes After *In-Vitro* Fertilization/ Intracytoplasmic Sperm Injection

*Qiumin Wang*[1,2†], *Honghong Wang*[1,2,3†], *Ping Li*[4†], *Xiufang Li*[1,2], *Ze Wang*[1,2], *Lei Yan*[1,2*] *and Yuhua Shi*[2,5*]

[1] Center for Reproductive Medicine, Shandong University, Jinan, China, [2] Shandong Provincial Clinical Medicine Research Center for Reproductive Health, Shandong University, Jinan, China, [3] Children's Hospital of Shanxi and Women Health Center of Shanxi, Taiyuan, China, [4] Women and Children's Hospital, School of Medicine, Xiamen University, Xiamen, China, [5] Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, Guangzhou, China

**Objective:** This study aims to evaluate the association between polycystic ovary syndrome (PCOS) phenotypes and adverse perinatal outcomes, comparing the characteristics, ovarian response, and assisted reproductive outcomes in patients with various PCOS phenotypes after *in-vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI).

**Methods:** This study comprised 6,732 patients who underwent the first cycle of IVF/ICSI treatment in our outpatient department from January 2017 to July 2018. Propensity score matching (PSM) was used in PCOS and non-PCOS groups to balance the influence of intergroup confounding factors. After the PSM procedure, 1,186 patients were included in the two groups, and the PCOS patients were further divided into four PCOS phenotype groups based on the Rotterdam criteria.

**Results:** Patients with various PCOS phenotypes had similar rates of biochemical pregnancy, clinical pregnancy, and live birth (all *P*-values > 0.05). The overall incidence of adverse pregnancy outcomes (including ectopic pregnancy, miscarriage, preterm birth) was significantly higher in PCOS phenotype A and D groups than in the control group (44% and 46.4% vs. 28.7%, $P = 0.027$). The rates of hypertensive disorder of pregnancy (HDP) were significantly higher in PCOS phenotype A and C groups than in the control group (9.3% and 12.5% vs. 3.1%, $P = 0.037$). After adjustment for potential confounders, the differences in adverse pregnancy outcomes persisted ($P = 0.025$).

**Conclusions:** The overall incidence of adverse pregnancy outcomes is higher in women with PCOS phenotypes A and D than in women with non-PCOS.

**Keywords: polycystic ovarian syndrome, phenotype, assisted reproductive technology, hypertensive disorder of pregnancy, adverse pregnancy outcomes**

OPEN ACCESS

***Edited by:***
*Yimin Zhu,*
*Zhejiang University, China*

***Reviewed by:***
*Rong Li,*
*Peking University Third Hospital, China*
*Guimin Hao,*
*The Second Hospital of Hebei Medical University, China*
*Yun Sun,*
*Shanghai Jiao Tong University, China*

***Correspondence:**
*Lei Yan*
*yanlei@sdu.edu.cn*
*Yuhua Shi*
*shiyuhua2003@126.com*

*†These authors have contributed equally to this work and share first authorship*

***Specialty section:***
*This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology*

***Received:*** *03 March 2022*
***Accepted:*** *28 April 2022*
***Published:*** *03 June 2022*

***Citation:***
*Wang Q, Wang H, Li P, Li X, Wang Z, Yan L and Shi Y (2022) Association of Polycystic Ovary Syndrome Phenotypes With Adverse Pregnancy Outcomes After In-Vitro Fertilization/ Intracytoplasmic Sperm Injection. Front. Endocrinol. 13:889029. doi: 10.3389/fendo.2022.889029*

# INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common endocrine disorder in women of reproductive age and the main cause of anovulatory infertility (1–3), which is characterized by obesity, hyperandrogenism, anovulation, insulin resistance, polycystic ovary, and infertility. The global prevalence of PCOS ranges from 6% to 21% (4); however, the etiology of PCOS is unclear (5). Moreover, because anovulation in women with PCOS often results in infertility (6), assisted reproductive technology (ART) is usually required for these women to become pregnant. According to the Rotterdam criteria, PCOS patients can be divided into the following four phenotypes: phenotype A—coexistence of clinical hyperandrogenism/hyperandrogenemia, oligomenorrhea/anovulation, and polycystic ovaries (HA+OA +PCO); phenotype B—clinical hyperandrogenism or hyperandrogenemia and oligomenorrhea/anovulation (HA +OA); phenotype C—clinical hyperandrogenism or hyperandrogenemia and polycystic ovaries (HA+PCO); and phenotype D: oligomenorrhea/anovulation and polycystic ovaries (OA+PCO) (7). For different PCOS phenotypes, the ovarian response to gonadotropin (Gn) is varied in controlled ovarian hyperstimulation (COH) (8), which in turn affects the outcome of ART.

Because PCOS patients have the characteristics of reproductive endocrine dysfunction and metabolic disorder (9), they were more prone to having pregnancy complications (10, 11), which increases the risk of adverse perinatal outcomes (12). Previous studies found that the risk of pregnancy-related complications and adverse pregnancy outcomes *via* ART was higher than *via* spontaneous conception (13–15), and a recent meta-analysis showed that patients with PCOS undergoing IVF were associated with higher risks of adverse pregnancy outcomes (16). However, studies on the association between various PCOS phenotypes after IVF/ICSI and adverse perinatal outcomes were relatively small.

The present study retrospectively analyzed the adverse perinatal outcomes of patients with various PCOS phenotypes who underwent IVF/ICSI.

# MATERIALS AND METHODS

## Study Patients

We screened patients who underwent their first IVF/ICSI cycle at the Center for Reproductive Medicine, Cheeloo College of Medicine, Shandong University between January 2017 and July 2018. All patients were divided into the PCOS group and the control group. PCOS was defined according to the Rotterdam consensus criteria (2004) (17); that is, PCOS was diagnosed if at least two of the following criteria were present: oligomenorrhea/anovulation (defined as delaying of >35 days or <8 spontaneous hemorrhagic episodes/year), clinical and/or biochemical hyperandrogenism [biochemical hyperandrogenism was defined as total testosterone levels above 48.1 ng/dl detected in patients with no clinical evidence of hyperandrogenism or menstrual disturbances and not taking hormonal medication, and hirsutism was defined as patients with a total score ≥6 by the modified Ferriman–Gallwey score (18)], and polycystic ovary on ultrasonography (≥12 small follicles measuring 2–9 mm in at least one ovary and/or ovarian volume ≥10 $cm^3$), and it is necessary to exclude other endocrine dysfunctions. Furthermore, the PCOS group was classified into four phenotype subgroups as follows (19): phenotype A—HA+OA+PCO, phenotype B—HA+OA, phenotype C—HA+PCO, and phenotype D—OA+PCO. Women in the control group had regular menstrual cycles (21–35 days), without evidence of HA or PCO. All patients with the following conditions were excluded: age >38 years old, serum FSH level >15 IU/L, diabetes, hypertension, abnormal parental karyotypes, severe intrauterine adhesion or uterine abnormality, chronic medical conditions that contraindicated pregnancy or with other endocrine dysfunction (such as Cushing's syndrome, primary hyperprolactinemia, thyroid dysfunction, congenital adrenal hyperplasia, androgen producing neoplasm), and history of recurrent spontaneous abortion (RSA) or unilateral oophorectomy.

In total, we identified 6,732 women who met the study criteria, consisting of 1,186 in the PCOS group and 5,546 in the control group. This study was approved by the Institutional Review Board of the Center for Reproductive Medicine, Cheeloo College of Medicine, Shandong University (2017-53).

## Measurement

All patients underwent clinical history (including but not limited to the menstrual cycle and infertility type), physical examination [including but not limited to body mass index (BMI), Ferriman–Gallwey score, and gynecologic examination], biochemical analysis [including but not limited to the levels of fasting blood glucose (FBG), follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol, progesterone, total testosterone (To), anti-Müllerian hormone (AMH) and thyroid-stimulating hormone (TSH), and prolactin], and transvaginal ultrasonography for calculated antral follicle count (AFC) on follicular phase. Blood samples were drawn for biochemical analyses on days 2–3 of a spontaneous or progestogen-induced menstrual cycle. All the hormonal assays were made at the Center for Reproductive Medicine Laboratory, Cheeloo College of Medicine, Shandong University.

## Treatment Protocol

According to a routine method (18), all patients received a standardized ovarian stimulation regimen; underwent oocyte retrieval, fertilization, and transfer embryos; and were provided luteal phase support. All patients underwent COH with standard long agonist protocol or antagonist protocol [as previously described (20, 21)]. As monitored on ultrasound and based on the level of serum sex hormones (including FSH, LH, $E_2$, progesterone), Gn doses were adjusted based on the ovarian response. Human chorionic gonadotropin (HCG) at a dose of 4,000 to 8,000 IU was administered when at least two follicles were ≥18 mm. Oocyte retrieval was performed 34–36 h later under transvaginal ultrasound guidance. According to sperm quality, IVF/ICSI was performed. The embryo quality was graded according to the number of blastomeres, percent fragmentation, and regularity. Embryos were transferred on day 3 or day 5 after oocyte retrieval according to the patient's

condition (such as embryo quality, abdominal distention, and endocrine examination results). Cycle cancellation is defined if the patient does not have a fresh embryo transfer after oocyte retrieval (and we excluded cycles canceled before HCG triggering). Luteal phase support was provided after oocyte retrieval for those women who planned to transfer fresh embryos, as previously described (18, 20). Fourteen days after embryo transfer, the serum HCG levels were measured. If conception occurred, the luteal phase support was maintained. Transvaginal ultrasonography was performed 35 days after embryo transfer.

## IVF/ICSI Outcomes

In this study, the primary outcome measures were adverse perinatal outcomes, while the secondary outcome measures included biochemical pregnancy, clinical pregnancy (CP), and live birth (LB). Adverse perinatal outcomes were categorized into adverse pregnancy outcomes and pregnancy complications. Adverse pregnancy outcomes included ectopic pregnancy, miscarriage, and premature birth, and pregnancy complications included hypertensive disorders of pregnancy (HDP), gestational diabetes mellitus (GDM), and others (postpartum hemorrhage, placenta previa, placental abruption, premature rupture of membrane, cardiac diseases complicating pregnancy). Ectopic pregnancy was considered as developing blastocyst implanted outside the endometrial cavity. Miscarriage was defined as clinical pregnancy lost before 28 weeks of gestation. Premature birth was defined as a baby born between the 28th and 37th week of pregnancy. In this study, HDP included gestational hypertension (333 cases) and preeclampsia (1 case). Gestational hypertension and preeclampsia were defined as previously described (22–24). GDM was defined as the variable severity of glucose intolerance with onset or first recognition during pregnancy (25). Biochemical pregnancy was defined as serum HCG level ≥10 IU/L. CP was defined as the presence of gestational sacs by ultrasonography. LB was defined as the delivery of any viable infant at 28 weeks or more of gestation. Additionally, the cycle cancellation rate was calculated as the number of canceled fresh embryo transfer cycles divided by the number of oocyte retrieval cycles. Embryos of grades I and II, with 7–10 cells on day 3, were defined as high-quality embryos, and high-quality embryo rate, defined as the number of high-quality embryo/number of zygotes, was calculated. Fertilization rate (FR) was calculated as the number of 2PN divided by the number of oocyte retrieval, and implantation rate (IR) was calculated as the number of observed gestational sacs divided by the number of transferred embryos.

## Statistical Analysis

Comparisons between groups were performed using one-way analysis of variance (with the LSD *post-hoc* test) for continuous variables and the chi-squared test (or Fisher's exact test when the expected frequencies were less than five) for categorical variables. The results were expressed as mean ± standard deviation (SD) for continuous variables and as percentages for categorical variables. The study was retrospective to balance basic patient characteristics (including age, infertility type, and stimulation protocol) between groups. We used 1:1 propensity score matching (PSM) to match control patients to PCOS patients, and 0 is the matching caliper of PSM in this study. In the PCOS subgroups, logistic regression was used to evaluate the relationship between PCOS phenotype and IVF/ICSI outcomes while adjusting for relevant confounders, and the results were expressed as odds ratios (OR) with 95% confidence intervals (CI).

All statistical analyses were performed using the Statistical Package for Social Sciences (version 26.0, SPSS Inc., Chicago, USA) and R software. $P$-value <0.05 was considered statistically significant.

# RESULTS

A total of 6,732 patients were recorded, with 1,186 in the PCOS group and 5,546 in the control group. After the PSM procedure, 1,186 patients were included in the control group, and there were 293 cases of phenotype A, 53 cases of phenotype B, 77 cases of phenotype C, and 763 cases of phenotype D in the PCOS groups (**Figure 1**).

## Patients' Characteristics

The basic characteristics of the patients among the five groups are shown in **Table 1**. The results showed significant differences in BMI, FBG, FSH, LH, LH/FSH ratio, To, AMH, and AFC among the five groups (all $P < 0.001$). Of these, BMI, FBG, LH, and To were higher in the PCOS phenotype A group than in the other groups (all $P < 0.001$). The basic characteristics before PSM are shown in **Supplementary Table 1**. In addition, we only compared the 2-h plasma glucose concentrations after OGTT in various PCOS phenotype groups, and the results showed no statistically significant differences between groups ($P = 0.633$, data not shown).

## Ovarian Response and Pregnancy Outcomes

The ovarian response and pregnancy outcomes of patients among the four PCOS phenotype groups and the control group are

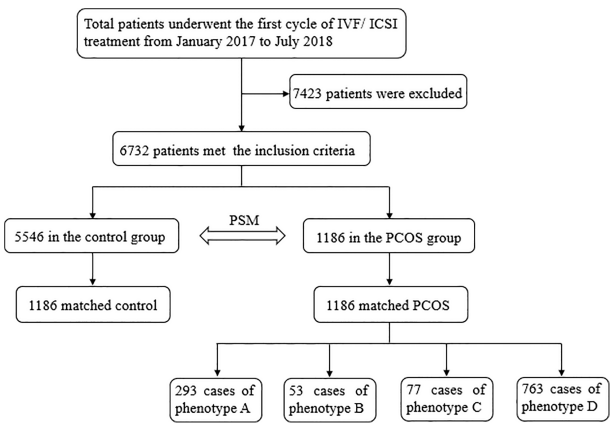



**FIGURE 1** | Flow chart database searching pathway and group divisions.

**TABLE 1** | Basic characteristics of the patients among the four PCOS phenotype groups and the control group.

| | Phenotype A (*n* = 293) | Phenotype B (*n* = 53) | Phenotype C (*n* = 77) | Phenotype D (*n* = 763) | Matched control (*n* = 1,186) | *P*-value* |
|---|---|---|---|---|---|---|
| Age (years) | 28.92 ± 3.36 | 28.87 ± 3.02 | 28.90 ± 3.12 | 29.49 ± 3.46 | 29.28 ± 3.40 | 0.095 |
| BMI (kg/m$^2$) | 25.79 ± 3.91$^{ce}$ | 24.78 ± 3.25$^{e}$ | 24.45 ± 3.75$^{ade}$ | 25.35 ± 3.88$^{ce}$ | 23.43 ± 3.54$^{ab,cd}$ | <0.001 |
| FBG (mmol/L) | 5.37 ± 0.74$^{e}$ | 5.22 ± 0.38 | 5.33 ± 0.41 | 5.32 ± 0.50$^{e}$ | 5.22 ± 0.45$^{ad}$ | <0.001 |
| FSH (IU/L) | 5.71 ± 1.36$^{e}$ | 6.08 ± 1.49$^{d}$ | 5.88 ± 1.17$^{e}$ | 5.57 ± 1.25$^{b,e}$ | 6.37 ± 1.67$^{acd}$ | <0.001 |
| LH (IU/L) | 11.68 ± 5.30$^{bcde}$ | 8.80 ± 4.67$^{ae}$ | 8.34 ± 5.55$^{ae}$ | 8.20 ± 4.94$^{ae}$ | 5.20 ± 2.95$^{abcd}$ | <0.001 |
| LH/FSH | 2.07 ± 0.89$^{bcde}$ | 1.49 ± 0.82$^{ae}$ | 1.42 ± 0.86$^{ae}$ | 1.48 ± 0.87$^{ae}$ | 0.86 ± 0.58$^{abcd}$ | <0.001 |
| To (ng/dl) | 62.28 ± 14.57$^{cde}$ | 60.00 ± 12.18$^{de}$ | 58.29 ± 11.31$^{ade}$ | 31.78 ± 10.15$^{abce}$ | 25.04 ± 10.72$^{abcd}$ | <0.001 |
| AMH (ng/ml) | 12.35 ± 6.03$^{bcde}$ | 7.05 ± 3.91$^{acde}$ | 8.86 ± 4.37$^{abe}$ | 9.37 ± 4.80$^{abe}$ | 4.43 ± 3.05$^{abcd}$ | <0.001 |
| AFC | 33.73 ± 11.64$^{bcde}$ | 16.70 ± 3.66$^{acd}$ | 27.74 ± 8.62$^{abe}$ | 28.98 ± 8.10$^{abe}$ | 15.33 ± 6.11$^{acd}$ | <0.001 |
| Infertility type, *n* (%) | | | | | | 0.222 |
| Primary | 176 (60.1) | 35 (66.0) | 57 (74.0) | 468 (61.3) | 737 (62.1) | |
| Secondary | 117 (39.9) | 18 (34.0) | 20 (26.0) | 295 (38.7) | 449 (37.9) | |

*BMI, body mass index; FBG, fasting blood glucose; FSH, follicle-stimulating hormone; To, total testosterone concentration; AMH, anti-Müllerian hormone; AFC, antral follicle count.*
[a]*Significantly different from phenotype A.*
[b]*Significantly different from phenotype B.*
[c]*Significantly different from phenotype C.*
[d]*Significantly different from phenotype D.*
[e]*Significantly different from the control group.*
**All P-values for quantitative variables were determined by post-hoc analysis (LSD).*

presented in **Table 2**. Significant differences in HCG dose, endometrial thickness, the number of follicles of diameter ≥14 mm and $E_2$ levels on the trigger day, the number of retrieved oocytes and frozen embryos, and high-quality embryo rate among groups were observed (all $P < 0.05$). Of these, the number of follicles of diameter ≥14 mm and $E_2$ levels on the trigger day and the number of retrieved oocytes were significantly higher in PCOS phenotype A and C groups compared with the other phenotype groups and the control group (all $P < 0.05$). It is worth noting that the high-quality embryo rate of PCOS phenotype A and D groups was lower than that of the other groups, especially the control group ($P = 0.019$). Although there were significant differences in Gn priming dose, stimulation duration, and the number of 2PN among the five groups (all $P < 0.001$), the total dose of Gn and FR were not statistically different (all $P > 0.05$). We can see that the cycle cancellation rate of the PCOS phenotype D group is lower than that of PCOS phenotype A and C groups and higher than that of PCOS phenotype B and control groups (62.8% and 68.8% vs. 53.9% vs. 39.6% and 34.8%, $P < 0.001$). The patients in the five groups had similar biochemical pregnancy rates, CPRs, ectopic pregnancy rates, miscarriage rates, premature birth rates, and LBRs (all $P > 0.05$). The data on ovarian response and pregnancy outcomes before PSM are shown in **Supplementary Table 2**. In addition, we compared the incidence of ovarian hyperstimulation syndrome (OHSS) in various PCOS phenotype groups after IVF-ET, and the results showed no statistically significant differences between groups ($P = 0.788$, data not shown).

## Adverse Perinatal Outcomes

The adverse perinatal outcomes of the five groups are displayed in **Table 3**. The adverse pregnancy outcome rate was higher in PCOS phenotype A and D groups than in the control group (44.0% and 46.4% vs. 28.7%, $P = 0.027$). Despite the differences in HDP rate of PCOS phenotype A and C groups and the control group (9.3% and 12.5% vs. 3.1%, $P = 0.037$), the incidence of total pregnancy complications, GDM, or other pregnancy complications was similar among the five groups. There was no difference between the groups for the rates of ectopic pregnancy, miscarriage, premature birth, cesarean section, and multiple births (all $P > 0.05$). The data on adverse perinatal outcomes before PSM are shown in **Supplementary Table 3**. In addition, the statistical power of the R×C square test was calculated *via* the "pwr" package in R software, where the effect size was determined as 0.55 using the ES.w2() function, and the statistical power was calculated as pwr.chisq.test(w = ES.w2(prob), $N = 799$, $df = 4$, sig.level = 0.05) >0.99, based on which we admit that the results in **Table 3** are accurate.

## Logistic Regression Assessment of Adverse Perinatal Outcomes

According to the previous results of adverse perinatal outcomes, a univariate logistic analysis of adverse pregnancy outcomes and HDP was performed. Compared with the control group, PCOS phenotypes A and D were the risk factors for adverse pregnancy outcomes [cOR (crude odds ratio)-A: 1.952, 95% CI-A: 1.185–3.216; cOR-D: 1.401, 95% CI-D: 1.001–1.960] and PCOS phenotype A was the risk factor for HDP (cOR: 3.228, 95% CI: 1.258–8.285). The factors with significant differences in the univariate analysis (these results are shown in **Supplementary Tables 4**, **5**) were included in the multivariate logistic regression analysis. After adjusting for confounding factors, PCOS phenotypes A and D were shown as independent risk factors for adverse pregnancy outcomes (aOR-A: 1.835, 95% CI-A: 1.095–3.075; aOR-D: 1.435, 95% CI-D: 1.025–2.008) (see **Table 4**).

# DISCUSSION

In this study, the relationship between PCOS phenotypes and pregnancy was retrospectively analyzed in patients who underwent the first cycle of IVF/ICSI treatment. The results revealed that the PCOS phenotype was correlated with adverse

**TABLE 2 |** Comparison of ovarian response and pregnancy outcomes among the four PCOS phenotype groups and the control group.

| | Phenotype A (*n* = 293) | Phenotype B (*n* = 53) | Phenotype C (*n* = 77) | Phenotype D (*n* = 763) | Matched control (*n* = 1,186) | *P*-value* |
|---|---|---|---|---|---|---|
| Stimulation protocol, *n* (%) | | | | | | 0.386 |
| Long agonist | 121 (41.3) | 26 (49.1) | 41 (53.2) | 332 (43.5) | 520 (43.8) | |
| Antagonist | 172 (58.7) | 27 (50.9) | 36 (46.8) | 431 (56.5) | 666 (56.2) | |
| Gn priming dose (IU) | 140.49 ± 28.87[bde] | 152.83 ± 36.50[a] | 143.99 ± 33.91[e] | 143.38 ± 30.53[e] | 158.27 ± 45.32[acd] | <0.001 |
| Total dose of Gn (IU) | 1,812.47 ± 947.69 | 1,834.67 ± 818.40 | 1,721.27 ± 887.01 | 1,827.65 ± 857.09 | 1,842.21 ± 758.18 | 0.781 |
| Stimulation duration (days) | 10.55 ± 2.52[e] | 10.15 ± 2.17 | 10.04 ± 2.40 | 10.48 ± 2.39[e] | 9.87 ± 1.81[ad] | <0.001 |
| HCG dose (IU) | 6,139.93 ± 1,756.85[bde] | 6,660.38 ± 1,640.16[ae] | 6,272.73 ± 1,675.18[e] | 6,570.12 ± 1,672.30[ae] | 7,265.18 ± 1,502.00[abcd] | <0.001 |
| Endometrial thickness on the trigger day (mm) | 10.43 ± 2.01[de] | 10.69 ± 2.62 | 10.67 ± 1.94 | 10.90 ± 1.92[a] | 10.96 ± 1.95[a] | 0.001 |
| No. of follicles of diameter ≥14 mm on the trigger day | 15.72 ± 6.04[bde] | 13.02 ± 4.85[acde] | 16.26 ± 5.97[bde] | 14.56 ± 5.57[abce] | 10.77 ± 4.73[abcd] | <0.001 |
| $E_2$ levels on the trigger day (pg/ml) | 4,882.83 ± 2,918.41[bde] | 4,161.73 ± 2,444.63[ae] | 4,790.87 ± 2,823.48[de] | 4,168.88 ± 2,488.18[ace] | 3,233.50 ± 1,826.45[abcd] | <0.001 |
| No. of retrieved oocytes | 15.82 ± 8.07[bde] | 12.60 ± 6.11[acd] | 16.17 ± 7.12[bde] | 14.56 ± 6.94[abce] | 11.08 ± 5.45[acd] | <0.001 |
| No. of 2PN | 8.44 ± 4.50[e] | 7.98 ± 4.61 | 8.92 ± 3.85[e] | 8.29 ± 3.98[e] | 6.90 ± 3.78[acd] | <0.001 |
| FR (%) | 0.61 ± 0.21[e] | 0.65 ± 0.24 | 0.63 ± 0.21 | 0.64 ± 0.21 | 0.64 ± 0.23[a] | 0.269 |
| High-quality embryo rate (%) | 34.20 ± 22.08[e] | 38.16 ± 20.45 | 38.48 ± 21.72 | 35.75 ± 22.06[e] | 38.45 ± 23.79[ad] | 0.019 |
| Cycle cancellation rate, *n* (%) | 184/293 (62.8)[bde] | 21/53 (39.6)[acd] | 53/77 (68.8)[bde] | 411/763 (53.9)[abce] | 413/1,186 (34.8)[acd] | <0.001 |
| No. of transferred embryos | 1.72 ± 0.45[e] | 1.72 ± 0.46[e] | 1.71 ± 0.46 | 1.64 ± 0.48[e] | 1.54 ± 0.50[abd] | <0.001 |
| No. of transferred high-quality embryos | 1.69 ± 0.52[e] | 1.72 ± 0.46[e] | 1.67 ± 0.57 | 1.61 ± 0.53[e] | 1.52 ± 0.53[abd] | 0.001 |
| IR (%) | 56.40 ± 44.67 | 57.80 ± 44.19 | 56.30 ± 44.99 | 55.00 ± 44.30[e] | 48.30 ± 44.52[d] | 0.077 |
| Biochemical pregnancy rate/ET cycles (%) | 84/109 (77.1)[e] | 23/32 (71.9) | 18/24 (75.0) | 258/352 (73.3)[e] | 521/773 (67.4)[ad] | 0.123 |
| CPR/ET cycles (%) | 75/109 (68.8)[e] | 22/32 (68.8) | 16/24 (66.7) | 233/352 (66.2)[e] | 453/773 (58.6)[ad] | 0.051 |
| LBR (%) | 59/109 (54.1) | 17/32 (53.1) | 15/24 (62.5) | 190/352 (54.0) | 386/773 (49.9) | 0.543 |

*Gn, gonadotropin; HCG, human chorionic gonadotropin; FR, fertilization rate; IR, implantation rate; CPR, clinical pregnancy rate; LBR, live birth rate.*
*[a]Significantly different from phenotype A.*
*[b]Significantly different from phenotype B.*
*[c]Significantly different from phenotype C.*
*[d]Significantly different from phenotype D.*
*[e]Significantly different from the control group.*
**All P-values for quantitative variables were determined by post-hoc analysis (LSD).*

**TABLE 3 |** Comparison of adverse perinatal outcomes among the four PCOS phenotype groups and the control group.

| | Phenotype A (*n* = 75) | Phenotype B (*n* = 22) | Phenotype C (*n* = 16) | Phenotype D (*n* = 233) | Matched control (*n* = 453) | *P*-value* |
|---|---|---|---|---|---|---|
| Adverse pregnancy outcome rate (%) | 33/75 (44.0)[e] | 10/22 (45.5) | 4/16 (25.0) | 84/233 (46.4)[e] | 130/453 (28.7)[a,d] | 0.027 |
| Ectopic pregnancy rate (%) | 2/75 (2.7) | 0/22 (0.0) | 0/16 (0.0) | 5/233 (2.1) | 13/453 (2.9) | 0.959 |
| Miscarriage rate (%) | 14/75 (18.7) | 5/22 (22.7) | 1/16 (6.3) | 39/233 (16.7) | 53/453 (11.7) | 0.121 |
| Premature birth rate (%) | 17/75 (22.7) | 5/22 (22.7) | 3/16 (18.8) | 40/233 (17.2) | 64/453 (14.1) | 0.266 |
| Pregnancy complication rate (%) | 13/75 (17.3) | 4/22 (18.2) | 2/16 (12.5) | 40/233 (17.2) | 57/453 (12.6) | 0.429 |
| HDP rate (%) | 7/75 (9.3)[e] | 1/22 (4.5) | 2/16 (12.5)[e] | 13/233 (5.6) | 14/453 (3.1)[a,c] | 0.037 |
| GDM rate (%) | 7/75 (9.3) | 2/22 (9.1) | 0/16 (0.0) | 21/233 (9.0) | 25/453 (5.5) | 0.257 |
| Rate of others (%) | 0/75 (0.0) | 1/22 (4.5) | 0/16 (0.0) | 7/233 (3.0) | 19/453 (4.2) | 0.343 |
| Rate of cesarean section (%) | 41/75 (54.7) | 15/22 (68.2) | 9/16 (56.3) | 129/232 (55.6) | 254/453 (56.1) | 0.851 |
| Multiple birth rate (%) | 18/59 (30.5) | 5/17 (29.4) | 5/15 (33.3) | 54/190 (28.4) | 90/386 (23.3) | 0.470 |

*HDP, hypertensive disorders of pregnancy; GDM, gestational diabetes mellitus.*
*[a]Significantly different from phenotype A.*
*[c]Significantly different from phenotype C.*
*[d]Significantly different from phenotype D.*
*[e]Significantly different from the control group.*
**All P-values for quantitative variables were determined by post-hoc analysis (LSD).*

**TABLE 4** | Logistic regression analysis of maternal and perinatal outcomes.

| Outcomes | Phenotypes | cOR (95% CI) | *P*-value | aOR (95% CI) | *P*-value |
|---|---|---|---|---|---|
| Adverse pregnancy outcomes | | | 0.030 | | 0.025 |
| | Control | Reference | – | | – |
| | PCOS phenotype A | 1.952 (1.185–3.216) | 0.009 | 1.835 (1.095–3.075) | 0.021 |
| | PCOS phenotype B | 2.071 (0.873–4.910) | 0.099 | 2.084 (0.873–4.974) | 0.098 |
| | PCOS phenotype C | 0.828 (0.262–2.615) | 0.748 | 0.538 (0.169–1.720) | 0.296 |
| | PCOS phenotype D | 1.401 (1.001–1.960) | 0.049 | 1.435 (1.025–2.008) | 0.035 |
| HDP | | | 0.085 | | 0.898 |
| | Control | Reference | – | | – |
| | PCOS phenotype A | 3.228 (1.258–8.285) | 0.015 | 1.415 (0.337–5.944) | 0.635 |
| | PCOS phenotype B | 1.493 (0.187–11.898) | 0.705 | 0.807 (0.080–8.127) | 0.856 |
| | PCOS phenotype C | 4.480 (0.928–21.624) | 0.062 | 2.573 (0.436–15.185) | 0.297 |
| | PCOS phenotype D | 1.853 (0.856–4.010) | 0.117 | 1.385 (0.614–3.125) | 0.433 |

*cOR, crude odds ratio; CI, confidence interval; aOR, adjusted odds ratio.*

*The indicators with statistical differences in adverse pregnancy outcomes included patient type, BMI, To, HCG dose, $E_2$ levels on the trigger day, no. of transferred embryos, and no. of transferred high-quality embryos, and the indicators with statistical differences in HDP included as patient type, BMI, and To.*

pregnancy outcomes (ectopic pregnancy, miscarriage, and premature birth), and PCOS phenotypes A and D were the independent risk factors for adverse pregnancy outcomes. Moreover, CPR and LBR in various PCOS phenotypes were comparable.

Adverse pregnancy outcomes have been the subject of considerable attention, and the relationship between PCOS and adverse pregnancy outcomes has been a topic of great interest in the assisted reproductive field. A meta-analysis of pregnancy-related outcomes and complications in PCOS patients reported that PCOS patients present a high risk of adverse pregnancy outcomes despite the fact that they achieved a better LBR (16). Previous studies concluded that PCOS increased the risk of adverse pregnancy outcomes by affecting the reproductive endocrine and metabolic functions (6, 10, 26, 27). In addition, women with PCOS present with an abnormal endometrial phenotype and function (28), which possibly explains some of the adverse pregnancy outcomes such as miscarriage and premature birth (29).

The results of this study, for the first time, showed that PCOS phenotypes A and D were the independent risk factors for adverse pregnancy outcomes. In other words, higher incidences of adverse pregnancy outcomes occurred in women with PCOS phenotypes A and D. It was found that these two phenotypes of PCOS exist with common characteristics: OA and PCO. We speculated that the higher rates of adverse pregnancy in patients with PCOS result from a combined action of OA and PCO. A menstrual disorder in PCOS patients mainly results from insulin resistance, and it can reflect the degree of metabolic dysfunction (30). Recent findings showed that the menstrual patterns of PCOS patients might be correlated with the higher rates of adverse pregnancy outcomes (27). The result of a retrospective study showed that amenorrhea in PCOS patients was an independent risk factor for adverse pregnancy outcomes. Also, oocyte maturation and fertility rate in women with anovulation were lower than in women with regular cycling, and the development rate of the embryo shared a similar trend (31). Another study involving dairy cattle with anovulation reported that anovulation results in significant alterations in gene expression. Specifically, transcripts linked to the control of energy metabolism and DNA repair were downregulated, whereas genes involved in apoptosis and autophagy were upregulated. It was also found that the risk factors for OA have a direct impact on embryo development and endometrial receptivity (32).

Moreover, several studies suggested that PCO were associated with poor oocyte quality, and they also found elevated levels of homocysteine in the blood of PCOS patients (33–35) and in the follicular fluid of patients with PCO (36). These findings suggested that abnormally high homocysteine levels of follicular fluid were related to the poor quality of oocytes and low fertilization rates, even to the poor quality of embryos and adverse pregnancy outcomes (36). In a previous study, Jia et al. reported that the quality of oocytes in PCO has decreased, which could be due to mtDNA hypermethylation and abnormal activation of one-carbon metabolism (37). In addition, we also found that the high-quality embryo rate of PCOS phenotype A and D groups was lower than that of the other groups, especially the control group. This result supports our speculation. The coexistence of OA and PCO may be associated with higher rates of adverse pregnancy by affecting the quality of oocyte and embryo.

At present, advanced maternal age (38, 39), high levels of BMI (40, 41), and a thin endometrium (42–44) as risk factors for adverse pregnancy outcomes are well recognized in the literature. Therefore, multivariate logistic regression analyses in our study were performed to exclude the potential influences of these confounding factors, but the effect of PCOS phenotypes A and D on adverse pregnancy outcomes persists. In addition, a recent meta-analysis suggested that HA has adverse effects on assisted reproductive outcomes in patients with PCOS (45). However, the contribution of HA to miscarriage is still debated (46, 47). The effect of HA on adverse pregnancy outcomes was not found in our study, but the aOR of PCOS phenotype A (with HA) was higher than that of PCOS phenotype D (without HA) in the logistic analysis of adverse pregnancy outcomes. It was hypothesized that HA may have a role in the incidence of adverse pregnancy outcomes in IVF/ICSI and that this effect would be weak. Simultaneously, OA and PCO were the primary influencers in adverse pregnancy outcomes. As we all know,

OHSS is also an important factor affecting adverse pregnancy outcomes (48), and patients with PCOS are at a greater risk to develop OHSS (49). In the present study, we compared the incidence of OHSS in various PCOS phenotype groups after IVF-ET, and the results showed no statistically significant differences between groups. These results were probably due to some PCOS patients with a higher OHSS risk canceling fresh embryo transfer and selecting all-embryo cryopreservation (50).

The results of our study highlight the need for individualized treatment and intensive follow-up after pregnancy in patients with PCOS phenotypes A and D, to decrease the incidence of adverse pregnancy outcomes. However, as with all retrospective data analyses, we were not able to completely rule out all potential confounders. Moreover, our study inevitably suffers from several limitations, even though we used PSM statistical methods to diminish bias. Although we have expanded the sample size compared with those reported in previous studies (8, 51), the sample size of some PCOS phenotypes is still the main limitation of the study. We think that one possible explanation could be the characteristics of the study population. Therefore, further prospective research with a sufficient sample size will be needed to confirm these findings in the future.

Taken together, our data revealed that PCOS phenotypes A and D were the independent risk factors for adverse pregnancy outcomes. Specifically, the higher incidences of adverse pregnancy outcomes occur in women with PCOS phenotypes A and D compared with women with non-PCOS. Therefore, for women with PCOS phenotypes A and D, individualized treatment during assisted reproduction and close follow-up after clinical pregnancy are necessary.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of the Center for Reproductive Medicine, Cheeloo College of Medicine, Shandong University. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

YS, LY, and QW conceived and designed this study. QW contributed to the statistical analysis and interpretation of data and drafting of the manuscript. HW and PL performed the statistical analysis and participated in the discussion. XL and ZW analyzed and interpreted the data. LY and QW participated in the discussion and critically revised the manuscript. All authors read and approved the final manuscript.

## FUNDING

This study was supported by the National Key R&D Program of China (2021YFC2700404, 2018YFC1003202).

## ACKNOWLEDGMENTS

The authors thank the clinicians, nurses, and laboratory staff for their contribution. Moreover, the authors thank the infertile couples who participated in this study.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.889029/full#supplementary-material

## REFERENCES

1. Giampaolino P, Morra I, Della Corte L, Sparice S, Di Carlo C, Nappi C, et al. Serum Anti-Mullerian Hormone Levels After Ovarian Drilling for the Second-Line Treatment of Polycystic Ovary Syndrome: A Pilot-Randomized Study Comparing Laparoscopy and Transvaginal Hydrolaparoscopy. *Gynecol Endocrinol* (2017) 33(1):26–9. doi: 10.1080/09513590.2016.1188280
2. Della Corte L, Foreste V, Barra F, Gustavino C, Alessandri F, Centurioni MG, et al. Current and Experimental Drug Therapy for the Treatment of Polycystic Ovarian Syndrome. *Expert Opin Investig Drugs* (2020) 29(8):819–30. doi: 10.1080/13543784.2020.1781815
3. Giampaolino P, De Rosa N, Della Corte L, Morra I, Mercorio A, Nappi C, et al. Operative Transvaginal Hydrolaparoscopy Improve Ovulation Rate After Clomiphene Failure in Polycystic Ovary Syndrome. *Gynecol Endocrinol* (2018) 34(1):32–5. doi: 10.1080/09513590.2017.1323204
4. Lizneva D, Suturina L, Walker W, Brakta S, Gavrilova-Jordan L, Azziz R. Criteria, Prevalence, and Phenotypes of Polycystic Ovary Syndrome. *Fertil Steril* (2016) 106(1):6–15. doi: 10.1016/j.fertnstert.2016.05.003
5. Giampaolino P, Foreste V, Di Filippo C, Gallo A, Mercorio A, Serafino P, et al. Microbiome and PCOS: State-Of-Art and Future Aspects. *Int J Mol Sci* (2021) 22(4):2048. doi: 10.3390/ijms22042048
6. Azziz R, Carmina E, Chen Z, Dunaif A, Laven JS, Legro RS, et al. Polycystic Ovary Syndrome. *Nat Rev Dis Primers* (2016) 2:16057. doi: 10.1038/nrdp.2016.57
7. Giampaolino P, Corte LD, Rosa ND, Mercorio A, Bruzzese D, Bifulco G. Ovarian Volume and PCOS: A Controversial Issue. *Gynecol Endocrinol* (2018) 34(3):229–32. doi: 10.1080/09513590.2017.1391205
8. Cela V, Obino MER, Alberga Y, Pinelli S, Sergiampietri C, Casarosa E, et al. Ovarian Response to Controlled Ovarian Stimulation in Women With Different Polycystic Ovary Syndrome Phenotypes. *Gynecol Endocrinol* (2018) 34(6):518–23. doi: 10.1080/09513590.2017.1412429
9. Gilbert EW, Tay CT, Hiam DS, Teede HJ, Moran LJ. Comorbidities and Complications of Polycystic Ovary Syndrome: An Overview of Systematic Reviews. *Clin Endocrinol (Oxf)* (2018) 89(6):683–99. doi: 10.1111/cen.13828
10. Bahri Khomami M, Boyle JA, Tay CT, Vanky E, Teede HJ, Joham AE, et al. Polycystic Ovary Syndrome and Adverse Pregnancy Outcomes: Current State

of Knowledge, Challenges and Potential Implications for Practice. *Clin Endocrinol (Oxf)* (2018) 88(6):761–9. doi: 10.1111/cen.13579
11. Li Y, Ruan X, Wang H, Li X, Cai G, Du J, et al. Comparing the Risk of Adverse Pregnancy Outcomes of Chinese Patients With Polycystic Ovary Syndrome With and Without Antiandrogenic Pretreatment. *Fertil Steril* (2018) 109 (4):720–7. doi: 10.1016/j.fertnstert.2017.12.023
12. Kelley AS, Smith YR, Padmanabhan V. A Narrative Review of Placental Contribution to Adverse Pregnancy Outcomes in Women With Polycystic Ovary Syndrome. *J Clin Endocrinol Metab* (2019) 104(11):5299–315. doi: 10.1210/jc.2019-00383
13. Henningsen AK, Pinborg A, Lidegaard Ø, Vestergaard C, Forman JL, Andersen AN. Perinatal Outcome of Singleton Siblings Born After Assisted Reproductive Technology and Spontaneous Conception: Danish National Sibling-Cohort Study. *Fertil Steril* (2011) 95(3):959–63. doi: 10.1016/j.fertnstert.2010.07.1075
14. Pinborg A, Wennerholm UB, Romundstad LB, Loft A, Aittomaki K, Söderström-Anttila V, et al. Why do Singletons Conceived After Assisted Reproduction Technology Have Adverse Perinatal Outcome? Systematic Review and Meta-Analysis. *Hum Reprod Update* (2013) 19(2):87–104. doi: 10.1093/humupd/dms044
15. Qin J, Wang H, Sheng X, Liang D, Tan H, Xia J. Pregnancy-Related Complications and Adverse Pregnancy Outcomes in Multiple Pregnancies Resulting From Assisted Reproductive Technology: A Meta-Analysis of Cohort Studies. *Fertil Steril* (2015) 103(6):1492–1508.e1491-1497. doi: 10.1016/j.fertnstert.2015.03.018
16. Sha T, Wang X, Cheng W, Yang Y. A Meta-Analysis of Pregnancy-Related Outcomes and Complications in Women With Polycystic Ovary Syndrome Undergoing IVF. *Reprod BioMed Online* (2019) 39(2):281–93. doi: 10.1016/j.rbmo.2019.03.203
17. Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group. Revised 2003 Consensus on Diagnostic Criteria and Long-Term Health Risks Related to Polycystic Ovary Syndrome. *Fertil Steril* (2004) 81(1):19–25. doi: 10.1016/j.fertnstert.2003.10.004
18. Chen ZJ, Shi Y, Sun Y, Zhang B, Liang X, Cao Y, et al. Fresh Versus Frozen Embryos for Infertility in the Polycystic Ovary Syndrome. *New Engl J Med* (2016) 375(6):523–33. doi: 10.1056/NEJMoa1513873
19. Norman RJ, Dewailly D, Legro RS, Hickey TE. Polycystic Ovary Syndrome. *Lancet* (2007) 370(9588):685–97. doi: 10.1016/S0140-6736(07)61345-2
20. Shi Y, Sun Y, Hao C, Zhang H, Wei D, Zhang Y, et al. Transfer of Fresh Versus Frozen Embryos in Ovulatory Women. *New Engl J Med* (2018) 378(2):126–36. doi: 10.1056/NEJMoa1705334
21. Liu F, Jiang Q, Sun X, Huang Y, Zhang Z, Han T, et al. Lipid Metabolic Disorders and Ovarian Hyperstimulation Syndrome: A Retrospective Analysis. *Front Physiol* (2020) 11:491892. doi: 10.3389/fphys.2020.491892
22. Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy. *Am J Obstet Gynecol* (2000) 183 (1):S1–s22.
23. Mol BWJ, Roberts CT, Thangaratinam S, Magee LA, de Groot CJM, Hofmeyr GJ. Pre-Eclampsia. *Lancet (London England)* (2016) 387(10022):999–1011. doi: 10.1016/S0140-6736(15)00070-7
24. Toyokawa S, Hasegawa J, Ikenoue T, Asano Y, Jojima E, Satoh S, et al. Weekend and Off-Hour Effects on the Incidence of Cerebral Palsy: Contribution of Consolidated Perinatal Care. *Environ Health Prev Med* (2020) 25(1):52. doi: 10.1186/s12199-020-00889-y
25. Baz B, Riveline JP, Gautier JF. Endocrinology of Pregnancy: Gestational Diabetes Mellitus: Definition, Aetiological and Clinical Aspects. *Eur J Endocrinol* (2016) 174(2):R43–51. doi: 10.1530/EJE-15-0378
26. Yu HF, Chen HS, Rao DP, Gong J. Association Between Polycystic Ovary Syndrome and the Risk of Pregnancy Complications: A PRISMA-Compliant Systematic Review and Meta-Analysis. *Medicine* (2016) 95(51):e4863. doi: 10.1097/MD.0000000000004863
27. Yu T, Wu D, Cao Y, Zhai J. Association Between Menstrual Patterns and Adverse Pregnancy Outcomes in Patients With Polycystic Ovary Syndrome. *Front Endocrinol* (2021) 12:740377. doi: 10.3389/fendo.2021.740377
28. Giudice LC. Endometrium in PCOS: Implantation and Predisposition to Endocrine CA. *Best Pract Res Clin Endocrinol Metab* (2006) 20(2):235–44. doi: 10.1016/j.beem.2006.03.005
29. Piltonen TT. Polycystic Ovary Syndrome: Endometrial Markers. *Best Pract Res Clin Obstet Gynaecol* (2016) 37:66–79. doi: 10.1016/j.bpobgyn.2016.03.008
30. Ezeh U, Ezeh C, Pisarska MD, Azziz R. Menstrual Dysfunction in Polycystic Ovary Syndrome: Association With Dynamic State Insulin Resistance Rather Than Hyperandrogenism. *Fertil Steril* (2021) 115(6):1557–68. doi: 10.1016/j.fertnstert.2020.12.015
31. Barnes FL, Kausche A, Tiglias J, Wood C, Wilton L, Trounson A. Production of Embryos From *In Vitro*-Matured Primary Human Oocytes. *Fertil Steril* (1996) 65(6):1151–6. doi: 10.1016/S0015-0282(16)58330-7
32. Santos JE, Bisinotto RS, Ribeiro ES. Mechanisms Underlying Reduced Fertility in Anovular Dairy Cows. *Theriogenology* (2016) 86(1):254–62. doi: 10.1016/j.theriogenology.2016.04.038
33. Yarali H, Yildirir A, Aybar F, Kabakçi G, Bükülmez O, Akgül E, et al. Diastolic Dysfunction and Increased Serum Homocysteine Concentrations may Contribute to Increased Cardiovascular Risk in Patients With Polycystic Ovary Syndrome. *Fertil Steril* (2001) 76(3):511–6. doi: 10.1016/S0015-0282(01)01937-9
34. Badawy A, State O, El Gawad SSA, El Aziz OA. Plasma Homocysteine and Polycystic Ovary Syndrome: The Missed Link. *Eur J Obstet Gynecol Reprod Biol* (2007) 131(1):68–72. doi: 10.1016/j.ejogrb.2006.10.015
35. Schachter M, Raziel A, Friedler S, Strassburger D, Bern O, Ron-El R. Insulin Resistance in Patients With Polycystic Ovary Syndrome is Associated With Elevated Plasma Homocysteine. *Hum Reprod* (2003) 18(4):721–7. doi: 10.1093/humrep/deg190
36. Berker B, Kaya C, Aytac R, Satiroglu H. Homocysteine Concentrations in Follicular Fluid are Associated With Poor Oocyte and Embryo Qualities in Polycystic Ovary Syndrome Patients Undergoing Assisted Reproduction. *Hum Reprod* (2009) 24(9):2293–302. doi: 10.1093/humrep/dep069
37. Jia L, Li J, He B, Jia Y, Niu Y, Wang C, et al. Abnormally Activated One-Carbon Metabolic Pathway is Associated With mtDNA Hypermethylation and Mitochondrial Malfunction in the Oocytes of Polycystic Gilt Ovaries. *Sci Rep* (2016) 6:19436. doi: 10.1038/srep19436
38. Frick AP. Advanced Maternal Age and Adverse Pregnancy Outcomes. *Best Pract Res Clin Obstet Gynaecol* (2021) 70:92–100. doi: 10.1016/j.bpobgyn.2020.07.005
39. Kawwass JF, Badell ML. Maternal and Fetal Risk Associated With Assisted Reproductive Technology. *Obstet Gynecol* (2018) 132(3):763–72. doi: 10.1097/AOG.0000000000002786
40. Lewandowska M, Sajdak S, Więckowska B, Manevska N, Lubiński J. The Influence of Maternal BMI on Adverse Pregnancy Outcomes in Older Women. *Nutrients* (2020) 12(9):2838. doi: 10.3390/nu12092838
41. Grieger JA, Bianco-Miotto T, Grzeskowiak LE, Leemaqz SY, Poston L, McCowan LM, et al. Metabolic Syndrome in Pregnancy and Risk for Adverse Pregnancy Outcomes: A Prospective Cohort of Nulliparous Women. *PloS Med* (2018) 15(12):e1002710. doi: 10.1371/journal.pmed.1002710
42. Liu X, Wang J, Fu X, Li J, Zhang M, Yan J, et al. Thin Endometrium is Associated With the Risk of Hypertensive Disorders of Pregnancy in Fresh IVF/ICSI Embryo Transfer Cycles: A Retrospective Cohort Study of 9,266 Singleton Births. *Reprod Biol Endocrinol* (2021) 19(1):55. doi: 10.1186/s12958-021-00738-9
43. Revel A. Defective Endometrial Receptivity. *Fertil Steril* (2012) 97(5):1028–32. doi: 10.1016/j.fertnstert.2012.03.039
44. Guo Z, Xu X, Zhang L, Zhang L, Yan L, Ma J. Endometrial Thickness is Associated With Incidence of Small-for-Gestational-Age Infants in Fresh In Vitro Fertilization-Intracytoplasmic Sperm Injection and Embryo Transfer Cycles. *Fertil Steril* (2020) 113(4):745–52. doi: 10.1016/j.fertnstert.2019.12.014
45. Ma L, Cao Y, Ma Y, Zhai J. Association Between Hyperandrogenism and Adverse Pregnancy Outcomes in Patients With Different Polycystic Ovary Syndrome Phenotypes Undergoing In Vitro Fertilization/Intracytoplasmic Sperm Injection: A Systematic Review and Meta-Analysis. *Gynecol Endocrinol* (2021) 37(8):694–701. doi: 10.1080/09513590.2021.1897096
46. Yang W, Yang R, Yang S, Li J, Tu B, Gao C, et al. Infertile Polycystic Ovary Syndrome Patients Undergoing *In Vitro* Fertilization With the Gonadotropin-Releasing Hormone-Antagonist Protocol: Role of

Hyperandrogenism. *Gynecol Endocrinol* (2018) 34(8):715–8. doi: 10.1080/09513590.2018.1431773
47. Sun YF, Zhang J, Xu YM, Cao ZY, Wang YZ, Hao GM, et al. High BMI and Insulin Resistance Are Risk Factors for Spontaneous Abortion in Patients With Polycystic Ovary Syndrome Undergoing Assisted Reproductive Treatment: A Systematic Review and Meta-Analysis. *Front Endocrinol* (2020) 11:592495. doi: 10.3389/fendo.2020.592495
48. Haas J, Baum M, Meridor K, Hershko-Klement A, Elizur S, Hourvitz A, et al. Is Severe OHSS Associated With Adverse Pregnancy Outcomes? Evidence From a Case-Control Study. *Reprod BioMed Online* (2014) 29(2):216–21. doi: 10.1016/j.rbmo.2014.04.015
49. Wang F, Dai W, Yang XH, Guo YH, Sun YP. Analyses of Optimal Body Mass Index for Infertile Patients With Either Polycystic or non-Polycystic Ovary Syndrome During Assisted Reproductive Treatment in China. *Sci Rep* (2016) 6:34538. doi: 10.1038/srep34538
50. Mourad S, Brown J, Farquhar C. Interventions for the Prevention of OHSS in ART Cycles: An Overview of Cochrane Reviews. *Cochrane Database Syst Rev* (2017) 1(1):Cd012103. doi: 10.1002/14651858.CD012103.pub2
51. Palomba S, Falbo A, Russo T, Tolino A, Orio F, Zullo F. Pregnancy in Women With Polycystic Ovary Syndrome: The Effect of Different Phenotypes and Features on Obstetric and Neonatal Outcomes. *Fertil Steril* (2010) 94(5):1805–11. doi: 10.1016/j.fertnstert.2009.10.043

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Endocrinology

ORIGINAL RESEARCH
published: 06 June 2022
doi: 10.3389/fendo.2022.878853

# Adverse Effects of Polycystic Ovarian Syndrome on Pregnancy Outcomes in Women With Frozen-Thawed Embryo Transfer: Propensity Score-Matched Study

*Zhexin Ni[1†], Shanshan Mei[2†], Siting You[3†], Yi Lin[4], Wen Cheng[1], Ling Zhou[1], Yanping Kuang[5*] and Chaoqin Yu[1,2*]*

*[1] Department of Traditional Chinese Medicine Gynecology, Changhai Hospital, Shanghai, China, [2] Department of Traditional Chinese Medicine Gynecology, Shanghai University of Traditional Chinese Medicine, Shanghai, China, [3] Central Laboratory, Changhai Hospital, Second Military Medical University, Shanghai, China, [4] Department of Traditional Chinese Medicine, Naval Medical University, Shanghai, China, [5] Department of Assisted Reproductive Medicine, Shanghai Ninth People's Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China*

OPEN ACCESS

**Edited by:**
*Yiping Shen,*
*Harvard Medical School,*
*United States*

**Reviewed by:**
*Mohd Ashraf Ganie,*
*Sher-I-Kashmir Institute of Medical Sciences, India*
*Fan Jin,*
*Zhejiang University, China*

***Correspondence:**
*Chaoqin Yu*
*chqyu81@163.com*
*Yanping Kuang*
*kuangyp@sh9hospital.org*

*[†]These authors have contributed equally to this work*

**Specialty section:**
*This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology*

**Received:** *18 February 2022*
**Accepted:** *26 April 2022*
**Published:** *06 June 2022*

**Citation:**
*Ni Z, Mei S, You S, Lin Y, Cheng W, Zhou L, Kuang Y and Yu C (2022) Adverse Effects of Polycystic Ovarian Syndrome on Pregnancy Outcomes in Women With Frozen-Thawed Embryo Transfer: Propensity Score-Matched Study. Front. Endocrinol. 13:878853. doi: 10.3389/fendo.2022.878853*

**Purpose:** This work aimed to evaluate the adverse effect of polycystic ovary syndrome (PCOS) on pregnancy outcomes of singletons after vitrification in women with frozen-thawed embryo transfer (FET).

**Methods:** Patients with/without PCOS who underwent FET from January 2013 and December 2018 were included. Propensity score matching (PSM) was used to reduce the influence of bias. Logistic regression was applied to identify the risk factors of adverse pregnancy outcomes of singletons in women with PCOS.

**Result:** After PSM, the PCOS group had shorter gestational age (P<0.001) and lower newborn birth weight than the non-PCOS group (P=0.045). Compared with the non-PCOS group, the PCOS group had an increased risk of gestational diabetes mellitus (GDM) and pregnancy-induced hypertension (PIH) (P<0.001), placenta and membrane abnormality (P<0.001), stillbirth (P<0.001), neonatal complication (P=0.014), and miscarriage rate (P<0.001). Neonatal complication was associated with parity (adjusted OR=1.202, 95% CI=1.002–1.443, P=0.048) and basal P level (adjusted OR=1.211, 95% CI=1.021–1.436, P=0.028). According to multivariable logistic regression analysis, the miscarriage rate was related to parity (adjusted OR=1.201, 95% CI=1.057–1.166, P=0.005) and basal E2 (adjusted OR=1.002, 95% CI=1.000–1.004, P=0.019) and P levels on the day of embryo transfer (adjusted OR=0.971, 95% CI=0.957–0.985, P<0.001).

**Conclusions:** Compared with non-PCOS women, women with PCOS have a higher risk of GDM and PIH, and neonatal complications and therefore require additional care during pregnancy and parturition.

**Keywords: PCOS, FET, propensity score-matched study, pregnancy, hormone**

# INTRODUCTION

PCOS is the most common hormonal disorder in women of reproductive age and accounts for 80% of women with anovulatory infertility (1, 2). Upon exclusion of other specific diagnoses, PCOS is characterized by a combination of androgen excess and ovarian dysfunction. In women with PCOS, ineffectual aromatization to estrogens and increased androgen level lead to a low FSH level, resulting in androgen excess and estrogen shortage (3). Abnormal hormone levels in women with PCOS may lead to poor pregnancy outcomes (4, 5). As one of the clinical manifestations of PCOS, obesity is also related to poor pregnancy outcomes in women with PCOS (6) and is usually associated with high circulating insulin levels, which in turn increase ovarian androgen production (7). The abnormal hormones prevent women of reproductive age from ovulation, the main cause of infertility caused by PCOS.

*In vitro* fertilization (IVF) has been widely used in infertility treatment for decades. Compared with spontaneous pregnancies, IVF pregnancies in women with PCOS are associated with increased risks of adverse pregnancy outcomes (8, 9). During the treatment, the serum levels of hormones change dramatically. A previous research showed that frozen-thawed embryo transfer (FET) is associated with preeclampsia in infertile women with PCOS (10), and other studies found that sex hormones such as testosterone and FSH are associated with pregnancy outcomes in IVF treatment (11, 12). However, the adverse effect of PCOS on pregnancy outcomes in women with FET has never been clarified.

Therefore, this study aims to evaluate the adverse effect of PCOS on pregnancy outcomes in frozen embryo transfer cycles. The risk predictors of adverse pregnancy outcomes in women with PCOS were also identified. Propensity score matching (PSM) was performed to exclude the confounding bias between women with and without PCOS. A logistic regression model was established for the precise evaluation of the risks factors of adverse pregnancy outcomes in women with PCOS. The findings would serve as a basis for the implementation of management measures during routine clinical practice.

# METHODS AND MATERIALS

## Study Population and Characteristics

Patients who underwent IVF/intracytoplasmic sperm injection (ICSI) with FET were identified, and the women who conceived singleton were selected. PCOS was diagnosed by two gynecologists on the basis of the 2003 Rotterdam criteria for the patients who met two of the following criteria (1): oligo- or anovulation, (2) clinical and/or biochemical signs of hyperandrogenism, and (3) polycystic ovaries and exclusion of other related etiologies (13). Patients with the following diseases were excluded: (1) congenital uterine malformations; (2) severe cerebrovascular, liver, heart, or kidney diseases; (3) gynecological cancers; (4) metabolic or endocrine disorders (diabetes or pituitary adenomas); and (5) autoimmune diseases, such as systemic lupus erythematosus or scleroderma. We only included the patients with fallopian tubal blockage or infertility couples due to paternal factors in the non-PCOS group to minimize the influence of confounding factors. Cases with missing information of cycles, embryo, and clinical pregnancy data were also excluded. The following clinical data were collected: maternal age, maternal BMI, paternal age, duration of infertility, parity, cycle method, sperm origin, fertilization method, scoring for cleavage-stage and blastocyst-stage embryo, number of embryo transferred, basal LH, basal E2, basal FSH, basal P, basal testosterone (T), serum levels of E2 and P on the day of embryo transfer, neonatal gender, gestational age, birth weight, ectopic pregnancy, GDM and PIH, placenta and membrane abnormality, birth defect, stillbirth, complications of labor and delivery, neonatal complication, and miscarriage. This study was approved by the Research Ethics Committee of the Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, and Changhai Hospital, Naval Medical University and complied with the Declaration of Helsinki. Owing to the retrospective design of this study, informed consent was waived.

## Laboratory Protocols and Embryo Assessment

Blood samples for basal LH, E2, FSH, P, and T assessment were collected in the morning after overnight fasting, preferably on days 2–5 of the menstrual cycle of women with regular menstruation or during withdrawal bleeding in women with amenorrhea. Hormonal assays were performed with UniCel DxI 800 Access Immunoassay System (Beckman Coulter, Brea, CA) using commercial kits following the manufacturers' protocol. E2 and P on the day of FET were measured to assess their effect on pregnancy outcomes. Conventional IVF or ICSI was conducted depending on semen parameters and previous fertilization histories. All embryos were incubated in oil under 5% $O_2$, 6% $CO_2$, and 37°C. Vitrification and thawing were performed as previously described (14). Embryo quality was assessed during cleavage (day 3) or blastocyst stage (day 5/6). The scoring system for cleavage-stage embryos was based on the Istanbul consensus workshop (15). The blastocysts was grouped into four categories based on inner cell mass and trophectoderm scoring (15–17).

## Statistical Analysis

All analyses were conducted in SPSS (version 26.0 IBM Corporation, Armonk, NY) and R software (http://www.r-project.org/). Student's t-test was used to compare continuous variables, which were expressed as mean ± standard deviation (SD). Chi-squared test or Fisher's exact test was employed to analyze categorical data. Kruskal–Wallis test was applied to assess the relationship between PCOS and embryo quality. Propensity score was used to match the following independent variables to balance the influence of confounding factors: maternal age, paternal age, sperm origin method, stage at cryopreservation, and number of embryos transferred. A 1:1 match between the PCOS group and the non-PCOS group was obtained by nearest neighbor matching with a caliper width of 0.01 and without replacement. PSM was

performed with R software using the MatchIt package. Univariable and multivariable logistic regression analyses were carried out to identify risk factors such as maternal BMI, parity, cycle method, fertilization method, basal LH, basal E2, basal FSH, basal P, total T, and E2 and P on the day of embryo transfer in adverse pregnancy outcomes. Hazard ratio (HR) and 95% confidence interval (95% CI) were calculated to assess the relationship between serum levels and pregnancy outcomes. All P values were two-tailed, and <0.05 was considered statistically significant.

# RESULTS

A retrospective cohort including 1384 patients with PCOS and 14606 patients without PCOS was enrolled at the Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine between January 2013 and December 2018. After PSM, 1376 patients with PCOS and 1376 patients without PCOS were included in the PCOS and non-PCOS groups, respectively. In the non-PCOS group, 1337 patients with fallopian tubal blockage and 39 patients with paternal factors. The baseline characteristics, PCOS-associated characteristics, and pregnancy outcomes of singletons conceived after FET before and after PSM were evaluated.

## Patient Baseline Characteristics

Before PSM, the baseline characteristics of the two groups were unevenly distributed. The maternal and paternal ages of the PCOS group were younger than those in the non-PCOS group (maternal age: 30.54 ± 3.51 *vs.* 32.61 ± 4.45, P<0.001; paternal age: 32.42 ± 4.32 *vs.* 34.53 ± 5.53, P<0.001). The spouses of the non-PCOS group were more inclined to testicular sperm extraction than ejaculation (P<0.001). After matching, the baseline characteristics of the two groups were not different except for the scoring of blastocyst-stage embryo. The maternal age, paternal age, parity, cycle method, sperm origin method, fertilization method, stage at cryopreservation, and number of embryos transferred were similar in the two groups as shown in **Table 1**.

## PCOS-Associated Characteristics

After matching, the maternal BMI differed between the PCOS and non-PCOS groups (23.58 ± 6.08 *vs.* 21.53 ± 5.03, P<0.001). Non-PCOS patients used natural cycles, and those with PCOS employed artificial cycles (P<0.001). The parity of the non-PCOS group was higher than that of the PCOS group (P=0.038). The number of patients in the non-PCOS group who underwent IVF+ICSI or ICSI was significantly higher than that in the PCOS group (P<0.001).

For sex hormonal panels, the serum levels of basal LH, basal FSH, and total T were higher in the PCOS group than in the non-PCOS group (basal LH: 5.42 ± 3.92 *vs.* 4.62 ± 3.42, P<0.001; total T: 0.04 ± 0.13 *vs.* 0.03 ± 0.12, P=0.048). The serum concentration of basal E2 and basal FSH were lower in the PCOS group than in the non-PCOS group (basal E2: 39.51 ± 51.17 *vs.* 60.08 ± 62.69, P<0.001; basal FSH: 5.07 ± 1.41 *vs.* 6.02 ± 3.78, P<0.001). Other hormonal indicators such as basal P and serum E2 and P levels on the day of embryo transfer were not different between the two groups. All the PCOS-associated characteristics are summarized in **Table 2**.

**TABLE 1** | The baseline characteristics of singletons conceived after frozen/thawed embryo transfer (FET).

| Characteristic | Before PSM (N=15990) | | | After PSM (N=2752) | | |
|---|---|---|---|---|---|---|
| | PCOS group (N=1384) | Non-PCOS group (N=14606) | P | PCOS group (N=1376) | Non-PCOS group (N=1376) | P |
| Maternal Age (y, mean ± SD) | 30.54 ± 3.51 | 32.61 ± 4.45 | <0.001 | 30.58 ± 3.49 | 30.63 ± 3.60 | 0.711 |
| Paternal age (y, mean ± SD) | 32.42 ± 4.32 | 34.53 ± 5.53 | <0.001 | 32.45 ± 4.31 | 32.35 ± 4.21 | 0.543 |
| Sperm origin | | | <0.001 | | | 0.052 |
| Ejaculation | 1374 (99.3%) | 14290 (97.8%) | | 1373 (99.8%) | 1366 (99.3%) | |
| Testicular sperm extraction | 10 (0.7%) | 316 (2.2%) | | 3 (0.2%) | 10 (0.7%) | |
| Stage at cryopreservation | | | 0.991 | | | 0.152 |
| Cleavage stage | 1138 (82.2%) | 12008 (82.2%) | | 1133 (82.3%) | 1161 (84.4%) | |
| Blastocyst | 246 (17.8%) | 2598 (17.8%) | | 243 (17.7%) | 215 (15.6%) | |
| Scoring for cleavage-stage embryo* | | | 0.447 | | | 0.134 |
| Good | 151 (13.3%) | 1776 (14.8%) | | 151 (13.3%) | 136 (11.7%) | |
| Fair | 830 (72.9%) | 8240 (68.6%) | | 825 (72.8%) | 845 (72.8%) | |
| Poor | 157 (13.8%) | 1992 (16.6%) | | 157 (13.9%) | 180 (15.5%) | |
| Scoring for blastocyst-stage embryo# | | | 0.097 | | | 0.023 |
| Excellent | 18 (7.3%) | 178 (6.9%) | | 18 (7.4%) | 10 (4.7%) | |
| Good | 39 (15.9%) | 377 (14.5%) | | 39 (16.1%) | 26 (12.1%) | |
| Average | 138 (56.1%) | 1353 (52.1%) | | 136 (55.9%) | 119 (55.3%) | |
| poor | 51 (20.7%) | 690 (26.6%) | | 50 (20.6%) | 60 (27.9%) | |
| No. of embryos transferred | | | 0.056 | | | 0.188 |
| 1 | 250 (18.1%) | 2953 (20.2%) | | 248 (18.0%) | 222 (16.1%) | |
| ≥2 | 1134 (81.9%) | 11653 (79.8%) | | 1128 (78%) | 1154 (83.9%) | |

**Scoring system for cleavage-stage embryos was based on the Istanbul consensus workshop; #Blastocyst was grouped into four categories based on inner cell mass and trophectoderm scoring (15–17).*

## Pregnancy Outcomes

Comparison of pregnancy outcomes between the two groups is shown in **Table 3**. The PCOS group displayed a shorter gestational age (P<0.001) and a slighter birth weight (P<0.001) than the non-PCOS group. For adverse pregnancy outcomes, the PCOS group had higher probabilities of GDM and PIH, (P<0.001), placenta and membrane abnormality (P<0.001), stillbirth (P<0.001), neonatal complications (P<0.001), and miscarriage (P<0.001) than the non-PCOS group. However, the PCOS group showed a lower probability of ectopic pregnancy(P<0.001) and birth defects (P<0.001) than the non-PCOS group.

## Relationship Between Serum Sex Hormones and Adverse Pregnancy Outcomes

Logistic regression was performed to clarify the association between serum hormones and adverse pregnancy outcomes in patients with PCOS who underwent IVF. **Table 4** shows that after the adjustment for basal LH, basal E2, basal FSH, basal P, E2, and P levels on the day of embryo transfer, no factor was associated with GDM and PIH. The parity in PCOS group was associated with neonatal complications (including congenital anomalies, urogenital defects, jaundice, and pneumonia) (adjusted OR=1.202, 95% CI=1.002–1.443, P=0.048). The serum levels of basal P (adjusted OR: 1.211, 95% CI= 1.021–1.436, P=0.028) was associated with neonatal complications in women with PCOS (**Table 5**). Multivariate logistic regression revealed that parity (adjusted OR=1.201, 95% CI=1.057–1.366, P=0.005) and basal E2 (OR: 1.003, 95% CI=1.001–1.006, P<0.018) were related to an increased risk of miscarriage. Meanwhile, a high serum P level on the day of embryo transfer was associated with a significantly decreased risk of miscarriage in the PCOS group (adjusted OR=0.971, 95% CI=0.957–0.985, P<0.001) (**Table 6**).

# DISCUSSION

## Main Findings

To our knowledge, this is the first propensity score-matched study to identify the risk factors of adverse pregnancy outcomes in women with PCOS who received IVF treatment. PCOS is a common endocrine disorder that affects about 6%–10% of women and is characterized by hypotestosteronemia, hyperinsulinemia, high LH/FSH ratio, and obesity (18). One of its most prevalent consequences is oligo/amenorrhea anovulation, leading to infertility problems in women of childbearing age (19). In the past decades, IVF has increased the pregnancy rate in women with PCOS compared with that in non-PCOS controls. However, an increased risk of developing unfavorable pregnancy complications was reported in women with PCOS (20). In one study, PCOS was considered as an independent risk factor associated with late miscarriage in women treated with IVF (21). Although the negative effects of PCOS on the assisted reproductive outcome of IVF/ICSI in women with PCOS have been widely investigated, the association between serum sex hormone levels and pregnancy outcome remains unclear. Thus, the influence of each hormone on pregnancy outcomes in women with PCOS cannot be accurately elucidated. In the present work, we compared the serum sex hormone levels and pregnancy outcomes between

**TABLE 2** | The PCOS-associated characteristics of singletons conceived after frozen/thawed embryo transfer (FET).

| Characteristics | Before PSM (N=15990) | | | After PSM (N=2752) | | |
|---|---|---|---|---|---|---|
| | PCOS group (N=1384) | Non-PCOS group (N=14606) | P | PCOS group (N=1376) | Non-PCOS group (N=1376) | P |
| Maternal BMI (Kg/m$^2$, mean ± SD) | 23.44 ± 3.74 | 21.65 ± 4.24 | <0.001 | 23.58 ± 6.08 | 21.53 ± 5.03 | <0.001 |
| Duration of infertility (y, mean ± SD) | 3.46 ± 2.55 | 3.41 ± 2.98 | 0.553 | 3.44 ± 2.49 | 3.37 ± 2.48 | 0.490 |
| Parity | | | <0.001 | | | 0.038 |
| first | 917 (66.3%) | 7618 (52.2%) | | 912 (66.3%) | 860 (62.5%) | |
| High order | 467 (33.7%) | 6988 (47.8%) | | 464 (33.7%) | 516 (37.5%) | |
| Cycle method | | | <0.001 | | | <0.001 |
| Natural cycle | 45 (3.3%) | 4021 (27.5%) | | 45 (3.3%) | 381 (27.7%) | |
| Artificial cycle | 1339 (96.7%) | 10585 (72.5%) | | 1331 (96.7%) | 995 (72.3%) | |
| Fertilization method | | | <0.001 | | | <0.001 |
| IVF | 817 (59.0%) | 9092 (62.2%) | | 811 (58.9%) | 806 (58.6%) | |
| ICSI | 298 (21.5%) | 4109 (28.1%) | | 298 (21.7%) | 391 (28.4%) | |
| IVF+ICSI | 269 (19.4%) | 1405 (9.6%) | | 267 (19.4%) | 179 (13.0%) | |
| Basal LH (mIU/mL, mean ± SD) | 5.43 ± 3.93 | 5.48 ± 6.35 | 0.740 | 5.42 ± 3.92 | 4.62 ± 3.42 | <0.001 |
| Basal E2 (pg/mL, mean ± SD) | 39.48 ± 51.04 | 62.52 ± 76.09 | <0.001 | 39.51 ± 51.17 | 60.08 ± 62.69 | <0.001 |
| Basal FSH (U/L, mean ± SD) | 5.08 ± 1.41 | 6.09 ± 3.55 | <0.001 | 5.07 ± 1.41 | 6.02 ± 3.78 | <0.001 |
| Basal P (ng/mL, mean ± SD) | 0.26 ± 0.31 | 0.31 ± 0.71 | 0.007 | 0.26 ± 0.31 | 0.29 ± 0.49 | 0.092 |
| Total T (ng/mL, mean ± SD) | 0.511 ± 0.29 | 0.26 ± 0.16 | <0.001 | 0.04 ± 0.13 | 0.03 ± 0.12 | 0.048 |
| Indicators on the day of embryo transferred | | | | | | |
| E2 (pg/mL, mean ± SD) | 260.56 ± 479.39 | 218.03 ± 343.61 | <0.001 | 146.97 ± 330.72 | 170.38 ± 389.62 | 0.089 |
| P (ng/mL, mean ± SD) | 9.95 ± 11.95 | 9.74 ± 11.93 | 0.535 | 9.97 ± 11.96 | 10.19 ± 12.05 | 0.629 |

**TABLE 3 |** The pregnancy outcomes of live born singletons in women with and without PCOS after FET.

| Outcomes | Before PSM | | | After PSM | | |
|---|---|---|---|---|---|---|
| | PCOS group (N=1387) | Non-PCOS group (N=14606) | P | PCOS group (N=1376) | Non-PCOS group (N=1376) | P |
| Gender* | | | 0.741 | | | <0.001 |
| Male | 641 (46.2%) | 6630 (45.4%) | | 635 (46.1%) | 577 (41.9%) | |
| Female | 586 (42.2%) | 5940 (40.7%) | | 585 (42.5%) | 731 (58.1%) | |
| Gestational age (wk) | | | <0.001 | | | <0.001 |
| <32 | 186 (13.4%) | 2075 (14.2%) | | 182 (13.2%) | 71 (5.2%) | |
| 32–36 | 124 (8.9%) | 769 (5.3%) | | 123 (8.9%) | 39 (2.8%) | |
| ≥37 | 1077 (77.6%) | 11762 (80.5%) | | 1071 (77.8%) | 1266 (92.0%) | |
| Birth weight | | | 0.097 | | | <0.001 |
| <2500g | 63 (4.5%) | 504 (3.5%) | | 63 (4.6%) | 29 (2.1%) | |
| 2500-4000g | 1092 (78.7%) | 11261 (77.1%) | | 1085 (78.9%) | 1164 (84.6%) | |
| >4000g | 75 (5.4%) | 865 (5.9%) | | 75 (5.5%) | 121 (8.8%) | |
| Ectopic pregnancy | 3 (0.2%) | 63 (0.4%) | 0.215 | 3 (0.2%) | 6 (0.4%) | <0.001 |
| GDM and PIH** | 206 (14.8%) | 1302 (8.9%) | <0.001 | 205 (14.9%) | 96 (7.0%) | <0.001 |
| Placenta and membrane abnormality# | 24 (1.7%) | 311 (2.1%) | 0.265 | 24 (1.7%) | 20 (1.5%) | <0.001 |
| Birth defects | 16 (1.2%) | 223 (1.5%) | 0.243 | 16 (1.2%) | 27 (2.0%) | <0.001 |
| Stillbirth | 2 (0.1%) | 5 (0.03%) | 0.122 | 2 (0.1%) | 0 | <0.001 |
| Complication of labor and delivery$ | 9 (0.6%) | 94 (0.6%) | 0.961 | 9 (0.6%) | 9 (0.6%) | 1.000 |
| Neonatal complication | 55 (4.0%) | 373 (2.6%) | 0.003 | 55 (4.0%) | 41 (3.0%) | <0.001 |
| Miscarriage | 157 (11.3%) | 1973 (13.5%) | 0.022 | 153 (11.1%) | 62 (4.5%) | <0.001 |

**Excluding excludes ectopic pregnancy and miscarriage cases; **GDM and PIH, Gestational Diabetes Mellitus and Pregnancy-Induced Hypertension; #Including placenta previa and premature rupture of membrane. $Including postpartum hemorrhage, amniotic fluid embolism, rupture of uterus and dysfunction of cord.*

women with and without PCOS after PSM. The results showed that the patients with PCOS had a higher maternal BMI, higher basal LH, and higher testosterone levels than the non-PCOS women. Our study also retrospectively analyzed the pregnancy outcomes of women with and without PCOS. Our primary finding is that patients with PCOS who received IVF had a short gestational age and an increased risk of GDM and PIH, neonatal complications, and miscarriage. Furthermore, parity and basal P were associated with neonatal complications. In women with PCOS who received IVF treatment, parity and high basal E2 level had a negative effect on miscarriage. Meanwhile, P level on the day of embryo transfer was a protective factor on miscarriage.

In our study, women with PCOS who underwent IVF had worse pregnancy outcomes compared with non-PCOS women. Among the patients with PCOS, those who underwent IVF had an increased risk of small gestational age and low birth weight compared with those who got pregnant spontaneously. Mostinckx et al. (22) compared the obstetric and neonatal outcome of *in vitro* maturation and controlled ovarian stimulation for assisted reproductive technology in patients with PCOS and found that women with PCOS who received *in*

**TABLE 4 |** Risk factors of GDM and PIH in women with PCOS.

| | Before matching | | | | After matching | | | |
|---|---|---|---|---|---|---|---|---|
| | Crude OR | P | Adjusted OR | P | Crude OR | P | Adjusted OR | P |
| Maternal BMI (Kg/m$^2$) | 1.000 (1.000-1.000) | 0.836 | 1.000 (1.000-1.000) | 0.850 | 1.011 (1.001-1.020) | 0.027 | 1.009 (0.999-1.020) | 0.072 |
| Parity (high order *vs.* first) | 1.037 (0.995-1.081) | 0.088 | 1.047 (1.003-1.092) | 0.037 | 1.053 (0.937-1.184) | 0.386 | 1.043 (0.925-1.176) | 0.489 |
| Cycle method (artificial *vs.* natural) | 1.278 (1.125-1.453) | <0.001 | 1.104 (0.959-1.271) | 0.168 | 0.835 (0.577-1.288) | 0.338 | 0.726 (0.489-1.076) | 0.111 |
| Fertilization method (ICSI *vs.* IVF) | 0.965 (0.858-1.085) | 0.550 | 0.973 (0.863-1.096) | 0.652 | 0.850 (0.640-1.130) | 0.263 | 0.869 (0.651-1.159) | 0.338 |
| Fertilization method (IVF+ICSI *vs.* IVF) | 1.097 (0.935-1.287) | 0.258 | 1.118 (0.950-1.316) | 0.180 | 0.928 (0.680-1.265) | 0.635 | 0.956 (0.697-1.312) | 0.071 |
| Basal LH (mIU/mL) | 0.982 (0.971-0.992) | 0.001 | 0.999 (0.987-1.010) | 0.842 | 0.985 (0.959-1.012) | 0.278 | 0.995 (0.943-1.050) | 0.855 |
| Basal E2 (pg/mL) | 0.997 (0.996-0.998) | <0.001 | 0.998 (0.997-0.999) | 0.001 | 0.998 (0.995-1.001) | 0.259 | 0.998 (0.994-1.002) | 0.277 |
| Basal FSH (U/L) | 0.994 (0.978-1.010) | 0.456 | 0.991 (0.974-1.009) | 0.332 | 0.994 (0.946-1.046) | 0.826 | 0.995 (0.943-1.050) | 0.855 |
| Basal P (ng/mL) | 0.932 (0.818-1.042) | 0.196 | 0.961 (0.865-1.067) | 0.453 | 0.627 (0.284-1.383) | 0.247 | 0.682 (0.307-1.518) | 0.349 |
| Total T (ng/mL) | 1.000 (0.764-1.310) | 0.998 | 1.007 (0.750-1.350) | 0.965 | 0.820 (0.439-1.531) | 0.532 | 0.860 (0.463-1.596) | 0.632 |
| Indicators on the day of embryo transfer | | | | | | | | |
| E2 (pg/mL) | 1.000 (1.000-1.000) | 0.058 | 1.000 (1.000-1.000) | 0.065 | 1.000 (1.000-1.000) | 0.405 | 0.998 (0.994-1.002) | 0.277 |
| P (ng/mL) | 1.006 (1.002-1.010) | 0.003 | 1.006 (1.002-1.010) | 0.006 | 1.008 (0.999-1.016) | 0.096 | 1.008 (0.999-1.017) | 0.079 |

**TABLE 5 |** Risk factors of neonatal complications in women with PCOS.

| | Before matching | | | | After matching | | | |
|---|---|---|---|---|---|---|---|---|
| | Crude OR | P | Adjusted OR | P | Crude OR | P | Adjusted OR | P |
| Maternal BMI (Kg/m$^2$) | 1.000 (1.000-1.000) | 0.943 | 1.000 (1.000-1.000) | 0.943 | 1.000 (0.965-1.036) | 0.996 | 0.999 (0.964-1.036) | 0.966 |
| Parity (high order *vs.* first) | 1.036 (0.959-1.120) | 0.372 | 1.054 (0.974-1.041) | 0.193 | 1.194 (0.999-1.427) | 0.051 | 1.202 (1.002-1.443) | 0.048 |
| Cycle method (artificial *vs.* natural) | 1.466 (1.143-1.880) | 0.003 | 1.379 (1.048-1.814) | 0.022 | 1.612 (0.787-3.303) | 0.192 | 1.585 (0.731-3.438) | 0.244 |
| Fertilization method (ICSI *vs.* IVF) | 1.197 (0.969-1.478) | 0.095 | 1.213 (0.979-1.504) | 0.078 | 1.045 (0.646-1.689) | 0.858 | 1.086 (0.665-1.774) | 0.742 |
| Fertilization method (IVF+ICSI *vs.* IVF) | 1.045 (0.762-1.433) | 0.786 | 1.085 (0.786-1.497) | 0.620 | 1.090 (0.632-1.880) | 0.757 | 1.192 (0.683-2.079) | 0.537 |
| Basal LH (mIU/mL) | 0.989 (0.971-1.007) | 0.243 | 0.997 (0.975-1.019) | 0.789 | 0.990 (0.948-1.035) | 0.660 | 1.001 (0.952-1.052) | 0.967 |
| Basal E2 (pg/mL) | 0.998 (0.997-1.000) | 0.060 | 0.999 (0.997-1.001) | 0.604 | 1.000 (1.000-1.001) | 0.060 | 0.999 (0.984-1.004) | 0.722 |
| Basal FSH (U/L) | 1.012 (0.987-1.036) | 0.352 | 1.011 (0.984-1.038) | 0.431 | 1.011 (0.952-1.074) | 0.714 | 1.010 (0.948-1.077) | 0.749 |
| Basal P (ng/mL) | 1.016 (0.895-1.153) | 0.807 | 1.027 (0.910-1.160) | 0.664 | 1.174 (0.998-1.381) | 0.053 | 1.211 (1.021-1.436) | 0.028 |
| Total T (ng/mL) | 1.083 (0.718-1.634) | 0.704 | 1.104 (0.716-1.701) | 0.655 | 1.749 (0.730-4.188) | 0.210 | 1.754 (0.737-4.171) | 0.204 |
| Indicators on the day of embryo transfer | | | | | | | | |
| E2 (pg/mL) | 1.000 (1.000-1.000) | 0.454 | 1.000 (1.000-1.000) | 0.471 | 1.000 (1.000-1.001) | 0.060 | 1.000 (1.000-1.001) | 0.080 |
| P (ng/mL) | 1.004 (0.996-1.012) | 0.316 | 1.003 (0.996-1.011) | 0.387 | 1.000 (0.983-1.016) | 0.957 | 0.999 (0.983-1.016) | 0.927 |

*vitro* maturation had a shorter gestational age than those who received controlled ovarian stimulation. By contrast, Liu and colleagues (20) reported that the gestational age was not significantly different between women with PCOS and without PCOS; however, the authors did not adjust for confounding factors when they analyzed the pregnancy outcomes of a cohort of 666 women with PCOS and 7012 controls using chi-square test. This phenomenon may explain the inconsistency between their results and ours. The newborn birthweight in the PCOS group was slightly lower than that in the non-PCOS group possibly due to the relatively short gestational age of women with PCOS. Our result was in line with the conclusion of previous studies. Sunkara et al. (23) found that women with PCOS had an increased risk of low birthweight; however, no specific risk factor was identified in their study. In another case-control research, Sir-Petermann and colleagues (24) observed that women with PCOS who had spontaneous pregnancy exhibited a higher prevalence of small gestational age and low birth weight compared with the control group. Han et al. (25) found that the incidence of low gestational age infants was higher in women with PCOS than in women with infertility due to tubal factors (25). A previous animal experiment showed that placenta insufficiency and prenatal exposure to sex steroids may be the reason for the small gestational age (26).

Obesity is one of the typical clinical characteristics of PCOS and may result from insulin resistance. Univariable logistic regression analysis found a positive correlation between maternal BMI and GDM and PIH in patients with PCOS who received IVF treatment. GDM and PIH are the two most common pregnancy complications. A high risk of GDM and PIH is frequently reported in women with PCOS (27, 28). GDM and PIH may result from some of the clinical characteristics of PCOS, such as polycystic ovaries, insulin resistance, and hyperandrogenism (29, 30). Early evidence suggested that obesity increases the risk of type 2 diabetes during pregnancy in patients with PCOS, and this finding was in agreement with our result (31). However, multivariable analysis indicated that maternal BMI was not associated with GDM and PIH. Kouhkan

**TABLE 6 |** Risk factors of miscarriage in women with PCOS.

| | Before matching | | | | After matching | | | |
|---|---|---|---|---|---|---|---|---|
| | Crude OR | P | Adjusted OR | P | Crude OR | P | Adjusted OR | P |
| Maternal BMI (Kg/m$^2$) | 1.000 (1.000-1.000) | 0.793 | 1.000 (1.000-1.000) | 0.835 | 1.006 (0.991-1.022) | 0.418 | 1.005 (0.987-1.023) | 0.590 |
| Parity (high order *vs.* first) | 1.169 (1.134-1.205) | <0.001 | 1.181 (1.145-1.218) | <0.001 | 1.176 (1.039-1.330) | 0.010 | 1.201 (1.057-1.366) | 0.005 |
| Cycle method (artificial *vs.* natural) | 0.999 (0.906-1.102) | 0.985 | 1.031 (0.927-1.147) | 0.569 | 0.855 (0.544-1.343) | 0.496 | 0.918 (0.577-1.461) | 0.718 |
| Fertilization method (ICSI *vs.* IVF) | 0.967 (0.877-1.066) | 0.496 | 1.045 (0.946-1.154) | 0.390 | 0.942 (0.675-1.313) | 0.723 | 1.008 (0.720-1.411) | 0.963 |
| Fertilization method (IVF+ICSI *vs.* IVF) | 0.972 (0.841-1.123) | 0.699 | 1.100 (0.949-1.274) | 0.207 | 1.056 (0.740-1.507) | 0.765 | 1.131 (0.785-1.629) | 0.509 |
| Basal LH (mIU/mL) | 0.998 (0.991-1.005) | 0.633 | 0.995 (0.987-1.003) | 0.238 | 0.978 (0.946-1.012) | 0.209 | 0.970 (0.932-1.011) | 0.148 |
| Basal E2 (pg/mL) | 1.000 (1.000-1.000) | 0.294 | 1.000 (1.000-1.001) | 0.155 | 1.001 (1.001-1.003) | 0.052 | 1.002 (1.000-1.004) | 0.019 |
| Basal FSH (U/L) | 1.011 (1.000-1.022) | 0.052 | 1.010 (0.999-1.023) | 0.087 | 0.948 (0.874-1.027) | 0.190 | 0.970 (0.893-1.053) | 0.467 |
| Basal P (ng/mL) | 1.036 (0.987-1.087) | 0.149 | 1.035 (0.987-1.086) | 0.155 | 0.815 (0.382-1.742) | 0.598 | 0.829 (0.472-1.454) | 0.513 |
| Total T (ng/mL) | 0.302 (0.209-0.436) | <0.001 | 0.346 (0.239-0.502) | <0.001 | 0.898 (0.446-1.809) | 0.763 | 0.952 (0.476-1.904) | 0.888 |
| Indicators on the day of embryo transfer | | | | | | | | |
| E2 (pg/mL) | 0.999 (0.999-1.000) | <0.001 | 0.999 (0.999-1.000) | <0.001 | 0.999 (0.999-1.000) | 0.076 | 1.000 (0.999-1.000) | 0.085 |
| P (ng/mL) | 0.986 (0.982-0.990) | <0.001 | 0.986 (0.982-0.990) | <0.001 | 0.970 (0.956-0.984) | <0.001 | 0.971 (0.957-0.985) | <0.001 |

and colleagues (27) identified PCOS history as a risk factor for GDM in women with treated PCOS. Regardless of spontaneous pregnancy or IVF, patients with PCOS have a significantly increased risk of GDM during pregnancy due to the metabolic disorders caused by insulin resistance that lead to obesity and diabetes (32, 33). For PIH, women with PCOS undergoing IVF treatment have a higher risk of PIH than non-PCOS women. In the current study, logistic regression showed that maternal BMI was positively related to the risk of PIH in women with PCOS who underwent IVF. This finding was in agreement with Joham et al. (34), who analyzed the data from the Australian Longitudinal Study on Women's Health and found that the incidence of hypertension was higher among obese women with PCOS than among lean women with PCOS. Palomba et al. (35) conducted a meta-analysis and found that women with PCOS had a three- to fourfold increased risk of PIH when BMI was not adjusted. According to the present multivariable logistic regression analysis, although obesity was not a risk factor of GDM and PIH, the patients with obesity must be given additional attention to prevent the occurrence of adverse pregnancy events.

In our study, we found that the risk of stillbirth was higher in the PCOS group than in the non-PCOS group. Our result is consistent with the findings of Valgeirsdottir et al. (36), who conducted a nationwide register-base cohort study and found that PCOS was associated with stillbirth. Two meta-analyses reported that PCOS is related to the risk of perinatal death, including stillbirth and early neonatal death (37, 38). However, the association between PCOS and stillbirth remains unclear.

The offspring of women with PCOS are at an increased risk of cardiovascular and other anomalies. Battaglia et al. (39) found that daughters born to patients with PCOS had an increased cardiovascular risk. Doherty and colleagues (40) demonstrated that the offspring of women with PCOS were at a high risk of postnatal hospitalizations, congenital anomalies, and urogenital defects. Our results suggested that the risk of neonatal complications in the offspring of women with PCOS is higher than that in the offspring of women with other infertile causes.

After PSM, the clinical miscarriage rate in women with PCOS was significantly different compared with that in non-PCOS women. However, we demonstrated that parity and serum basal E2 level were related to an increased risk of miscarriage, and a high serum P level on the day of embryo transfer was associated with a significantly decreased risk of miscarriage in the PCOS group. Progesterone is an essential hormone for maintaining early pregnancy and decidualized endometrium, relaxing uterine smooth muscle, improving uterine blood supply, and regulating immunity in early pregnancy (41). Supplement progesterone is widely used for miscarriage prevention and treatment of assisted reproductive technology. Miscarriage occurs in 15%–20% of all clinical pregnancies, and most of the causes remain unknown (42). In our article, we demonstrated that the serum progesterone level on the day of embryo transfer was a protective factor for miscarriage, and the estrogen level was related to an increased risk of miscarriage. Women with PCOS have a high risk rate of miscarriage; the increase in estradiol caused by endocrine disorders works synergistically with factors such as hyperinsulinemia, free insulin-like growth factors, and obesity, thus ultimately leading to infertility and miscarriage (43). Nevertheless, only a few studies focused on the prognosis of serum estrogen and progesterone levels on the day of embryo transfer for the pregnancy outcomes in patients with PCOS; large-scale studies are needed in the future. Compared with the non-PCOS group, the PCOS group had greater percentage of first-time pregnancy. However, parity was a risk factor of neonatal complication and miscarriage in our study. To date, limited studies have focused on parity and the occurrence of adverse pregnancy events in patients with PCOS. Additional research is needed to clarify this relationship in the future.

## Strengths and Weaknesses

The strengths of our study are as follows. First is the large cohort that focused on women with PCOS who underwent FET, which was adjusted the confounding factors by propensity score matching. Maternal age, paternal age, sperm origin, stage at cryopreservation, and number of embryos transferred were matched to reduce the potential bias when analyzing the risk factors between women with and without PCOS. Second, we conducted a comprehensive analysis to identify the risk factors of adverse pregnancy outcomes in women with PCOS. The sex hormone levels in women with PCOS greatly differ from those in women without PCOS. In this work, we analyzed the association of the sex hormones in patients with PCOS with GDM and PIH, neonatal complications, and miscarriage. We revealed the relationship between factors, such as maternal BMI, duration of infertility, parity, cycle method, fertilization method, basal serum of FSH, P, and E2, and adverse pregnancy events in patients with PCOS. However, this study also has several limitations. First, selection and information bias cannot be ruled out because of the retrospective design of this study. Second, this research is a single-center study, and the cases are all Chinese national. Hence, the relationship between the sex hormones and pregnancy outcomes must be validated in multiple centers. Third, PCOS patients are treated with drugs while undergoing IVF treatment, which can affect the serum level of sex hormones. Such drug treatment will lead to the development of patients with PCOS on the bright side, leading us to underestimate the impact of PCOS disease on IVF results. Fourth, owing to the observational nature of this study, the positive results are just a statistical correlation. Hence, causality cannot be established. Further investigation is needed to determine the underlying mechanism of the relationship between sex hormones and pregnancy outcomes in women with PCOS who received IVF treatment.

# CONCLUSION

This study evaluated the incidence of adverse pregnancy outcomes in women with and without PCOS. The results showed that women with PCOS have a higher risk of GDM

and PIH, stillbirth, neonatal complications, and miscarriage than non-PCOS women. Neonatal complication was associated with parity and basal P level. Parity and basal estradiol were an increased risk factor of miscarriage, and serum progesterone level on the day of embryo transfer was a protective factor in women with PCOS.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

CY and YK conceived the study. ZN, SM, and SY extracted and analyzed the data. YL and WC contributed to the acquisition, analysis, and interpretation of the data. ZN and SM wrote the first draft of the manuscript which was revised by SY and LZ. This study was supervised by CY and YK. All authors contributed to the article and approved the submitted version.

## FUNDING

This work was supported by National Natural Science Foundation of China (82004408), Leading Project of Traditional Chinese Medicine of Shanghai Science and Technology Committee (19401930200) and Shanghai Sailing Program (20YF1448600).

## ACKNOWLEDGMENTS

The authors thank the nurses and laboratory staff of the Department of Assisted Reproduction for their contribution to this work. Moreover, the authors thank the infertile couples who participated in this study.

## REFERENCES

1. ESHRE/ASRM. Consensus on Infertility Treatment Related to Polycystic Ovary Syndrome. *Hum Reprod* (2008) 23(3):462–77. doi: 10.1093/humrep/dem426
2. Balen AH, Morley LC, Misso M, Franks S, Legro RS, Wijeyaratne CN, et al. The Management of Anovulatory Infertility in Women With Polycystic Ovary Syndrome: An Analysis of the Evidence to Support the Development of Global WHO Guidance. *Hum Reprod Update* (2016) 22(6):687–708. doi: 10.1093/humupd/dmw025
3. Turhan NO, Artini PG, D'Ambrogio G, Droghini F, Battaglia C, Genazzani AD, et al. A Comparative Study of Three Ovulation Induction Protocols in Polycystic Ovarian Disease Patients in an *In Vitro* Fertilization/Embryo Transfer Program. *J Assist Reprod Genet* (1993) 10(1):15–20. doi: 10.1007/bf01204435
4. Singh N, Mishra N, Dogra Y. Do Basal Luteinizing Hormone and Luteinizing Hormone/Follicle-Stimulating Hormone Ratio Have Significance in Prognosticating the Outcome of *In Vitro* Fertilization Cycles in Polycystic Ovary Syndrome? *J Hum Reprod Sci* (2021) 14(1):21–7. doi: 10.4103/jhrs.JHRS_96_20
5. Jayasena CN, Franks S. The Management of Patients With Polycystic Ovary Syndrome. *Nat Rev Endocrinol* (2014) 10(10):624–36. doi: 10.1038/nrendo.2014.102
6. Cena H, Chiovato L, Nappi RE. Obesity, Polycystic Ovary Syndrome, and Infertility: A New Avenue for GLP-1 Receptor Agonists. *J Clin Endocrinol Metab* (2020) 105(8):e2695-709. doi: 10.1210/clinem/dgaa285
7. Rachoń D, Teede H. Ovarian Function and Obesity–Interrelationship, Impact on Women's Reproductive Lifespan and Treatment Options. *Mol Cell Endocrinol* (2010) 316(2):172–9. doi: 10.1016/j.mce.2009.09.026
8. Bosdou JK, Anagnostis P, Goulis DG, Lainas GT, Tarlatzis BC, Grimbizis GF, et al. Risk of Gestational Diabetes Mellitus in Women Achieving Singleton Pregnancy Spontaneously or After ART: A Systematic Review and Meta-Analysis. *Hum Reprod Update* (2020) 26(4):514–44. doi: 10.1093/humupd/dmaa011
9. Tang T, Glanville J, Hayden CJ, White D, Barth JH, Balen AH. Combined Lifestyle Modification and Metformin in Obese Patients With Polycystic Ovary Syndrome. A Randomized, Placebo-Controlled, Double-Blind Multicentre Study. *Hum Reprod* (2006) 21(1):80–9. doi: 10.1093/humrep/dei311
10. Chen ZJ, Shi Y, Sun Y, Zhang B, Liang X, Cao Y, et al. Fresh Versus Frozen Embryos for Infertility in the Polycystic Ovary Syndrome. *N Engl J Med* (2016) 375(6):523–33. doi: 10.1056/NEJMoa1513873
11. Qin Y, Zhao Z, Sun M, Geng L, Che L, Chen ZJ. Association of Basal Serum Testosterone Levels With Ovarian Response and *In Vitro* Fertilization Outcome. *Reprod Biol Endocrinol* (2011) 9:9. doi: 10.1186/1477-7827-9-9
12. Xu H, Deng K, Luo Q, Chen J, Zhang X, Wang X, et al. High Serum FSH Is Associated With Brown Oocyte Formation and a Lower Pregnacy Rate in Human IVF Parctice. *Cell Physiol Biochem* (2016) 39(2):677–84. doi: 10.1159/000445658
13. Group REASPCW. Revised 2003 Consensus on Diagnostic Criteria and Long-Term Health Risks Related to Polycystic Ovary Syndrome. *Fertil Steril* (2004) 81(1):19–25. doi: 10.1016/j.fertnstert.2003.10.004
14. Kuwayama M, Vajta G, Kato O, Leibo SP. Highly Efficient Vitrification Method for Cryopreservation of Human Oocytes. *Reprod BioMed Online* (2005) 11(3):300–8. doi: 10.1016/s1472-6483(10)60837-1
15. Gardner DK, Surrey E, Minjarez D, Leitz A, Stevens J, Schoolcraft WB. Single Blastocyst Transfer: A Prospective Randomized Trial. *Fertil Steril* (2004) 81(3):551–5. doi: 10.1016/j.fertnstert.2003.07.023
16. Tannus S, Cohen Y, Henderson S, Al Ma'mari N, Shavit T, Son WY, et al. Fresh Transfer of Day 5 Slow-Growing Embryos Versus Deferred Transfer of Vitrified, Fully Expanded Day 6 Blastocysts: Which Is the Optimal Approach? *Hum Reprod* (2019) 34(1):44–51. doi: 10.1093/humrep/dey351
17. Zhao YY, Yu Y, Zhang XW. Overall Blastocyst Quality, Trophectoderm Grade, and Inner Cell Mass Grade Predict Pregnancy Outcome in Euploid Blastocyst Transfer Cycles. *Chin Med J (Engl)* (2018) 131(11):1261–7. doi: 10.4103/0366-6999.232808
18. Azziz R. Polycystic Ovary Syndrome. *Obstet Gynecol* (2018) 132(2):321–36. doi: 10.1097/aog.0000000000002698
19. Ożegowska K, Plewa S, Mantaj U, Pawelczyk L, Matysiak J. Serum Metabolomics in PCOS Women With Different Body Mass Index. *J Clin Med* (2021) 10(13):1–17. doi: 10.3390/jcm10132811
20. Liu S, Mo M, Xiao S, Li L, Hu X, Hong L, et al. Pregnancy Outcomes of Women With Polycystic Ovary Syndrome for the First *In Vitro* Fertilization Treatment: A Retrospective Cohort Study With 7678 Patients. *Front Endocrinol (Lausanne)* (2020) 11:575337. doi: 10.3389/fendo.2020.575337
21. Yang AM, Xu X, Han Y, Wei JJ, Hao GM, Cui N, et al. Risk Factors for Different Types of Pregnancy Losses: Analysis of 15,210 Pregnancies After Embryo Transfer. *Front Endocrinol (Lausanne)* (2021) 12:683236. doi: 10.3389/fendo.2021.683236
22. Mostinckx L, Segers I, Belva F, Buyl R, Santos-Ribeiro S, Blockeel C, et al. Obstetric and Neonatal Outcome of ART in Patients With Polycystic Ovary Syndrome: IVM of Oocytes Versus Controlled Ovarian Stimulation. *Hum Reprod* (2019) 34(8):1595–607. doi: 10.1093/humrep/dez086

23. Sunkara SK, La Marca A, Seed PT, Khalaf Y. Increased Risk of Preterm Birth and Low Birthweight With Very High Number of Oocytes Following IVF: An Analysis of 65 868 Singleton Live Birth Outcomes. *Hum Reprod* (2015) 30 (6):1473–80. doi: 10.1093/humrep/dev076
24. Sir-Petermann T, Hitchsfeld C, Maliqueo M, Codner E, Echiburú B, Gazitúa R, et al. Birth Weight in Offspring of Mothers With Polycystic Ovarian Syndrome. *Hum Reprod* (2005) 20(8):2122–6. doi: 10.1093/humrep/dei009
25. Han AR, Kim HO, Cha SW, Park CW, Kim JY, Yang KM, et al. Adverse Pregnancy Outcomes With Assisted Reproductive Technology in non-Obese Women With Polycystic Ovary Syndrome: A Case-Control Study. *Clin Exp Reprod Med* (2011) 38(2):103–8. doi: 10.5653/cerm.2011.38.2.103
26. Manikkam M, Crespi EJ, Doop DD, Herkimer C, Lee JS, Yu S, et al. Fetal Programming: Prenatal Testosterone Excess Leads to Fetal Growth Retardation and Postnatal Catch-Up Growth in Sheep. *Endocrinology* (2004) 145(2):790–8. doi: 10.1210/en.2003-0478
27. Kouhkan A, Khamseh ME, Moini A, Pirjani R, Valojerdi AE, Arabipoor A, et al. Predictive Factors of Gestational Diabetes in Pregnancies Following Assisted Reproductive Technology: A Nested Case-Control Study. *Arch Gynecol Obstet* (2018) 298(1):199–206. doi: 10.1007/s00404-018-4772-y
28. Bahri Khomami M, Earnest A, Loxton D, Teede HJ, Joham AE. Predictors of Hypertensive Disorders in Pregnancy in Women With and Without Polycystic Ovary Syndrome: The Australian Longitudinal Study of Women's Health. *Clin Endocrinol (Oxf)* (2021) 95(2):323–31. doi: 10.1111/cen.14451
29. Cenk Sayin N, Gücer F, Balkanli-Kaplan P, Ali Yüce M, Yardim T. Insulin Resistance and Lipid Profile in Women With Polycystic Appearing Ovaries: Implications With Regard to Polycystic Ovary Syndrome. *Gynecol Endocrinol* (2003) 17(5):387–96. doi: 10.1080/09513590312331290278
30. Qin JZ, Pang LH, Li MJ, Fan XJ, Huang RD, Chen HY. Obstetric Complications in Women With Polycystic Ovary Syndrome: A Systematic Review and Meta-Analysis. *Reprod Biol Endocrinol* (2013) 11:56. doi: 10.1186/1477-7827-11-56
31. Ollila MM, West S, Keinänen-Kiukaanniemi S, Jokelainen J, Auvinen J, Puukka K, et al. Overweight and Obese But Not Normal Weight Women With PCOS are at Increased Risk of Type 2 Diabetes Mellitus-a Prospective, Population-Based Cohort Study. *Hum Reprod* (2017) 32(2):423–31. doi: 10.1093/humrep/dew329
32. Boudreaux MY, Talbott EO, Kip KE, Brooks MM, Witchel SF. Risk of T2DM and Impaired Fasting Glucose Among PCOS Subjects: Results of an 8-Year Follow-Up. *Curr Diabetes Rep* (2006) 6(1):77–83. doi: 10.1007/s11892-006-0056-1
33. Gambineri A, Patton L, Altieri P, Pagotto U, Pizzi C, Manzoli L, et al. Polycystic Ovary Syndrome Is a Risk Factor for Type 2 Diabetes: Results From a Long-Term Prospective Study. *Diabetes* (2012) 61(9):2369–74. doi: 10.2337/db11-1360
34. Joham AE, Kakoly NS, Teede HJ, Earnest A. Incidence and Predictors of Hypertension in a Cohort of Australian Women With and Without Polycystic Ovary Syndrome. *J Clin Endocrinol Metab* (2021) 106(6):1585–93. doi: 10.1210/clinem/dgab134
35. Palomba S, de Wilde MA, Falbo A, Koster MP, La Sala GB, Fauser BC. Pregnancy Complications in Women With Polycystic Ovary Syndrome. *Hum Reprod Update* (2015) 21(5):575–92. doi: 10.1093/humupd/dmv029
36. Valgeirsdottir H, Kunovac Kallak T, Sundström Poromaa I, Jonsson M, Roos N, Lindström L, et al. Polycystic Ovary Syndrome and Risk of Stillbirth: A Nationwide Register-Based Study. *BJOG* (2021) 128(13):2073–82. doi: 10.1111/1471-0528.16890
37. Yu HF, Chen HS, Rao DP, Gong J. Association Between Polycystic Ovary Syndrome and the Risk of Pregnancy Complications: A PRISMA-Compliant Systematic Review and Meta-Analysis. *Med (Baltimore)* (2016) 95(51):e4863. doi: 10.1097/md.0000000000004863
38. Boomsma CM, Eijkemans MJ, Hughes EG, Visser GH, Fauser BC, Macklon NS. A Meta-Analysis of Pregnancy Outcomes in Women With Polycystic Ovary Syndrome. *Hum Reprod Update* (2006) 12(6):673–83. doi: 10.1093/humupd/dml036
39. Battaglia C, Mancini F, Cianciosi A, Busacchi P, Persico N, Paradisi R, et al. Cardiovascular Risk in Normal Weight, Eumenorrheic, Nonhirsute Daughters of Patients With Polycystic Ovary Syndrome: A Pilot Study. *Fertil Steril* (2009) 92(1):240–9. doi: 10.1016/j.fertnstert.2008.05.018
40. Doherty DA, Newnham JP, Bower C, Hart R. Implications of Polycystic Ovary Syndrome for Pregnancy and for the Health of Offspring. *Obstet Gynecol* (2015) 125(6):1397–406. doi: 10.1097/aog.0000000000000852
41. Wahabi HA, Fayed AA, Esmaeil SA, Bahkali KH. Progestogen for Treating Threatened Miscarriage. *Cochrane Database Syst Rev* (2018) 8(8):Cd005943. doi: 10.1002/14651858.CD005943.pub5
42. Cohain JS, Buxbaum RE, Mankuta D. Spontaneous First Trimester Miscarriage Rates Per Woman Among Parous Women With 1 or More Pregnancies of 24 Weeks or More. *BMC Pregnancy Childbirth* (2017) 17 (1):437. doi: 10.1186/s12884-017-1620-1
43. Giudice LC. Endometrium in PCOS: Implantation and Predisposition to Endocrine CA. *Best Pract Res Clin Endocrinol Metab* (2006) 20(2):235–44. doi: 10.1016/j.beem.2006.03.005


**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Endocrinology

ORIGINAL RESEARCH
published: 10 June 2022
doi: 10.3389/fendo.2022.905263

# Follicular-Phase GnRH Agonist Protocol Is Another Choice for Polycystic Ovary Syndrome Patients With Lower LH/FSH and Lower AMH Levels Without Increasing Severe OHSS Risk

*Rui Gao[1,2,3†], Xin Liao[2,4†], Wanrong Huang[5], Rujun Zeng[1,2], Lang Qin[1,2*] and Peng Bai[6*]*

*[1] The Reproductive Medical Center, Department of Obstetrics and Gynecology, West China Second University Hospital, Sichuan University, Chengdu, China, [2] Key Laboratory of Birth Defects and Related Diseases of Women and Children of the Ministry of Education, West China Second University Hospital, Sichuan University, Chengdu, China, [3] West China School of Medicine, Sichuan University, Chengdu, China, [4] Department of the Central Operating Unit, West China Second University Hospital, Sichuan University/West China School of Nursing, Sichuan University, Chengdu, China, [5] Department of Dermatology, The First Hospital of China Medical University, Shenyang, China, [6] Department of Forensic Genetics, West China School of Basic Medical Sciences & Forensic Medicine, Sichuan University, Chengdu, China*

**Purpose:** To explore another choice for a controlled ovarian stimulation (COS) protocol that does not increase severe ovarian hyperstimulation syndrome (OHSS) risk among polycystic ovarian syndrome (PCOS) patients with specific clinical features.

**Methods:** A retrospective study was performed. Two hundred and fifty-nine participants were divided into two groups, group 1 (fixed GnRH antagonist protocol, n = 295) and group 2 (follicular-phase GnRH agonist protocol, n = 69) according to COS protocols. The basic characteristics and laboratory indicators between these two groups were compared. The severe OHSS rate and clinical pregnancy rate were selected as indicators to evaluate the risks and benefits of the two COS protocols. Subgroup analyses for the severe OHSS rate and clinical pregnancy rate were performed based on baseline luteinizing hormone/follicle-stimulating hormone (bLH/FSH) and anti-Mullerian hormone (AMH) levels.

**Results:** The severe OHSS rate was statistically higher in group 2 than in group 1 (11.6% vs. 3.7%, p = 0.008), but the biochemical pregnancy rate and clinical pregnancy rate showed no statistical difference between the groups (71.9% vs. 60.3% and 62.5% vs. 54.3%). In the higher bLH/FSH subgroup (≥1.33) and the higher serum AMH level subgroup (>3.4 ng/ml), severe OHSS incidence was statistically higher in group 2 compared to group 1, but this incidence was lower in the bLH/FSH subgroup (<1.33) and the subgroup with lower serum AMH levels (≤3.4 ng/ml); a difference in severe OHSS risk was not observed. There was no statistical difference between the two groups regarding clinical pregnancy rate in any subgroup.

OPEN ACCESS

***Edited by:***
*Yimin Zhu,*
*Zhejiang University, China*

***Reviewed by:***
*Junhao Yan,*
*Shandong University, China*
*Diao Feiyang,*
*Nanjing Medical University, China*
*Jiming Chen,*
*Changzhou No. 2 People's Hospital, China*
*Hong Cai,*
*Hangzhou First People's Hospital, China*

***Correspondence:**
*Peng Bai*
*535946167@qq.com*
*Lang Qin*
*cacier@163.com*

*†These authors have contributed equally to this work*

***Specialty section:***
*This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology*

***Received:*** *26 March 2022*
***Accepted:*** *09 May 2022*
***Published:*** *10 June 2022*

***Citation:***
*Gao R, Liao X, Huang W, Zeng R, Qin L and Bai P (2022) Follicular-Phase GnRH Agonist Protocol Is Another Choice for Polycystic Ovary Syndrome Patients With Lower LH/FSH and Lower AMH Levels Without Increasing Severe OHSS Risk. Front. Endocrinol. 13:905263. doi: 10.3389/fendo.2022.905263*

**Conclusion:** The limited evidence from this study indicates that in PCOS patients with lower bLH/FSH levels (<1.33) and lower serum AMH levels (≤3.4 ng/ml), a follicular-phase GnRH agonist protocol may be another choice that does not increase the risk of severe OHSS.

**Keywords: controlled ovarian stimulation (COS), polycystic ovarian syndrome, *in vitro* fertilization, intracytoplasmic sperm injection (ICSI), ovarian hyperstimulation syndrome (OHSS), clinical pregnancy rate (CPR)**

## INTRODUCTION

Polycystic ovary syndrome (PCOS) is one of the most common and heterogeneous endocrinological problems among women of reproductive age (1). PCOS affects more than 10% of women around the world (1), and the prevalence of PCOS in Chinese women aged 19–45 years old is 5.6% (2). The clinical manifestations of PCOS are complicated and individualized. According to the Rotterdam criteria, a diagnosis of PCOS must include at least two of the following three features: oligo-anovulation, clinical and/or biochemical hyperandrogenism, and polycystic ovaries on ultrasonography, excluding other endocrinopathies (3). Thus, irregular menstruation, amenorrhea, hairiness, acne, a higher baseline antral follicle count (AFC; the presence of 12 or more follicles in each ovary measuring 2–9 mm in diameter), or increased ovarian volume (>10 ml) are always observed in PCOS patients. In addition, other clinical manifestations include a higher baseline luteinizing hormone/follicle-stimulating hormone ratio (bLH/FSH) (4, 5), higher levels of anti–Müllerian hormone (AMH) (5, 6), insulin resistance (7), and obesity, all of which are common among PCOS patients despite not being included in the diagnosis criteria for PCOS.

About 80% of anovulation infertility is caused by PCOS according to the European Society of Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM) (3). For PCOS patients suffering infertility, assisted reproductive technology (ART) is an important strategy for achieving pregnancy (8). Controlled ovarian stimulation (COS) is an essential step for *in vitro* fertilization (IVF) and/or intracytoplasmic sperm injection (ICSI), as its purpose is to induce the maturation of more oocytes, thus maximizing the chance of successful pregnancy. Ovarian hyperstimulation syndrome (OHSS) is an uncommon but serious complication associated with COS, and evidence from well-designed cohort or case–control studies indicates that PCOS is a risk factor of OHSS; it may also be related to higher ovarian reserve markers such as elevated AMH levels, peak estradiol levels, and higher AFC (9). Previous studies have demonstrated that a gonadotropin-releasing hormone (GnRH) antagonist protocol can reduce the risk of OHSS (10–12). Thus, the GnRH antagonist protocol is recommended as a first-line COS protocol for PCOS patients, according to the World Health Organization (WHO) (13). No high-quality, randomized, controlled trials providing direct evidence for COS selection in PCOS patients have, however, been performed, and some studies also suggest that the GnRH antagonist protocol might result in lower cumulative live birth rates (cLBRs) (14) and lower ongoing pregnancy rates (12) compared to a GnRH agonist protocol used in fresh embryo transfer cycles. Failure in IVF/ICSI is a psychological stressor for women, and patients with poor IVF/ICSI outcomes are more likely to suffer anxiety and depression (15, 16). Improving clinical pregnancy rates, ongoing pregnancy rates, and LBR without increasing the incidence of OHSS is a key point of IVF/ICSI, as this balances the risk of OHSS against clinical benefit.

Some demographic characteristics and biomarkers were found to have predictive value for severe OHSS, and among them, convenient peripheral blood biomarkers associated with ovarian reserves such as AMH and bLH/FSH have been widely used to predict the risk of OHSS. For example, a previous study showed that an AMH value over 3.4 ng/ml is an independent risk factor for severe OHSS, but for PCOS patients with AMH values less than or equal to 3.4 ng/ml, severe OHSS is acceptable (9). Thus, whether the GnRH antagonist protocol leads to lower severe OHSS rates compared to the GnRH agonist protocol in PCOS patients with lower AMH and lower bLH/FSH levels is a point of confusion that has not been investigated by previous studies. In addition, the possibility of clinical pregnancy is worth considering, especially for patients with a history of recurrent IVF/ICSI failure. Clinical pregnancy outcomes directly depend on COS protocols in IVF/ICSI and fresh embryo transfer cycles, but for PCOS patients, the available COS protocols are restricted because of severe OHSS risks. The effect of follicular-phase GnRH agonist protocols on clinical pregnancy outcomes in PCOS patients with lower AMH and lower bLH/FSH levels have not been explored. Therefore, in this retrospective study, the severe OHSS rate and clinical pregnancy rate of PCOS patients receiving a fixed GnRH antagonist protocol and a follicular-phase GnRH agonist protocol—based on subgroups classified by AMH and bLH/FSH levels—were evaluated, thereby providing more evidence for the selection of individualized COS protocols for PCOS patients.

## MATERIALS AND METHODS

### Participants and Study Design

A retrospective cohort study was performed by analyzing the records of PCOS patients who had entered their first cycle undergoing standard IVF/ICSI due to infertility at the Reproductive Center of West China Second University Hospital, Sichuan University, Chengdu, China, from June 2020 to June 2021. Infertility is defined as a disease of the reproductive

system characterized by the failure to achieve a clinical pregnancy after 12 months or more of regular, unprotected sexual intercourse. Only patients who underwent a fixed GnRH antagonist protocol and follicular GnRH agonist protocol were included. Patients were divided into two groups according to their COS protocols. PCOS among the patients was diagnosed according to the Rotterdam criteria. Patients with a history of genital tuberculosis, a history of recurrent pregnancy loss, a history of ovarian surgery, evidence for hyperprolactinoma or hypothyroidism, and other associated infertility factors, except for tubal factors, were excluded. Patients receiving preimplantation genetic diagnoses were also excluded. Ultimately, 295 patients received the fixed GnRH antagonist protocol (group 1), and 69 patients received the follicular phase GnRH agonist protocol (group 2). All these patients met the inclusion criteria and were included in this study. This study was performed according to the World Medical Association Declaration of Helsinki and was approved by the Ethics Committee of West China Second University Hospital.

## Controlled Ovarian Stimulation Protocol

In the fixed GnRH antagonist protocol, patients were started on intramuscular injections of recombinant FSH (injection Gonal-f, Merck Serono Specialties, Italy) from the second day of their menstrual cycle. The starting dose was between 150 and 225 IU/day. A GnRH antagonist (injection Cetrotide acetate, Aeterna Zentaris, Canada) was administered at a dose of 0.25 mg/day from the sixth day of the menstrual cycle until the ovulation trigger day. In the follicular phase GnRH agonist protocol, transvaginal ultrasounds (TVS) were performed on the second or third day of the menstrual cycle to assess AFC, and the intramuscular injection of the GnRH agonist (Triptorelin; Ferring, Kiel, Germany) was commenced at 3.75 mg if no follicle reached 10 mm in diameter. Twenty-eight days later, a serum sex hormone assessment and TVS were performed, and pituitary downregulation was completed if the patients met the following criteria: E2 ≤ 30 pg/ml, LH ≤ 5 IU/l, ovarian follicle diameter ≤ 5 mm, and endometrial thickness (ET) ≤ 5 mm. The intramuscular injection of recombinant FSH was provided (150 to 225 IU/day) for ovarian stimulation based on patient age, BMI, and AFC. The dose of recombinant FSH was adjusted every 3–4 days according to the ovarian response until the trigger day. These cycles were cancelled in patients with no follicle greater than 10 mm in diameter after 10 days of recombinant FSH stimulation. For all these protocols, when at least two follicles reached 18 mm or three follicles reached 17 mm, the final stage of triggering ovulation was performed using human chorionic gonadotropin (hCG; Lizhu Pharmaceutical Trading, Zhuhai, China) at doses from 8,000 to 10,000 IU. For women at a high risk for OHSS, low doses of hCG (4,000 to 5,000 IU) were used to trigger ovulation. Serum sex hormone levels and ET were measured on the trigger day.

## Oocyte Retrieval and Embryo Transfer

Oocyte retrieval was performed 36–38 h after triggering ovulation by transvaginal-guided, single-lumen needle aspiration. Oocyte assessment was performed by the standard morphology criteria (17), and a nuclear maturity assessment was performed for cases subjected to ICSI. Conventional IVF or ICSI was performed depending on semen parameters and previous fertilization history. Ultrasound guidance was used for all embryo transfers and was performed 3 or 5 days after oocyte retrieval. Embryo transfer was cancelled for severe OHSS or high OHSS risk (peak E2 > 4,500 pg/ml) patients, and all embryos were frozen. Severe OHSS was diagnosed *via* clinical evidence of ascites and/or hydrothorax, severe dyspnea, oliguria/anuria, intractable nausea/vomiting, severe hemoconcentration (Hct > 55%), a white cell count over $25 \times 10^9$/l, creatinine clearance (CrCl) < 50 ml/min, creatinine (Cr) > 1.6 mg/dl, sodium (Na+) <135 mEq/l, potassium (K+) > 5 mEq/l, and elevated liver enzymes according to the ARSM guidelines for OHSS (9).

All patients were given luteal phase support *via* the intramuscular injection of progesterone at 100 mg/day. Two weeks after embryo transfer, pregnancy was assessed by serum β-hCG assay (where serum β-hCG > 50 IU/l was regarded as biochemical pregnancy) and confirmed *via* TVS after another 2 weeks (the presence of the gestational sac was regarded as clinical pregnancy). The measurement of E2, progesterone, LH, FSH, and βhCG was done by fully automated electrochemiluminescence technology (Roche Cobas e411 analyzer, Hitachi, Tokyo, Japan).

## Information Collection

Basic patient information on age; height; weight; BMI; the duration of infertility, the type of infertility (primary infertility or secondary infertility); basic serum FSH, LH, E2, and P levels (*via* detection on the second day of the menstrual cycle); serum levels of AMH and T; and AFC were collected from hospital records. PCOM was defined as the presence of 12 or more follicles in each ovary measuring 2–9 mm in diameter and/or increased ovarian volume (>10 ml). Information associated with COS was also collected, including information on the rate of OHSS, the Gn starting dose, the total number of Gn days, the total Gn dose, and serum E2, P, and LH levels on the day of hCG, ET on the day of hCG, the number of follicles greater than or equal to 14 mm in diameter, the number of oocytes retrieved, the number of MII oocytes, the 2PN number, the fertilization rate, the cleavage rate, the number of available D3 embryos, the number of high-quality D3 embryos, the number of available blastocysts, the number of high-quality blastocysts, and the rate of cancelled cycles. The MII oocyte rate was defined as the percentage of MII oocytes among the total number of oocytes retrieved. The cleavage rate was defined as the percentage of cleavage embryos among the total number of zygotes. The high-quality D3 embryo rate was defined as the percentage of high-quality D3 embryos among the total number of normal cleavage embryos. The available D3 embryo rate was defined as the percentage of available D3 embryos among the total number of cleavage embryos. The high-quality blastocyst rate was defined as the percentage of high-quality blastocysts among the total number of cleavage embryos used in blastocyst cultures. Moreover, the available blastocyst rate was defined as the percentage of available blastocysts among the number of cleavage embryos used in blastocyst cultures. Embryo grading

was done by standard morphology assessment according to modified Veecks' scoring (18). Blastocysts graded as AA, AB+, AB−, B+A, B−A, B+B+, and BB were classified as high-quality embryos. Fertilization was defined as the presence of pronuclei 16–18 h after insemination/injection. Primary outcome measures consisted of the severe OHSS rate and the clinical pregnancy rate (defined as the presence of a gestational sac per ET).

## Statistical Analysis

Multiple imputation by chained equations was used for missing values in the covariates of the adjusted statistical models. This was performed in the study population and was conducted separately for each group. Continuous variables were expressed as medians (interquartile range) and were compared *via* the Mann–Whitney U test. Categorical measurements are presented as a percentage, and these rates were compared *via* the chi-squared test; if numbers were less than 5 in at least 20% of the cells, Fisher's exact test was performed. The study population was stratified by bLH/FSH (<1.33 and ≥1.33) and AMH (≤3.4 and >3.4 ng/ml), both of which were reported as risk factors for severe OHSS in previous studies. In addition, cutoff values were also made according to previous studies or guidelines (4, 9, 19–21), and if events in two or more subgroups were zero, the related indicator was excluded from subgroup analysis. The differences between two groups were presented as odds ratios (OR) and 95% confidence intervals (CI). P values less than 0.05 were considered statistically significant, but P values less than 0.1 were also noted. Statistical analyses were performed by SPSS, version 25.0 (SPSS Inc., Chicago, IL, UPL).

# RESULTS

## Basic Participant Information

Basic information on the participants in this study is shown in **Table 1**. There were no statistical differences in age, height, weight, BMI, the duration of infertility, the type of infertility, T serum levels, serum sex hormone levels on days 2–3 of the menstrual cycle, and the fertilization type between the two groups. The prevalence of irregular menstruation was, however, higher in group 1 than in group 2 (90.2% vs. 81.2%, p = 0.035), and the levels of serum AMH were significantly higher in group 1 than in group 2 [10.42 (6.24–145.23) vs. 6.03 (3.61–10.20), p < 0.001]. Also, the prevalence of PCOM was higher in group 1 than in group 2 (68.1% vs. 46.4, p = 0.001).

## Laboratory and Clinical Outcomes Between the Two Groups

The clinical outcomes for the PCOS patients in group 1 and group 2 are shown in **Table 2**. The durations of Gn stimulation were shorter in group 1 than in group 2 [10 (9–11) vs. 12 (10–13), p < 0.001], and the total Gn doses in group 1 were also lower [1700.0 (1375.0–2175.0) vs. 2175.0 (1725.0–2800.0), p < 0.001]. On the trigger day, serum E2, P, and LH levels were higher in group 1 than in group 2 [p = 0.001, 0.014 and p < 0.001], but there was no statistical difference in single ET and the number of follicles greater than or equal to 14 mm in diameter. The numbers of oocytes retrieved were similar in both groups [14 (10–20) vs. 13 (10–20), p = 0.71], and there was no statistical difference in the ICSI fertilization rate, cleaved oocyte rate, blastocyst formation rate, available blastocyst rate, high-quality

**TABLE 1** | Basic information on the patients in this study.

| | Total (n = 364) | Group 1 (n = 295) | Group 2 (n = 69) | P-value |
|---|---|---|---|---|
| **Age (year)** | 29 (27–32) | 29 (27–32) | 29 (27–32) | 0.73 |
| **Height (cm)** | 159.0 (155.0–162.0) | 160.0 (155.0–162.0) | 158.0 (1555.0–162.0) | 0.44 |
| **Weight (kg)** | 57.0 (52.0–63.0) | 57.0 (52.0–63.0) | 56.5 (53.5–62.25) | 0.58 |
| **BMI (kg/m$^2$)** | 22.82 (20.58–24.94) | 22.46 (20.50–24.97) | 22.89 (20.83–24.30) | 0.48 |
| **Duration of infertility (year)** | 3 (2–5) | 3 (2–5) | 3 (2–5) | 0.96 |
| **Type of infertility [n(%)]** | | | | |
| Primary | 247 (67.9) | 198 (67.1) | 49 (71.0) | 0.53 |
| Secondary | 117 (32.1) | 97 (32.9) | 20 (29.0) | |
| **Irregular menstruation [n(%)]** | | | | |
| Yes | 322 (88.5) | 266 (90.2) | 56 (81.2) | 0.035 |
| No | 42 (11.5) | 29 (9.8) | 13 (18.8) | |
| **Serum levels of T (ng/mL)** | 0.40 (0.31–0.55) | 0.41 (0.32–0.55) | 0.37 (0.28–0.53) | 0.15 |
| **Serum levels of AMH (ng/mL)** | 9.75 (5.40–14.76) | 10.42 (6.24–14.52) | 6.03 (3.61–10.20) | < 0.001 |
| **Serum levels on days 2–3 of the menstrual cycle** | | | | |
| E2 (pg/mL) | 39.4 (30.3–51.0) | 39.5 (30.7–48.6) | 39.2 (26.1–57.5) | 0.90 |
| P (ng/mL) | 0.44 (0.31–0.58) | 0.44 (0.32–0.55) | 0.47 (0.27–0.59) | 1.00 |
| LH (IU/L) | 7.5 (4.9–12.1) | 7.9 (5.0–11.8) | 6.6 (3.7–12.9) | 0.19 |
| FSH (IU/L) | 6.4 (5.5–7.7) | 6.4 (5.6–7.7) | 6.3 (5.0–7.9) | 0.37 |
| LH/FSH | 1.26 (0.79–1.88) | 1.24 (0.81–1.88) | 1.30 (0.58–1.88) | 0.47 |
| **PCOM [n(%)]** | 233 (64.0) | 201 (68.1) | 32 (46.4) | 0.001 |
| **Fertilization type [n(%)]** | | | | |
| IVF | 311 (85.4) | 247 (83.7) | 64 (92.8) | 0.12 |
| ICSI | 12 (3.3) | 10 (3.4) | 2 (2.9) | |
| IVF+ICSI | 41 (11.3) | 38 (12.9) | 3 (4.3) | |

*T, free androgen; AMH, anti-Mullerian hormone; E2, estrogen; P, progesterone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; PCOM, polycystic ovarian morphology; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection.*

**TABLE 2** | Comparison of clinical outcomes between the two groups.

| | Total (n = 364) | Group 1 (n = 295) | Group 2 (n = 69) | P-value |
|---|---|---|---|---|
| **Duration of Gn (days)** | 10 (9–12) | 10 (9–11) | 12 (10–13) | < 0.001 |
| **Total Gn dose (IU)** | 1,800.0 (1,425.0–2,250.0) | 1,700.0 (1,375.0–2,175.0) | 2,175.0 (1,725.0–2,800.0) | < 0.001 |
| **On the trigger day** | | | | |
| E2 (pg/mL) | 4,134.3 (2,749.0–6,424.0) | 4,552.4 (2,813.0–6,652.0) | 2,972.1 (2,400.8–4,865.2) | 0.001 |
| P (ng/mL) | 0.92 (0.65–1.31) | 0.96 (0.68–1.33) | 0.75 (0.57–1.19) | 0.014 |
| LH (IU/L) | 1.6 (0.7–3.0) | 2.0 (1.0–3.4) | 0.5 (0.3–0.7) | < 0.001 |
| Single ET (mm) | 5.0 (4.5–5.9) | 5.0 (4.5–5.8) | 5.5 (4.5–6.0) | 0.23 |
| No. of follicles ≥ 14 mm | 9 (8–12) | 9 (8–13) | 10 (8–11) | 0.84 |
| **No. of oocytes retrieved** | 14 (10–20) | 14 (10–20) | 13 (10–20) | 0.71 |
| **IVF fertilization rate [n(%)]** | 4,116/5,252 (78.4) | 3,276/4,236 (77.3) | 840/1,016 (82.7) | < 0.001 |
| **ICSI fertilization rate [n(%)]** | 424/473 (89.6) | 392/434 (90.3) | 32/39 (82.1) | 0.10 |
| **Cleaved oocyte rate [n(%)]** | 4,474/4,540 (98.5) | 3,612/3,668 (98.5) | 862/872 (98.9) | 0.40 |
| **Available D3 embryo rate [n(%)]** | 3,150/4,474 (70.4) | 2,578/3,612 (71.4) | 572/862 (66.4) | 0.004 |
| **High-quality D3 embryo rate [n(%)]** | 1,673/3,395 (49.3) | 1,370/2,721 (50.3) | 303/674 (45.0) | 0.012 |
| **Blastocyst formation rate [n(%)]** | 1,605/2,259 (71.0) | 1,349/1,890 (71.4) | 256/369 (69.4) | 0.44 |
| **Available blastocyst rate [n(%)]** | 1,396/1,605 (87.0) | 1,176/1,349 (97.2) | 220/256 (85.9) | 0.59 |
| **High-quality blastocyst rate [n(%)]** | 466/1,605 (29.0) | 387/1,349 (29.7) | 79/256 (30.9) | 0.48 |
| **Embryo transfer cancelled [n(%)]** | 216 (59.3) | 179 (60.7) | 37 (53.6) | 0.28 |
| **Severe OHSS rate [n(%)]** | 19 (5.2) | 11 (3.7) | 8 (11.6) | 0.008 |
| **Biochemical pregnancy rate [n(%)]** | 93/148 (62.8) | 70/116 (60.3) | 23/32 (71.9) | 0.23 |
| **Clinical pregnancy rate [n(%)]** | 83/148 (56.1) | 63/116 (54.3) | 20/32 (62.5) | 0.41 |

*Gn, gonadotropin; E2, estrogen; P, progesterone; LH, luteinizing hormone; ET, endometrial thickness; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; OHSS, ovarian hyperstimulation syndrome.*

blastocyst rate, or embryo transfer cancellation rate among the two groups. The IVF fertilization and severe OHSS rates were, however, higher in group 2 than in group 1 [82.7% vs. 77.3% and 11.6% vs. 3.7%; p < 0.001 and p = 0.008]. The available D3 embryo rate and the high-quality D3 embryo rate were higher in group 1 than in group 2 [71.4% vs. 66.4% and 50.3% vs. 45.0%; p = 0.004 and 0.012]. Also, although the biochemical pregnancy rate and clinical pregnancy rate showed no statistical differences among the two groups (p = 0.23 and 0.41), these measures still demonstrated a lower trend in group 1 than in group 2 (60.3% vs. 71.9% and 54.3% vs. 62.5%).

## A Subgroup Analysis of the Severe OHSS Rate

The results of a subgroup analysis for the severe OHSS rate are shown in **Table 3**. A subgroup analysis based on bLH/FSH shows that, in patients with bLH/FSH levels of at least 1.33, the severe OHSS rate was different between the two groups [OR (95% CI): 5.08 (1.64–15.76), p = 0.005] but was similar in patients with bLH/FSH levels less than 1.33 [OR (95% CI): 1.09 (0.11–10.05), p = 0.94]. The study population was further divided into two subgroups by serum AMH levels; in the AMH > 3.4-ng/ml subgroup, the severe OHSS rate was higher in group 2 [OR (95% CI): 4.42 (1.66–11.76), p = 0.003], but in the AMH ≤ 3.4-ng/ml subgroup, the corresponding statistics could not be calculated because of zero events.

## A Subgroup Analysis of the Clinical Pregnancy Rate

The results of a subgroup analysis of the clinical pregnancy rate are shown in **Table 4**. In patients with lower bLH/FSH (<1.33) and lower AMH (≤3.4 ng/ml) levels, group 2 had a higher clinical pregnancy rate compared to group 1 (61.1% vs. 51.3 and 77.8% vs. 64.3%), but this difference did not reach statistical significance [OR (95% CI): 0.67 (0.24–1.92) and 0.51 (0.08–3.49); p = 0.45 and 0.49]. In patients with higher bLH/FSH (≥1.33) and higher AMH (>3.4 ng/ml) levels, the clinical pregnancy rate was similar

**TABLE 3** | A subgroup analysis of the severe OHSS rate.

| | Group 1 (n = 295) | | Group 2 (n = 69) | | OR (95% CI) | P-value |
|---|---|---|---|---|---|---|
| | Total | Events (%) | Total | Events (%) | | |
| **bLH/FSH** | | | | | | |
| <1.33 | 161 | 4 (2.5) | 37 | 1 (2.7) | 1.09 (0.11–10.05) | 0.94 |
| ≥1.33 | 134 | 7 (5.2) | 32 | 7 (21.9) | 5.08 (1.64–15.76) | 0.005 |
| **AMH** | | | | | | |
| ≤3.4 ng/ml | 20 | 1 (5.0) | 13 | 0 (0) | – | – |
| >3.4 ng/ml | 275 | 10 (3.6) | 56 | 8 (14.3) | 4.42 (1.66–11.76) | 0.003 |

*LH, luteinizing hormone; FSH, follicle-stimulating hormone; AMH, anti-Mullerian hormone; OR, odds ratio; CI, confidence interval.*
*Group 1 = "0"; group 2 = "1."*

**TABLE 4** | A subgroup analysis of the clinical pregnancy rate.

| | Group 1 (n = 116) | | Group 2 (n = 32) | | OR (95% CI) | P-value |
|---|---|---|---|---|---|---|
| | Total | Events (%) | Total | Events (%) | | |
| **bLH/FSH** | | | | | | |
| <1.33 | 76 | 39 (51.3) | 18 | 11 (61.1) | 0.67 (0.24–1.92) | 0.45 |
| ≥1.33 | 40 | 24 (60.0) | 14 | 9 (64.3) | 0.83 (0.24–2.95) | 0.78 |
| **AMH** | | | | | | |
| ≤3.4 ng/ml | 14 | 9 (64.3) | 9 | 7 (77.8) | 0.51 (0.08–3.49) | 0.49 |
| >3.4 ng/ml | 102 | 54 (52.9) | 23 | 13 (56.5) | 0.87 (0.35–2.15) | 0.76 |

*LH, luteinizing hormone; FSH, follicle-stimulating hormone; AMH, anti-Mullerian hormone; OR, odds ratio; CI, confidence interval.*
*Group 1 = "0"; group 2 = "1."*

in both groups [64.3% vs. 60.0% and 56.5% vs. 52.9%, OR (95% CI): 0.83 (0.24–2.95) and 0.87 (0.35–2.15); p = 0.78 and 0.76].

## DISCUSSION

As the most common endocrinal disorder characterized by oligo-anovulation, hyperandrogenemia, and polycystic ovaries on ultrasonography, PCOS seriously affects female reproductive health. In addition to reproductive disorders, women with PCOS are also at high risk for other long-term health problems, metabolic complications, and psychological problems, such as type II diabetes mellitus, cardiovascular disease, and anxiety (2). Hence, the diagnosis, prediction, treatment, and prognosis of PCOS deserve the attention of clinicians. For women suffering from PCOS with oligo-anovulation, carefully conducted and monitored pharmacological ovulation induction can be considered. Clomiphene citrate (CC) and letrozole are used as first-line pharmacotherapy, and gonadotropins and laparoscopic surgery appear to be a good alternative as a second-line treatment (13). As PCOS increasingly causes infertility (about 80% of anovulation infertility is caused by PCOS (3)), ART has been widely used in PCOS to help patients achieve pregnancy as a third-line treatment.

COS is an important step in IVF/ICSI, embryo transfer cycles, and, in particular, fresh embryo transfer. The fixed GnRH antagonist protocol and follicular phase GnRH agonist protocol are two important and classical COS protocols with different advantages and disadvantages. A rare but severe complication associated with COS is severe OHSS, which is regarded as related to the overreaction of the ovaries to Gn. Elevated serum AMH levels, multi-follicular development, and a high number of oocytes retrieved are acknowledged risk factors of severe OHSS (9). Higher bLH/FSH has also recently been shown to be associated with severe OHSS (4, 20). PCOS has been regarded as a risk factor of severe OHSS because some clinical features of PCOS typically reflect high ovarian sensitivity, such as high AMH levels and high bLH/FSH levels. The GnRH antagonist protocol can reduce the incidence of severe OHSS compared to the follicular phase GnRH agonist protocol; thus, it has been regarded as a first-line COS protocol for PCOS patients (13). It must be recognized, however, that the GnRH antagonist protocol results in lower clinical pregnancy and lower live birth rates than the GnRH agonist protocol in the general population (12, 14). Compared to the GnRH antagonist protocol, the follicular phase GnRH protocol may achieve better clinical outcomes, which can be explained by its positive effect on endometrial receptivity (22). It can be concluded that the follicular phase GnRH protocol may be an option for PCOS patients with lower AMH levels and lower bLH/FSH levels, especially for patients with a history of poor clinical outcomes.

In this study, we found that the severe OHSS rate in the follicular phase GnRH agonist group was significantly higher than that of the fixed GnRH antagonist group, but no statistical differences were observed in the biochemical pregnancy rates and the clinical pregnancy rates of the two groups. These results are generally consistent with those of previous studies (10, 11, 23). In this study, however, PCOS patients were innovatively stratified into subgroups according to their bLH/FSH and serum AMH levels. It was found that in PCOS women with higher bLH/FSH and higher serum AMH levels, severe OHSS incidence was higher in the follicular phase GnRH agonist group, but among PCOS women with lower bLH/FSH and lower AMH levels, severe OHSS incidence between the two groups was similar. These results indicate that bLH/FSH and serum AMH levels are worth considering when selecting COS protocols for PCOS patients. Regarding subgroup analyses of clinical pregnancy rates, it seems that no statistically valuable indicator has shown to be a good reference for COS selection in PCOS. Combining the results of these two subgroup analyses, the wild guess that follicular GnRH agonist protocols may be considered as an alternative choice for PCOS patients with lower bLH/FSH and lower serum AMH levels was entertained in this study, as such protocols do not increase the risk of severe OHSS. This assumption must be based, however, on the close observation of OHSS risk, and other OHSS risk factors must be fully considered. The cutoff values of bLH/FSH and serum AMH levels that predict severe OHSS should be verified with large-sample studies, and highly sensitive cutoff values should be selected.

We attempted to explain the results of this study by reviewing related physiological mechanisms. LH and FSH are both pituitary gonadotropin hormones essential for female fertility, and they are regulated by the frequency of pulsatile GnRH. According to the two-cell theory, LH stimulates follicular theca cells to produce androstenedione, and FSH stimulates the

synthesis of aromatase in granulosa cells, thus catalyzing the conversion of androstenedione to estradiol. LH and FSH work together to stimulate sex hormone secretion and oocyte development in the ovaries. On days 2–3 of the menstrual cycle, the dominant follicle continues to mature in physiological status under FSH. Increased LH levels can trigger ovarian follicular theca cells to secrete more androgen during this period, and FSH can trigger granulosa cells to convert extra androgens to estrogen (24). Thus, high bLH/FSH has been shown to impair the formation of follicles (25). In PCOS patients receiving GnRH antagonist protocols, endogenous LH was not suppressed during the early stages of Gn stimulation; thus, potential ovarian overstimulation may be inhibited by endogenous LH. In PCOS patients receiving follicular phase GnRH agonist protocols, however, endogenous LH generation is inhibited, and the body loses the potential mechanism of inhibiting ovarian overstimulation due to the downregulated pituitary function. AMH is produced in granulosa cells by pre-antral and small antral follicles and is highly correlated with bLH/FSH in PCOS women (26, 27). For this reason, AMH is also considered to represent ovarian reactivity. For PCOS patients with higher bLH/FSH levels or higher serum AMH levels, the ovaries are more like to respond to Gn, and the GnRH antagonist can thus restrict this reactivity *via* endogenous LH. However, for PCOS women with lower bLH/FSH levels and lower serum levels of AMH, the reactivity of their ovaries to Gn is not as obvious. These mechanisms may explain why the incidence of early-stage OHSS does not show significant differences among PCOS patients with lower bLH/FSH levels and lower AMH levels between the two COS protocols. Also, late-stage OHSS is strongly associated with pregnancy and is restricted to cycles in which clinical pregnancy occurred. PCOS is a strong risk factor of late-stage OHSS because the risk factor for high bLH/FSH levels and high AMH levels among PCOS patients with lower bLH/FSH and lower AMH levels is offset to some extent. Therefore, for PCOS patients with lower bLH/FSH levels and lower serum AMH levels, the follicular phase GnRH agonist protocol may be a viable choice.

It is worth emphasizing that there are some limitations that restrict the credibility of this study. Its results must therefore be carefully interpreted. The most important limitation is its small sample size, which is especially true for its PCOS patients who received the follicular phase GnRH agonist protocol. Because of guidelines published in recent years (13), GnRH antagonist protocols have been widely used in PCOS patients even though the individual differences of PCOS are not discussed in these guidelines. This could explain why there were significantly fewer patients in this study's follicular phase GnRH agonist group as compared to its GnRH antagonist group. In addition, severe OHSS is a rare complication of COS, and severe OHSS events in some subgroups are numbered as low as zero. All these factors restricted the sample size of this study, but considering the rarity of the resulting events and the interpretability of the results, these results are still worth reporting. Another limitation is due to the inherent nature of retrospective studies, as some potential confounding factors were not excluded in this study. In addition, other clinical outcomes such as the miscarriage rate and the live birth rate were not acquired. In the opinion of this study's authors, the greatest value of this study is that it provides another choice for controlled ovarian stimulation for PCOS patients with lower bLH/FSH and lower serum AMH levels. The results of this study must be validated by prospective studies with larger samples in the future.

In conclusion, GnRH antagonist protocols should serve as first-line COS protocols for PCOS patients undergoing IVF/ICSI and fresh embryo transfer cycles, but the limited evidence of this study suggests that for PCOS patients with lower bLH/FSH (<1.33) and lower serum AMH levels (≤3.4 ng/ml), follicular phase GnRH agonist protocols may be another safe choice that does not increase severe OHSS risks. The results of this study must be interpreted with caution.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of West China Second University Hospital. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

All authors contributed to this study's conception and design. Material preparation, data collection, and analysis were performed by RG, XL, WH, RZ, and LQ. The first draft of the manuscript was written by RG and PB, and all authors commented on previous versions of the manuscript. All authors contributed to the article and approved the submitted version.

## REFERENCES

1. Goodarzi MO, Dumesic DA, Chazenbalk G, Azziz R. Polycystic Ovary Syndrome: Etiology, Pathogenesis and Diagnosis. *Nat Rev Endocrinol* (2011) 7(4):219–31. doi: 10.1038/nrendo.2010.217
2. Li R, Zhang Q, Yang D, Li S, Lu S, Wu X, et al. Prevalence of Polycystic Ovary Syndrome in Women in China: A Large Community-Based Study. *Hum Reprod* (2013) 28(9):2562–9. doi: 10.1093/humrep/det262
3. Chang J, Azziz R, Legro R, Dewailly D, Franks S, Tarlatzis R, et al Revised 2003 Consensus on Diagnostic Criteria and Long–Term Health Risks Related to Polycystic Ovary Syndrome. *Fertil Steril* (2004) 81(1):19–25. doi: 10.1016/j.fertnstert.2003.10.004
4. Tarlatzis BC, Grimbizis G, Pournaropoulos F, Bontis J, Lagos S, Spanos E, et al. The Prognostic Value of Basal Luteinizing Hormone: Follicle–Stimulating Hormone Ratio in the Treatment of Patients With Polycystic Ovarian Syndrome by Assisted Reproduction Techniques. *Hum Reprod* (1995) 10(10):2545–9. doi: 10.1093/oxfordjournals.humrep.a135742

5. Dewailly D, Robin G, Peigne M, Decanter C, Pigny P, Catteau–Jonard S. Interactions Between Androgens, Fsh, Anti–Müllerian Hormone and Estradiol During Folliculogenesis in the Human Normal and Polycystic Ovary. *Hum Reprod Update* (2016) 22(6):709–24. doi: 10.1093/humupd/dmw027
6. Homburg R, Ray A, Bhide P, Gudi A, Shah A, Timms P, et al. The Relationship of Serum Anti–mullerian Hormone With Polycystic Ovarian Morphology and Polycystic Ovary Syndrome: A Prospective Cohort Study. *Hum Reprod* (2013) 28(4):1077–83. doi: 10.1093/humrep/det015
7. Diamanti–Kandarakis E, Dunaif A. Insulin Resistance and the Polycystic Ovary Syndrome Revisited: An Update on Mechanisms and Implications. *Endocr Rev* (2012) 33(6):981–1030. doi: 10.1210/er.2011-1034
8. Xiao ZN, Peng JL, Yang J and Xu WM. Flexible Gnrh Antagonist Protocol Versus Progestin-Primed Ovarian Stimulation (PPOS) Protocol in Patients With Polycystic Ovary Syndrome: Comparison of Clinical Outcomes and Ovarian Response. *Curr Med Sci* (2019) 39(3):431–6. doi: 10.1007/s11596-019-2055-x
9. Pfeifer S, Butts S, Dumesic D, Fossum G, Gracia C, La Barbera A, et al. Prevention and Treatment of Moderate and Severe Ovarian Hyperstimulation Syndrome: A Guideline. *Fertil Steril* (2016) 106(7):1634–47. doi: 10.1016/j.fertnstert.2016.08.048
10. Trenkić M, Popović J, Kopitović V, Bjelica A, Živadinović R, Pop–Trajković S. Flexible Gnrh Antagonist Protocol vs. Long Gnrh Agonist Protocol in Patients With Polycystic Ovary Syndrome Treated for IVF: Comparison of Clinical Outcome and Embryo Quality. *Ginekol Pol* (2016) 87(4):265–70. doi: 10.17772/gp/62205
11. Xiao J, Chen S, Zhang C, Chang S. Effectiveness of Gnrh Antagonist in the Treatment of Patients With Polycystic Ovary Syndrome Undergoing IVF: A Systematic Review and Meta Analysis. *Gynecol Endocrinol* (2013) 29(3):187–91. doi: 10.3109/09513590.2012.736561
12. Lambalk CB, Banga FR, Huirne JA, Toftager M, Pinborg A, Homburg R, et al. Gnrh Antagonist Versus Long Agonist Protocols in IVF: A Systematic Review and Meta-Analysis Accounting for Patient Type. *Hum Reprod Update* (2017) 23(5):560–79. doi: 10.1093/humupd/dmx017
13. Balen AH, Morley LC, Misso M, Franks S, Legro RS, Wijeyaratne CN, et al. The Management of Anovulatory Infertility in Women With Polycystic Ovary Syndrome: An Analysis of the Evidence to Support the Development of Global WHO Guidance. *Hum Reprod Update* (2016) 22(6):687–708. doi: 10.1093/humupd/dmw025
14. Zhang W, Xie D, Zhang H, Huang J, Xiao X, Wang B, et al. Cumulative Live Birth Rates After the First ART Cycle Using Flexible Gnrh Antagonist Protocol vs. Standard Long Gnrh Agonist Protocol: A Retrospective Cohort Study in Women of Different Ages and Various Ovarian Reserve. *Front Endocrinol (Lausanne)* (2020) 11:287. doi: 10.3389/fendo.2020.00287
15. Litzky JF, Marsit CJ. Epigenetically Regulated Imprinted Gene Expression Associated With IVF and Infertility: Possible Influence of Prenatal Stress and Depression. *J Assist Reprod Genet* (2019) 36(7):1299–313. doi: 10.1007/s10815-019-01483-0
16. Massarotti C, Gentile G, Ferreccio C, Scaruffi P, Remorgida V and Anserini P. Impact of Infertility and Infertility Treatments on Quality of Life and Levels of Anxiety and Depression in Women Undergoing *in Vitro* Fertilization. *Gynecol Endocrinol* (2019) 35(6):485–9. doi: 10.1080/09513590.2018.1540575
17. Lin YC, Chang SY, Lan KC, Huang HW, Chang CY, Tsai MY, et al. Human Oocyte Maturity *in Vivo* Determines the Outcome of Blastocyst Development in Vitro. *J Assist Reprod Genet* (2003) 20(12):506–12. doi: 10.1023/B:JARG.0000013651.37866.0c
18. Veeck L. *An Atlas of Human Gametes and Conceptuses*. London: Parthenon (1999).
19. Su NJ, Huang CY, Liu J, Kang DY, Wang SL, Liao LJ, et al. Association Between Baseline Lh/Fsh and Live-Birth Rate After Fresh-Embryo Transfer in Polycystic Ovary Syndrome Women. *Sci Rep* (2021) 11(1):20490. doi: 10.1038/s41598-021-99850-4
20. Le MT, Le VNS, Le DD, Nguyen VQH, Chen C and Cao NT. Exploration of the Role of Anti-Mullerian Hormone and Lh/Fsh Ratio in Diagnosis of Polycystic Ovary Syndrome. *Clin Endocrinol (Oxf)* (2019) 90(4):579–85. doi: 10.1111/cen.13934
21. Schirmer DA3rd, Kulkarni AD, Zhang Y, Kawwass JF, Boulet SL, Kissin DM. Ovarian Hyperstimulation Syndrome After Assisted Reproductive Technologies: Trends, Predictors, and Pregnancy Outcomes. *Fertil Steril* (2020) 114(3):567–78. doi: 10.1016/j.fertnstert.2020.04.004
22. Geng Y, Xun Y, Hu S, Lai Q, Jin L. Gnrh Antagonist Versus Follicular–Phase Single–Dose Gnrh Agonist Protocol in Patients of Normal Ovarian Responses During Controlled Ovarian Stimulation. *Gynecol Endocrinol* (2019) 35(4):309–13. doi: 10.1080/09513590.2018.1528221
23. Kollmann M, Martins WP, Lima ML, Craciunas L, Nastri CO, Richardson A, et al. Strategies for Improving Outcome of Assisted Reproduction in Women With Polycystic Ovary Syndrome: Systematic Review and Meta-Analysis. *Ultrasound Obstet Gynecol* (2016) 48(6):709–18. doi: 10.1002/uog.15898
24. Bildik G, Akin N, Seyhan A, Esmaeilian Y, Yakin K, Keles I, et al. Luteal Granulosa Cells From Natural Cycles Are More Capable of Maintaining Their Viability, Steroidogenic Activity and Lh Receptor Expression Than Those of Stimulated IVF Cycles. *Hum Reprod* (2019) 34(2):345–55. doi: 10.1093/humrep/dey353
25. Xie L, Zhang D, Ma H, He H, Xia Q, Shen W, et al. The Effect of Berberine on Reproduction and Metabolism in Women With Polycystic Ovary Syndrome: A Systematic Review and Meta-Analysis of Randomized Control Trials. *Evid Based Complement Alternat Med* (2019) 2019:7918631. doi: 10.1155/2019/7918631
26. Racoubian E, Aimagambetova G, Finan RR, Almawi WY. Age-Dependent Changes in Anti-Müllerian Hormone Levels in Lebanese Females: Correlation With Basal Fsh and Lh Levels and Lh/Fsh Ratio: A Cross-Sectional Study. *BMC Womens Health* (2020) 20(1):134. doi: 10.1186/s12905-020-00998-4
27. Dietz de Loos A, Hund M, Buck K, Meun C, Sillman J, Laven JSE. Antimüllerian Hormone to Determine Polycystic Ovarian Morphology. *Fertil Steril* (2021) 116(4):1149–57. doi: 10.1016/j.fertnstert.2021.05.094


**Conflict of Interest:** The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers | Frontiers in Endocrinology

ORIGINAL RESEARCH
published: 15 June 2022
doi: 10.3389/fendo.2022.877471

# Intracytoplasmic Sperm Injection May Not Improve Clinical Outcomes Despite Its Positive Effect on Embryo Results: A Retrospective Analysis of 1130 Half-ICSI Treatments

*Nan Peng*[1,2,3,4,5†], *Shuiying Ma*[1,2,3,4,5†], *Cheng Li*[1,2,3,4,5], *Hui Liu*[1,2,3,4,5], *Haibin Zhao*[1,2,3,4,5], *Lian-Jie Li*[1,2,3,4,5], *Qing Li*[1,2,3,4,5,6] *and Mei Li*[1,2,3,4,5,6*]

[1] Center for Reproductive Medicine, Cheeloo College of Medicine, Shandong University, Jinan, China, [2] Key Laboratory of Reproductive Endocrinology of Ministry of Education, Shandong University, Jinan, China, [3] Shandong Key Laboratory of Reproductive Medicine, Jinan, China, [4] Shandong Provincial Clinical Research Center for Reproductive Health, Jinan, China, [5] National Research Center for Assisted Reproductive Technology and Reproductive Genetics, Shandong University, Jinan, China, [6] The Second Hospital, Cheeloo College of Medicine, Shandong University, Jinan, China

OPEN ACCESS

**Edited by:**
*Yiping Shen,*
*Harvard Medical School, United States*

**Reviewed by:**
*Ahmad Mustafa Metwalley,*
*Women's Health Fertility Clinic, Saudi Arabia*
*Diao Feiyang,*
*Nanjing Medical University, China*

***Correspondence:**
*Mei Li*
*lee_mei@163.com*

†*These authors have contributed equally to this work and share first authorship*

**Specialty section:**
*This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology*

**Received:** *16 February 2022*
**Accepted:** *05 May 2022*
**Published:** *15 June 2022*

**Citation:**
*Peng N, Ma S, Li C, Liu H, Zhao H, Li L-J, Li Q and Li M (2022) Intracytoplasmic Sperm Injection May Not Improve Clinical Outcomes Despite Its Positive Effect on Embryo Results: A Retrospective Analysis of 1130 Half-ICSI Treatments. Front. Endocrinol. 13:877471. doi: 10.3389/fendo.2022.877471*

**Objective:** To explore the clinical application value of half-ICSI treatment for infertility in assisted reproductive technology.

**Method:** A retrospective analysis of 1130 half-ICSI treatments was conducted at the Affiliated Reproductive Hospital of Shandong University from January 2011 to December 2015. Patients with low fertilization rates in previous cycles, primary infertility for >5 years with unexplained reason, or secondary infertility for >5 years without fallopian tube factor were involved in this study. The 2PN rate, high-quality embryo rate, oocyte utilization rate, and clinical outcomes were compared between IVF insemination group (IVF group) and ICSI insemination group (ICSI group). The clinical outcome of half-ICSI insemination treatment, grouped according primary and secondary infertility, was also analyzed.

**Results:** Compared with IVF, ICSI resulted in a significantly higher 2PN rate (74.8% vs. 62.9%), high-quality embryo rate (54.6% vs. 51.7%), and oocyte utilization rate (35.9% vs. 32.8%; P<0.05). Among the 884 fresh-embryo transfer cycles, there were no notable differences in clinical pregnancy rate, live birth rate, or neonatal abnormality rate between the IVF and ICSI groups. Among the 792 primary infertility cycles, ICSI resulted in a significantly higher 2PN rate, high-quality embryo rate, and oocyte utilization rate compared with IVF (75.3% vs. 62.4%, 54.3% vs. 50.8%, 36.4% vs. 32.6%, P<0.05). For the 338 secondary infertility cycles, ICSI resulted in a significantly higher 2PN rate (73.6% vs. 63.9%, P<0.05) compared with IVF, but there were no notable differences in other laboratory results. Moreover, the biochemical pregnancy rate of the ICSI group was significantly lower than for IVF in secondary infertility cycles (49.3% vs. 65.6%; P<0.05). A total of 89 cycles (7.9%) with complete IVF fertilization failure showed a low second polar body (2PB) rate (33.6%) after a 5-h short-time fertilization period, including 34 cycles (3.0%) with no 2PB oocytes observed in the IVF group.

**Conclusion:** ICSI insemination improved laboratory results compared with IVF insemination, however, fresh-embryo transfer of ICSI originated embryos did not improve clinical pregnancy and live birth rates. Rescue ICSI has been successfully applied in clinical IVF insemination to avoid fertilization failure. Therefore, as an extra intervention, it is suggested that ICSI be used judiciously.

**Keywords: half-ICSI, 2PN, high-quality embryo, oocyte utilization rate, clinical outcome, live birth**

## INTRODUCTION

Oocyte fertilization is a critical step in assisted reproductive technology (ART). A low fertilization rate or complete fertilization failure may occur in some infertility treatment cycles, and the subsequent repeated assisted pregnancy therapy may result in psychological and economic pressure on patients. The incidence of complete fertilization failure ranges from 10% to 20% (1). In 2003, Jaroudi et al. (2) first expressed that intracytoplasmic sperm injection (ICSI) and conventional *in vitro* fertilization (IVF) are complementary techniques in the management of unexplained infertility. Nevertheless, despite the high fertilization rate (3), it is still not recommended that ICSI be a blanket fertilization method in ART, as ICSI treatment is more invasive and costly than IVF.

Recently, increasing evidence has shown the positive role of half-ICSI in ART. Half-ICSI results in more high-quality embryos for transfer and improves the rate of pregnancy for patients with a high risk of fertilization failure (4). In 2010, Guo et al. (5) reported that half-ICS treatment may be useful for patients with unexplained infertility and primary infertility, but not for patients with oligo-asthenozoospermia, teratozoospermia, or secondary infertility. However, controversy exists between some studies. Sauerbrun-Cutler et al. (6) report that in a split sibling oocyte cohort, although ICSI had a higher fertilization rate and more high-quality day-2 embryos, it had a lower blastulation rate.

The purpose of this study was to further evaluate the effect of half-ICSI treatment in ART. We conducted a retrospective analysis of 1130 half-ICSI insemination treatments at our center from January 2011 to December 2015. We evaluated the effects of different insemination methods on the clinical outcomes of these patients, in order to provide a reference for more focused clinical treatment in the future.

## MATERIALS AND METHODS

### Patients

A total of 1130 half-ICSI patients were enrolled in this study from January 2011 to December 2015. Half-ICSI treatment was given to patients with the following infertility backgrounds: patients with a fertilization rate between 30% and 50% in previous IVF cycles; patients with primary unexplained infertility for >5 years, or secondary infertility for >5 years without fallopian tube problems. All the patients involved in half-ICSI treatments had at least eight oocytes retrieved and the semen profile was normal.

### Ovarian Stimulation

All patients underwent controlled ovarian hyperstimulation (COH). Ovarian stimulation protocols included controlled ovarian hyperstimulation after gonadotropin-releasing hormone (GnRH) agonist down-regulation or an antagonist protocol. Recombinant follicle-stimulating hormone (rFSH, PUREGON; MSD Organon, Oss, Netherlands) was started on day 1–3 of the menstrual cycle. The dose adjustment of gonadotropin, monitoring of the ovarian response, and the timing for triggering the final oocyte maturation during ovarian stimulation was performed under the discretion of the supervising clinician. Oocyte retrieval was performed 34–36 h after the administration of human chorionic gonadotropin (hCG) at a dose of 4000–10 000 IU.

### Oocyte Insemination, Embryo Culture, and Embryo Transfer

Oocytes were inseminated approximately 3–6 h after follicular aspiration using a conventional insemination method and ICSI. The oocytes were divided into two groups equally and randomly. One group underwent IVF insemination and the other group underwent ICSI insemination. If the total number of oocytes is odd, one more oocyte was divided into the ICSI group. Short-time insemination in the IVF group was used, but no rescue ICSI was performed on the oocytes. Sequential culture media from Vitrolife (G-IVF, G1 and G2; Scandinavian IVF Science, Goteborg, Sweden) were used in all steps. Embryos were cultured separately in pre-equilibrated culture media overlaid with mineral oil. The culture dishes were housed in 37 °C tri-gas tabletop incubators (K-system, Denmark) containing 5% $O_2$ and 6% $CO_2$, balanced with $N_2$. Two or three high-quality embryos were selected for fresh transfer on day 3. For patients who could only accept a single embryo transfer, a single blastocyst was selected and transferred on day 5. High-quality embryos in the IVF group were selected for transfer as a priority. High-quality embryos in the ICSI group were selected for transfer if there was only one or no high-quality embryo in the IVF group. Supernumerary embryos were cultured for blastocyst cryopreservation. Morphologic criteria were used for day-3 embryo scoring based on the amount of anucleate fragments expelled during early cleavage, and on developmental speed (7). Embryos transferred and cryopreserved by vitrification on day 5 were assessed to be above grade 4BC according to Garden and Lane criteria (8). Embryo transfer was performed using a Wallace catheter under ultrasound guidance.

## Outcome Measures

In both the ICSI insemination group (ICSI group) and the IVF insemination group (IVF group), the 2PN rate of matured oocytes, the high-quality day-3 embryo rate, and the utilized oocyte rate, including embryos transferred and embryos vitrified, were calculated.

When embryos originated from different insemination groups in fresh-embryo transfer cycles, the clinical results were calculated separately. All the fresh-embryo transfer cycles were divided into three groups: the IVF group, where IVF insemination embryos were transferred; the ICSI group, where ICSI insemination embryos were transferred; and the IVF plus ICSI group, where IVF embryos and ICSI embryos were both transferred at the same time. A serum hCG level>10 IU/L at 14 days after embryo transfer was diagnosed as a biochemical pregnancy and cardiac activity 7 weeks after embryo transfer was defined as a clinical pregnancy. Live birth was defined as the delivery of a live-born infant at≥28 weeks of gestation. Preterm birth rate and neonatal abnormalities were also calculated.

## Statistical Analysis

All analyses were performed using SPSS Statistics (version 22.0). Statistical analyses were conducted using the t-test and chi-square test. P-value was bilateral and P<0.05 was considered statistically significant. Statistical analysis was performed using a $\chi 2$ test. A P-value<0.05 was considered statistically significant.

# RESULTS

## Description of the Study Patients

Patient characteristics are listed in **Table 1**. A total of 1130 patients were involved in this study. Their average age was 32.4 ± 4.1. Of these, 792 cases were primary infertility and 382 cases were secondary infertility. Finally, 884 cycles from these patients underwent fresh embryo transplantation. Cryopreservation was performed in 202 cycles, and 45 cycles were completely abandoned with no embryo transferred or frozen. The ovarian stimulation protocols used in the study included super long-term, long-term, short-term, antagonist, microstimulation protocol, and other protocols.

**TABLE 1** | Characteristics of the 1130 half-ICSI patients.

| Characteristic | No. cycles | Value |
|---|---|---|
| Age (y) (mean ± STD) | | 32.43 ± 4.05 |
| Infertility factors | | |
| Primary | 792 (70.1%) | |
| Secondary | 338 (30.0%) | |
| Regimen of ovarian hyperstimulation | | |
| super long-term protocol | 12 (1.1%) | |
| long-term protocol | 954 (84.4%) | |
| short-term protocol | 104 (9.2%) | |
| Antagonist protocol | 41 (3.6%) | |
| other protocol | 16 (1.4%) | |
| microstimulation protocol | 3 (0.3%) | |
| Result of treatment | | |
| Fresh embryo-transfer cycles | 884 (78.2%) | |
| Cryopreserved cycles | 202 (17.9%) | |
| Complete abandoned cycles | 45 (4.0%) | |

## Embryo Development Analysis of the Half-ICSI Treatment Cycles

The 2PN rate, high-quality embryo rate, and utilized oocyte rate of ICSI embryos were significantly higher than for IVF embryos (74.8%vs. 62.9%, 54.6%vs. 51.7%, 35.9%vs. 32.8%, P<0.05; **Table 2**). Additionally, for the 792 primary infertility patients, the 2PN rate, high-quality embryo rate, and utilized oocyte rate of ICSI embryos were significantly higher than for IVF embryos (75.3%vs. 62.4%, 54.3%vs. 50.8%, 36.4%vs32.6%, P<0.05). For the 338 secondary infertility patients, the 2PN rate of ICSI insemination embryos was significantly higher than for IVF insemination embryos (73.6%vs. 63.9%, P<0.05), but the high-quality embryo rate and utilized oocyte rate did not differ between IVF embryos and ICSI embryos (**Table 3**).

## Clinical Outcome Analysis of the Half-ICSI Treatment Cycles

A total of 884 patients underwent fresh embryo transfer. Biochemical pregnancy rates of the ICSI group and IVF plus ICSI group were both lower than for the IVF group (53.6%vs. 64.2%, 55.5%vs. 64.2%, P<0.05; **Table 4**). However, the clinical pregnancy rate, live birth rate, preterm birth rate, and the neonatal abnormality rate did not differ among the three groups. For primary infertility patients, all clinical indexes showed no clear difference. For secondary infertility patients, the biochemical pregnancy rate of the ICSI group and IVF plus ICSI group were both lower than for the IVF group (49.3%vs. 65.5%, 47.6%vs. 65.5%, P<0.05), and the clinical pregnancy rate, the live birth rate, and the preterm birth rate did not differ among the three groups (**Table 5**). There was no notable difference in neonatal abnormalities among the different transfer groups. The clinical outcome of patients with fresh embryo transplantation ≤ 35 years old was also analyzed (**Supplementary Tables 1**, **2**). The result is in accordance with the total fresh embryo transfer infertility patients.

## Embryo and Clinical Outcome of the 89 Complete IVF Fertilization Failure Cycles

A total of 89 cycles (7.9%) with complete IVF fertilization failure showed a low second polar body expulsion (2PB) rate (33.6%)

**TABLE 2** | Embryo outcome of the total 1130 half-ICSI patients.

| Characteristic | IVF | ICSI |
|---|---|---|
| Matured oocytes: no. | 6728 | 7014 |
| 2PN: no. (%) | 4230 (62.9) | 5245 (74.8)* |
| High-quality embryo: no. (%) | 2185 (51.7) | 2862 (54.6)* |
| Transferred embryo: no. | 978 | 782 |
| Vitrified embryo: no. | 1227 | 1737 |
| Utilized oocytes: no. (%) | 2205 (32.8) | 2519 (35.9)* |

*Values are presented as number (%).*
**P <0.05 ICSI compared to IVF groups.*

**TABLE 3 |** Embryo outcome of the 792 primary infertility and 338 secondary infertility patients.

| | Primary infertility | | Secondary infertility | |
|---|---|---|---|---|
| **Characteristic** | **IVF** | **ICSI** | **IVF** | **ICSI** |
| Matured oocytes: no. | 4752 | 4947 | 1976 | 2067 |
| 2PN: no. (%) | 2967 (62.4) | 3724 (75.3)* | 1264 (63.9) | 1521 (73.6)* |
| High-quality embryo: no. (%) | 1507 (50.8) | 2021 (54.3)* | 678 (53.7) | 841 (55.3) |
| Transferred embryo: no. | 650 | 547 | 328 | 235 |
| Vitrified embryo: no. | 897 | 1256 | 330 | 481 |
| Utilized oocytes: no. (%) | 1547 (32.6) | 1803 (36.4)* | 658 (33.3) | 716 (34.6) |

*Values are presented as number (%).*
**P <0.05 ICSI compared to IVF groups.*

**TABLE 4 |** Clinical outcome of half-ICSI patients with fresh embryo-transfer cycles.

| **Characteristic** | **IVF** | **ICSI** | **IVF+ICSI** |
|---|---|---|---|
| Fresh embryo transfer cycles | 363 | 274 | 247 |
| No. fresh embryo transferred per cycle (mean ± STD) | 2.0 ± 0.3 | 1.9 ± 0.5 | 2.2 ± 0.4 |
| Age (y) (mean ± STD) | 32.5 ± 3.8 | 32.4 ± 4.1 | 33.0 ± 4.5 |
| Live birth: no. (%) | 164 (45.2) | 114 (41.6) | 100 (40.5) |
| Singleton live birth per woman | 115 (31.7%) | 86 (31.4%) | 71 (28.7%) |
| Twin live birth per woman | 49 (13.5%) | 28 (10.2%) | 29 (11.8%) |
| Biochemical pregnancy: no. (%) | 233 (64.2) | 147 (53.6)[a] | 137 (55.5)[b] |
| Clinical pregnancy: no. (%) | 200 (55.1) | 132 (48.2) | 120 (48.6) |
| Preterm birth [no./total no. (%)] | 11/164 (6.7) | 9/114 (7.9) | 6/100 (6.0) |
| Neonatal abnormalities [no./total no. (%)] | 5/213 (2.3) | 4/142 (2.8) | 2/129 (1.6) |

*Values are presented as number (%).*
*[a]P<0.05 ICSI compared to IVF groups.*
*[b]P<0.05 IVF+ICSI compared to IVF groups.*

after a 5-h short-time fertilization period, which included 34 cycles (3.0%) with no 2PB oocytes observed in the IVF group. The 2PN rate, high-quality embryo rate and utilized oocyte rate of ICSI embryos were 71.1%, 62.9%, and 45.0%, respectively. The live birth rate, biochemical pregnancy rate, clinical pregnancy rate, preterm birth rate, nd the neonatal abnormality rate were 47.1%, 60%,55.7%, 3.0%, and 4.9%, respectively (**Table 6**).

**TABLE 6 |** Embryo and clinical outcome of 89 cycles of complete IVF infertility failure.

| **Characteristic** | **IVF** | **ICSI** |
|---|---|---|
| No. of matured oocytes | 417 | 478 |
| 2PN: no. (%) | 0 | 340 (71.1) |
| High-quality embryo: no. (%) | 0 | 214 (62.9) |
| No. of embryos transferred | 0 | 124 |
| No. of vitrified embryos | 0 | 91 |
| Utilized oocytes: no. (%) | 0 | 215 (45.0) |
| Fresh embryo transfer cycle | 0 | 70 |
| Live birth: no. (%) | – | 33 (47.1) |
| Singleton live birth per woman | – | 25 (35.7%) |
| Twin live birth per woman | – | 8 (11.4%) |
| Biochemical pregnancy: no. (%) | – | 42 (60) |
| Clinical pregnancy: no. (%) | – | 39 (55.7) |
| Preterm birth: [no./total no. (%)] | – | 1/33 (3.0) |
| Neonatal abnormalities: [no./total no. (%)] | – | 2/41 (4.9) |
| Second polar body expulsion: no. (%) | 140(33.6) | |

## DISCUSSION

Oocyte fertilization is a complex process affected by a series of factors, including state of oocyte maturation, sperm maturation, and vitality or fusion of genetic material. Any abnormality in these steps leads to fertilization failure. For oligozoospermia and asthenozoospermia patients, ICSI greatly improves oocyte fertilization rate. In recent years, several studies have found that ICSI fertilization can also be effective in improving the fertilization rate of patients with unexplained infertility (3). Based on existing research, we adopted the half-ICSI treatment for some patients. The selected patients exhibited: primary or secondary fertilization failure for >5 years, or had a low IVF fertilization rate in past cycles. Our results showed that for primary infertility patients, ICSI resulted in a significantly higher 2PN rate, high-quality embryo rate, and oocyte utilization rate compared with the IVF group. However, biochemical pregnancy rate, clinical pregnancy rate, and live birth rate did not differ among the IVF group, ICSI group, and IVF plus ICSI group. The application of ICSI improved embryo quality but did not ultimately increase clinical pregnancy rate. We speculated that although ICSI guarantees embryo

**TABLE 5 |** Clinical outcome of primary infertility and secondary infertility half-ICSI patients with fresh embryo-transfer cycles.

| | Primary infertility (608 cycles) | | | Secondary infertility (276 cycles) | | |
|---|---|---|---|---|---|---|
| **Characteristic** | **IVF** | **ICSI** | **IVF+ICSI** | **IVF** | **ICSI** | **IVF+ICSI** |
| Fresh embryo transfer cycle: no. (%) | 244 (40.1) | 201 (33.1) | 163 (26.8) | 119 (43.1) | 73 (26.4) | 84 (30.4) |
| No. fresh embryo transferred per cycle (mean ± STD) | 2.0 ± 0.3 | 1.9 ± 0.4 | 2.1 ± 0.3 | 2.0 ± 0.2 | 1.9 ± 0.5 | 2.2 ± 0.4 |
| Age (y) (mean ± STD) | 32.0 ± 3.7 | 31.9 ± 4.0 | 32.7 ± 4.5 | 33.6 ± 3.8 | 33.7 ± 4.0 | 33.8 ± 4.5 |
| Live birth: no. (%) | 107 (43.9) | 86 (42.8) | 66 (40.5) | 57 (47.9) | 28 (38.4) | 34 (40.5) |
| Singleton live birth per woman | 75 (30.7%) | 65 (32.3%) | 48 (29.4%) | 40 (33.6%) | 21 (28.8%) | 23 (27.4%) |
| Twin live birth per woman | 32 (13.2%) | 21 (10.5%) | 18 (11.1%) | 17 (14.3%) | 7 (9.6%) | 11 (13.1%) |
| Biochemical pregnancy: no. (%) | 155 (63.5) | 111 (55.2) | 97 (59.5) | 78 (65.5) | 36 (49.3)[a] | 40 (47.6)[b] |
| Clinical pregnancy: no. (%) | 135 (55.3) | 100 (49.8) | 81 (49.7) | 65 (54.6) | 32 (43.8) | 39 (46.5) |
| Preterm birth: [no./total no. (%)] | 6/107 (5.6) | 5/86 (5.8) | 3/66 (4.5) | 5/57 (8.8) | 4/28 (14.3) | 3/34 (8.8) |
| Neonatal abnormalities: [no./total no. (%)] | 5/139 (3.6) | 3/107 (2.8) | 2/84 (2.4) | 0/74 (0.0) | 1/35 (2.9) | 0/45 (0.0) |

*Values are presented as number (%).*
*[a]P<0.05 ICSI compared to IVF groups.*
*[b]P<0.05 IVF+ICSI compared to IVF groups.*

fertilization and early development, it does not improve the later developmental potential of embryos after transfer into the uterus.

For secondary infertility patients, ICSI only resulted in a significantly higher 2PN rate compared with IVF. The high-quality embryo rate and the utilized oocyte rate did not differ between IVF and ICSI groups. Moreover, ICSI embryos had a lower biochemical pregnancy rate. Therefore, according to our results, ICSI is not necessary for secondary infertility patients. Furthermore, the ICSI procedure may decrease embryo implantation capacity.

ICSI was first used for male-factor infertility, but in recent years, it has also been used in non-male-factor infertility cycles (9). The application of ICSI reduces the risk of complete fertilization failure, but does not increase the cumulative live birth rate in non-male factor infertility (10). Recently, accumulating information has shown the risk of ICSI on offspring, including congenital malformations, chromosomal abnormalities, and epigenetic syndromes (11–13). Cai et al. (14) reported that a sex ratio imbalance following blastocyst transfer is also associated with ICSI but not with IVF. A previous study also reported that for male-factor infertility, ICSI affects the fertility of male offspring, decreasing semen quality and quantity in young adults conceived by ICSI (15). Considering these risk, ICSI should be used with caution.

Short-time insemination and immediate rescue ICSI have been widely used in recent years, which has decreased complete fertilization failure. ICSI can be performed on oocytes that do not discharge a second polar body within 4–6 hours post-insemination (16). In our study, a total of 89 cycles with complete IVF fertilization failure showed a low second polar body (2PB) expulsion rate (33.6%) after a 5-h short-time fertilization period, which included 34 cycles with no 2PB oocytes in the IVF group. For this group of patients, immediate rescue ICSI after short-time insemination could avoid complete IVF fertilization failure.

This retrospective study had a large sample size and was conducted at one IVF laboratory. The laboratory results were valuable in comparing the effect of ICSI and IVF treatment for sibling oocytes. However, based on the benefit to patients, the embryo transfer selection order decreased the clinical significance of this study. A further study on cumulative pregnancy rate in the future may provide more conclusive answers. A second limitation was the absence of exclusively IVF or exclusively ICSI matched groups based on the same infertility factors. Hence, further studies, including prospective, randomized, controlled trials, are required to evaluate the clinical significance of half-ICSI.

In conclusion, this study demonstrated that half-ICSI insemination may be successful for primary infertility patients; however, for secondary infertility patients, ICSI is not necessary and may be an excessive intervention. For patients with a lower fertilization failure rate in conventional IVF, the use of short-time insemination and rescue ICSI would be key. With concern for the safety of ART, we suggest that half-ICSI is not necessary for patients with normal semen and that ICSI should be used with more caution.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Center for Reproductive Medicine, Cheeloo College of Medicine, Shandong University.

## AUTHOR CONTRIBUTIONS

ML put forward the study question and designed the research. NP analyzed the data. NP and SM drafted the manuscript and ML revised it critically. All authors were involved in the acquisition of data and have approved the version to be published.

## FUNDING

The National Natural Science Foundation of China (81401266), The National Natural Science Foundation of China (82171842) and the Natural Science Foundation of Shandong Province (ZR2013HQ053). The funding sources had no role in the study design, collection, analysis, or interpretation of the data, writing the manuscript, or the decision to submit the paper for publication.

## ACKNOWLEDGMENTS

We thank all the participants of this study. We also thank all research staff at the Center for Reproductive Medicine, Cheeloo College of Medicine, Shandong University, for their cooperation throughout the study.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.877471/full#supplementary-material

## REFERENCES

1. Kuczyński W, Dhont M, Grygoruk C, Pietrewicz P, Redzko S, Szamatowicz M. Rescue ICSI of Unfertilized Oocytes After IVF. *Hum Reprod* (2002) 17:2423–7. doi: 10.1093/humrep/17.9.2423

2. Jaroudi K, Al-Hassan S, Al-Sufayan H, Al-Mayman H, Qeba M, Coskun S. Intracytoplasmic Sperm Injection and Conventional *In Vitro* Fertilization Are Complementary Techniques in Management of Unexplained Infertility. *J Assist Reprod Genet* (2003) 20:377–81. doi: 10.1023/a:1025433128518

3. Johnson LN, Sasson IE, Sammel MD, Dokras A. Does Intracytoplasmic Sperm Injection Improve the Fertilization Rate and Decrease the Total Fertilization Failure Rate in Couples With Well-Defined Unexplained Infertility? A Systematic Review and Meta-Analysis. *Fertil Steril* (2013) 100:704–11. doi: 10.1016/j.fertnstert.2013.04.038
4. Wang YQ, Yang J, Xu WM. Indications and Clinical Outcomes of Half-ICSI in 99 Cases. *Nat J Androl* (2009) 15:814–8.
5. Guo XY, Zhang JY, Lin DW, Yu Y, Ge MX. Fertilization Rates Following 109 Half Intracytoplasmic Sperm Injection Cycles for *In Vitro* Fertilization-Embryo Transfer. *J South Med Univ* (2010) 30:1920–2. doi: 10.12122/j.issn.1673-4254.2010.08.046
6. Sauerbrun-Cutler M, Huber WJ, Has P, Shen C, Hackett R, Alvero R, et al. Is Intracytoplasmic Sperm (ICSI) Better Than Traditional *In Vitro* Fertilization (IVF): Confirmation of Higher Blastocyst Rates Per Oocyte Using a Split Insemination Design. *J Assist Reprod Genet* (2020) 37:1661–7. doi: 10.1007/s10815-020-01819-1
7. Puissant F, Van Ryssellberge M, Barlow P, Deweze J, Leroy F. Embryo Scoring as a Prognostic Tool in IVF Treatment. *Hum Reprod* (1987) 2:705–8. doi: 10.1093/oxfordjournals.humrep.a136618
8. Gardner DK, Lane M. Culture and Selection of Viable Blastocysts: A Feasible Proposition for Human IVF? *Hum Reprod* (1997) 3:367–82. doi: 10.1093/humupd/3.4.367
9. Dieke AC, Mehta A, Kissin DM, Nangia AK, Warner L, Boulet SL. Intracytoplasmic Sperm Injection Use in States With and Without Insurance Coverage Mandates for Infertility Treatment, United States, 2000-2015. *Fertil Steril* (2018) 109:691–7. doi: 10.1016/j.fertnstert.2017.12.027
10. Li Z, Wang AY, Bowman M, Hammarberg K, Farquhar C, Johnson L, et al. ICSI Does Not Increase the Cumulative Live Birth Rate in non-Male Factor Infertility. *Hum Reprod* (2018) 33:1322–30. doi: 10.1093/humrep/dey118
11. Davies MJ, Moore VM, Willson KJ, Van Essen P, Priest K, Scott H, et al. Reproductive Technologies and the Risk of Birth Defects. *N Engl J Med* (2012) 366:1803–13. doi: 10.1056/nejmoa1008095
12. Xiong X, Dickey RP, Buekens P, Shaffer JG, Pridjian G. Use of Intracytoplasmic Sperm Injection and Birth Outcomes in Women Conceiving Through *In Vitro* Fertilization. *Paediatr Perinat Epidemiol* (2017) 31:108–15. doi: 10.1111/ppe.12339
13. Esteves SC, Roque M, Bedoschi G, Haahr T, Humaidan P. Intracytoplasmic Sperm Injection for Male Infertility and Consequences for Offspring. *Nat Rev Urol* (2018) 15:535–62. doi: 10.1038/s41585-018-0051-8
14. Cai H, Ren W, Wang H. Sex Ratio Imbalance Following Blastocyst Transfer Is Associated With ICSI But Not With IVF: An Analysis of 14,892 Single Embryo Transfer Cycles. *Assist Reprod Genet* (2022) 39:211–8. doi: 10.1007/s10815-021-02387-8
15. Belva F, Bonduelle M, Tournaye H. Endocrine and Reproductive Profile of Boys and Young Adults Conceived After ICSI. *Curr Opin Obstet Gynecol* (2019) 31:163–9. doi: 10.1097/GCO.0000000000000538
16. Chen RC, Kattera S. Rescue ICSI of Oocytes That Failed to Extrude the Second Polar Body 6 H Post-Insemination in Conventional IVF. *Hum Reprod* (2003) 18:2118–21. doi: 10.1093/humrep/deg325

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers | Frontiers in Endocrinology

ORIGINAL RESEARCH
published: 15 June 2022
doi: 10.3389/fendo.2022.870008

# Predictive Factors for Recovery Time in Conceived Women Suffering From Moderate to Severe Ovarian Hyperstimulation Syndrome

*Kai Huang*[1†], *Ying Shi*[1†], *Gezi Chen*[2], *Hao Shi*[1] *and Jun Zhai*[1*]

[1] *Center for Reproductive Medicine, Henan Key Laboratory of Reproduction and Genetics, Henan Provincial Obstetrical and Gynecological Diseases (Reproductive Medicine) Clinical Research Center, Henan Engineering Laboratory of Preimplantation Genetic Diagnosis and Screening, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China,* [2] *Department of Obstetrics, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China*

**Objective:** This study aimed to evaluate potential predictors for recovery time in pregnant patients with moderate to severe ovarian hyperstimulation syndrome (OHSS).

**Methods:** A total of 424 pregnant patients with moderate to severe OHSS who underwent *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI) were retrospectively identified. The clinical features and laboratory findings within 24 h after admission were collected. Treatment for OHSS was carried out according to standard procedures, including fluid replacement therapy, human albumin, aspirin, low-molecular-weight heparin, and paracentesis, when necessary. Patients were discharged from the hospital when the tmorning hematocrit was <40% and no obvious clinically relevant symptoms existed, such as abdominal distension, abdominal pain, and shortness of breath. Meanwhile, ultrasound indicating little pleural or abdominal effusion and biochemical abnormalities returning to normal were required. Spearman's correlation analysis was used to assess the association between the blood-related parameters and recovery time. Multiple linear regression models were used to assess the relationship between the clinical or laboratory parameters and recovery time.

**Results:** The median recovery time of these patients was 11 days. In Spearman's correlation test, leukocytes, hemoglobin, platelets, hematocrit, creatinine, prothrombin time (PT), fibrinogen (Fib), D-dimer, and fibrinogen degradation products (FDPs) were positively correlated with recovery time. On the other hand, albumin and thrombin time (TT) were negatively correlated with recovery time. Multiple linear regression analysis showed that polycystic ovary syndrome (PCOS), hemoglobin, platelets, albumin, and Fib were significantly associated with the recovery time of patients with OHSS ($p = 0.023$, $p < 0.001$, $p = 0.007$, $p < 0.001$, and $p = 0.019$, respectively).

**Conclusions:** In pregnant patients with OHSS, PCOS and hypoalbuminemia were associated with a significantly longer recovery time. Meanwhile, the recovery time was longer when patients have high levels of hemoglobin, platelets, and Fib.

**Keywords: ovarian hyperstimulation syndrome (OHSS), clinical features, recovery time, potential predictors, coagulation function**

OPEN ACCESS

***Edited by:***
*Richard Ivell,*
*University of Nottingham,*
*United Kingdom*

***Reviewed by:***
*LIANG HU,*
*Central South University, China*
*Mengge LI,*
*Central South University, China*

***Correspondence:**
*Jun Zhai*
*bestzhai2005@163.com*

*[†]These authors have contributed equally to this work and share first authorship*

***Specialty section:***
*This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology*

***Received:*** *05 February 2022*
***Accepted:*** *11 April 2022*
***Published:*** *15 June 2022*

***Citation:***
*Huang K, Shi Y, Chen G, Shi H and Zhai J (2022) Predictive Factors for Recovery Time in Conceived Women Suffering From Moderate to Severe Ovarian Hyperstimulation Syndrome. Front. Endocrinol. 13:870008. doi: 10.3389/fendo.2022.870008*

## INTRODUCTION

Ovarian hyperstimulation syndrome (OHSS) is a self-limiting disease classically encountered in patients who undergo controlled ovarian hyperstimulation (COH) cycles. The mild manifestations of OHSS include nausea, vomiting, abdominal distension, and shortness of breath. In severe cases, ascites and pleural fluid may occur, causing respiratory, circulatory, and coagulation dysfunction, and especially thrombosis may endanger a patient's life (1).

Evidence from initial investigations indicates that some women are at increased risk of OHSS. The risk factors include young age (2, 3), body mass index (BMI) (4, 5), diagnosis of polycystic ovary syndrome (PCOS) (3, 6) high anti-müllerian hormone (AMH) levels (7, 8), large number and size of follicles in the ovary (8, 9), high serum estradiol (E2) concentrations (3, 9, 10) high number of retrieved oocytes (3, 9, 11), pregnancy following fresh embryo transfer (12), and a history of OHSS (13). The exact cause of OHSS is currently complex and remains subject to controversy. Latest research has demonstrated that OHSS is related to age, BMI, ovarian function, and the ovulation stimulation protocol.

Evidence has shown that OHSS occurs only after exposure to human chorionic gonadotropin (hCG), which has a significantly longer half-life than that of luteinizing hormone (LH) and a higher receptor affinity, thus causing extensive luteinization in the granulosa cells within the corpus luteum (14). This, in turn, leads to the production of vasoactive substances, including vascular endothelial growth factor (VEGF), renin–angiotensin system, interleukin 6, interleukin 1b, angiotensin II, insulin-like growth factor 1, and transforming growth factor b, of which VEGF is the most important in causing increased vascular permeability and hemoconcentration (15–17). VEGF stimulates endothelial cell mitogenesis and renders capillaries highly permeable to high-molecular-weight proteins (15). The pathophysiology of OHSS is characterized by arteriolar vasodilation and an increase in capillary permeability, leading to the leakage of fluid from the vascular compartment, with third space fluid accumulation and intravascular dehydration, causing intravascular volume depletion, hemoconcentration, hypoalbuminemia, electrolyte imbalance, and even thrombosis.

Whereas treatment for OHSS is largely supportive, prevention is crucial. Most current studies have been devoted to prevention and treatment strategies for OHSS, with relatively little attention paid to its clinical prognosis. This study provides clinicians with potential predictors of time to cure by describing some clinical features and laboratory findings in pregnant patients with OHSS.

## MATERIALS AND METHODS

### Study Population

This retrospective study was performed in the Reproductive Medical Center of The First Affiliated Hospital of Zhengzhou University, Henan Province, China. Patients who underwent *in vitro* fertilization (IVF)/intracytoplasmic single sperm injection (ICSI) after assisted conception with late-onset moderate to severe OHSS between January 2018 and December 2020 were selected. The access and processing of patient data was approved by the ethics committee under a protocol for retrospective studies. The inclusion criteria were as follows: 1) diagnosis of OHSS according to the Golan criteria; 2) patients with IVF/ICSI-assisted pregnancy in the first cycle; 3) patients given a long-acting gonadotropin-releasing hormone (GnRH) agonist by subcutaneous injection; 4) patients with a positive pregnancy test; and 5) age <35 years. The exclusion criteria included: 1) women treated with antithrombotic drugs; 2) women with known coagulopathies; and 3) uncertain laboratory results and missing laboratory data. To analyze the relationship between the recovery time of patients with OHSS and the blood parameters such as leukocyte, hemoglobin, platelet, hematocrit, creatinine, total protein, albumin, prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), fibrinogen (Fib), D-dimer, and fibrinogen degradation products (FDPs) within 24 h after admission.

Treatment of OHSS usually involves fluid replacement to maintain intravascular perfusion and supportive care, such as low-molecular-weight dextrose and hydroxyethyl starch. The patient's blood count, coagulation profile, electrolytes, creatinine, and albumin were observed. Depending on the patient's condition, albumin was given intravenously, and anticoagulant drugs were given to patients with thrombotic tendency and hypercoagulable state to prevent thrombosis (18). Moreover, when the patient has large quantities of pleural and ascites, puncture and drainage were performed under ultrasound guidance. The details of the patients' treatments are shown in **Table 1**.

A patient is clinically cured when the morning hematocrit was <40% and no obvious clinically relevant symptoms existed, such as abdominal distension, abdominal pain, and shortness of breath (19). On the other hand, ultrasound should indicate no pleural and abdominal effusion or a small amount of effusion, and the leukocyte count, creatinine, albumin, alanine transaminase (ALT), aspartate transaminase (AST), electrolytes, and other biochemical indicators should return to normal. The discharge criteria were considered the patients' cure criteria.

### Laboratory Variables

The patients underwent basic blood routine, liver and kidney function, blood coagulation function, D-dimer, FDP, and other tests 24 h after admission. The levels of these parameters were measured using the Roche HP800 automatic biochemical analyzer and Sysmex series automatic blood analyzer.

**TABLE 1** | Conventional intervention for ovarian hyperstimulation syndrome (OHSS).

| Treatment | *n* | % |
|---|---|---|
| Heparin | 46 | 10.8 |
| Albumin | 321 | 75.7 |
| Paracentesis | | |
| Peritoneal puncture | 219 | 51.7 |
| Pleural puncture | 52 | 12.3 |

## Controlled Ovarian Hyperstimulation Protocol

On the second to the third day of menstruation, the patients were given a long-acting GnRH agonist (Diphereline, 3.75 mg; Beaufour-Ipsen, Dreux, France) by subcutaneous injection. After 30 days, when the follicle-stimulating hormone (FSH) level was <5 IU/L, the LH level was <3 IU/L, and the antral follicle was nearly 5 mm in diameter, COH was initiated. We determined the individualized dosage of gonadotropin (Gn) (GONAL-f; Merck Serono, Darmstadt, Germany) according to the patient's age, BMI, and ovarian reserve. The Gn dosage was maintained or adjusted according to the follicle growth and serum hormone levels during the course of the drug administration. When one dominant follicle was ≥20 mm in diameter and at least three dominant follicles were ≥17 mm in diameter, a trigger injection of hCG (recombinant hCG alpha for injection; Merck Serono) was administered on the same night. After 36–37 h of the trigger injection, we performed transvaginal oocyte retrieval; the luteal phase support was routinely given approximately 14 days after oocyte retrieval.

Two fresh cleavage embryos or one blastocyst was transferred on day 3 or 5 after egg retrieval. The transplant was cancelled if the patient was deemed at high risk of OHSS, the P level on the day of hCG was >3 ng/ml, or a uterine effusion was demonstrated.

## Statistical Analysis

All statistical analyses were conducted using IBM SPSS Statistics for Windows, version 26.0 (IBM Corp., Armonk, NY, USA). Descriptive variables were expressed as the mean and standard deviation (SD) if the data were normally distributed, as median and interquartile range (IQR) if the data were not normally distributed, or as frequency and percentage for nominal data. Spearman's correlation analysis was used to evaluate the associations between the variables of interest and the clinical outcomes. Multiple linear regression analysis was used when the outcomes were continuous variables. A bilateral $p$-value <0.05 was considered as significant.

# RESULTS

A total of 424 pregnant patients who developed moderate to severe OHSS after IVF/ICSI treatment were included in this study. **Table 2** summarizes the basic information of these patients. The median recovery time of these patients was 11 days. A detailed distribution of the recovery times is shown in **Figure 1**.

The patients' primary laboratory findings are shown in **Table 3**. Spearman's correlation coefficients were calculated between the patients' recovery times and the laboratory indices examined on the day of their admission to the hospital. The levels of leukocytes, hemoglobin, platelets, hematocrit, creatinine, albumin, PT, Fib, TT, D-dimer, and FDPs were correlated with the time to healing. Leukocytes, hemoglobin, platelets, hematocrit, creatinine, PT, Fib, D-dimer, and FDP were positively correlated with recovery time. On the contrary, albumin and TT were negatively correlated with recovery time.

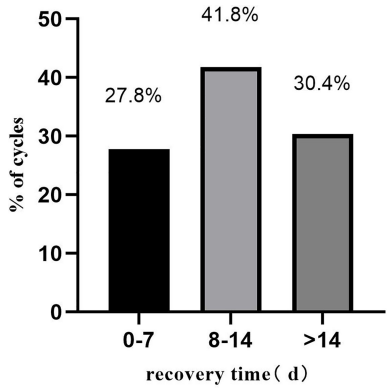



**FIGURE 1** | The recovery time distribution of the 424 OHSS patients.

All data with $p < 0.05$ in the above-mentioned correlation analysis and the high-risk factors affecting OHSS reported in the literature were included in the multiple linear regression. The results showed that PCOS, hemoglobin, platelets, albumin, and Fib significantly influenced the patients' recovery times ($p = 0.023$, $p < 0.001$, $p = 0.007$, $p < 0.001$, and $p = 0.019$, respectively) (**Table 4**). Moreover, scatter diagrams were used to clearly describe the relationship between the recovery time and the levels of hemoglobin, platelets, albumin, and Fib (**Figure 2**). The remaining indicators were not highly correlated with the recovery time of patients.

# DISCUSSION

OHSS is a major iatrogenic complication that arises during the process of assisted reproductive technology (ART), affecting 0.5%–2% of IVF cycles. There are two distinct types of OHSS: early-onset OHSS occurs in response to the hCG trigger within 7 days of ovulation, while late-onset OHSS is caused by the rising hCG hormone levels produced by the placenta in conception cycles (10). The present study focused on the recovery time of conceived women with OHSS requiring hospitalization.

To date, there is no universally accepted definition of OHSS recovery. The definition used in this research was based on a previous study (19). Patients were discharged from the hospital when the morning hematocrit was <40% and no obvious clinically relevant symptoms existed, such as abdominal distension, abdominal pain, and shortness of breath. On the other hand, ultrasound indicating little pleural or abdominal effusion and biochemical abnormalities returning to normal were required. The median recovery time in our study population was 11 days. This is much longer than that reported in the literature, partly due to the particular population and our strict release criteria.

Current studies have revealed that the incidence of OHSS was associated with numerous clinical and laboratory parameters. The concentration of E2 on the day of hCG >4,500 pg/ml and the number of oocytes retrieved >15 are commonly proposed to be

**TABLE 2 |** Baseline characteristics and cycle outcomes.

| Variables | Measures |
|---|---|
| Age (years) | 30 (27–31) |
| BMI (kg/m$^2$) | 20.8 (19.6–22.8) |
| AMH (μg/L) | 4.27 (2.83–6.33) |
| Type of infertility (*n*, %) | |
| Primary infertility | 241 (56.8%) |
| Secondary infertility | 183 (43.2%) |
| Fertilization method (*n*, %) | |
| IVF | 321 (75.7%) |
| ICSI | 103 (24.3%) |
| PCOS (*n*, %) | 51 (12%) |
| Gn use duration (days) | 13 (12–14) |
| Gn dosage (IU) | 1,662.5 (1,350–2,162.5) |
| Serum LH level on hCG day (mIU/ml) | 0.91 (0.5–1.79) |
| Serum E2 level on hCG day (pg/ml) | 3,437 (2,370.5–4,769) |
| Serum P level on hCG day (ng/ml) | 0.81 (0.51–1.25) |
| No. of oocytes retrieved | 13 (11–17) |
| Cleavage embryo or blastocyst (day 3 or 5) | |
| Day 3 | 315 (74.3%) |
| Day 5 | 109 (25.7%) |
| Severity of OHSS (*n*, %) | |
| Moderate | 181 (42.7%) |
| Severe | 243 (57.3) |
| Recovery time (d) | 11 (7–15) |
| Pregnancy rate (n, %) | 386 (91.0%) |
| Multiple pregnancy rate (n, %) | 151 (35.6%) |

*BMI, body mass index; AMH, anti-müllerian hormone; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; PCOS, polycystic ovary syndrome; LH, luteinizing hormone; E2, estradiol; P, progesterone; OHSS, ovarian hyperstimulation syndrome; hCG, human chorionic gonadotropin.*

risk factors for OHSS (3). However, our results showed that the median concentration of E2 on the day of hCG was 3,437 pg/ml and that the median number of oocytes retrieved was 13. This is consistent with the study of Wiser et al. because the freeze-all strategy was carried out in women at high risk of OHSS after retrieval of oocytes (20). In addition, the concentration of E2 on the day of hCG is not the main reason for the late-onset OHSS: pregnancy remains the main cause (21). Despite scholars having proposed multiple pregnancy as a predictive factor for recovery time from OHSS, our research found no differences between patients with singleton and multiple pregnancies.

As demonstrated in **Table 4**, the presence of PCOS was negatively correlated with the recovery time of pregnant OHSS patients, which is in accordance with the result of Nouri et al. (19). PCOS appeared to be the major predisposing factor for OHSS in a large number of studies (2). The explanation might be that PCOS cases are known to produce three times more follicles and oocytes than do normal ovulation patients when stimulated according to similar protocols (6). However, the reason for the prolonged recovery time of patients with PCOS remains unknown. VEGF is thought to be the major mediator of OHSS (22). An increased expression of VEGF mRNA in women with PCOS has been reported, and this may be responsible for the prolonged recovery time (23). Nevertheless, this is completely unproven. Further investigations are necessary to confirm this hypothesis. It is our suggestion that more stringent embryo transfer criteria should be administered or that patients with PCOS undergo whole embryo cryopreservation.

The second predictive factor for recovery time is serum albumin. Hypoalbuminemia is associated with a significantly longer recovery time. The mechanisms underlying the potential effect of serum albumin on OHSS are unknown. Some studies have suggested that the binding properties of albumin are beneficial in neutralizing vascular permeability mediators (24). Other studies have shown that serum albumin could maintain the intravascular volume in the event of capillary leakage, thus avoiding hypovolemia and hemoconcentration (25). Therefore, the level of serum albumin may reflect the severity of OHSS.

Studies have demonstrated that the levels of leukocytes, platelets, hematocrit, Fib, D-dimer, and FDP in OHSS patients were higher in routine laboratory tests (26). As shown in **Table 3**, this is consistent with our study. Moreover, in multiple linear regression analysis, hemoglobin, platelets, and Fib were

**TABLE 3 |** Relationship between the laboratory findings and recovery time of patients with ovarian hyperstimulation syndrome (OHSS).

| Laboratory findings | Normal range | Measures | Spearman's correlation | |
|---|---|---|---|---|
| | | | Correlation coefficient | *p*-value |
| Leukocyte (10$^9$/L) | 3.5–9.5 | 12.97 (10.6–15.91) | 0.401 | <0.001 |
| Hemoglobin (g/L) | 115–150 | 143.65 (135.25–153) | 0.494 | <0.001 |
| Platelets (10$^9$/L) | 125–350 | 340.5 (293–386) | 0.245 | <0.001 |
| Hematocrit (L/L) | 0.35–0.45 | 0.429 (0.402–0.458) | 0.478 | <0.001 |
| Creatinine (mmol/L) | 20–115 | 58.75 (52–66.18) | 0.352 | <0.001 |
| ALT (U/L) | 0–40 | 26 (15.25–41) | –0.027 | 0.577 |
| AST (U/L) | 0–58 | 22.5 (17–32.75) | –0.071 | 0.146 |
| Albumin (g/L) | 35–55 | 37.5 (34.9–40.1) | –0.202 | <0.001 |
| PT (s) | 8.8–13.6 | 10.3 (9.9–10.8) | 0.151 | 0.002 |
| APTT (s) | 26–40 | 28.3 (26.6–30.2) | –0.075 | 0.125 |
| Fib (g/L) | 2–4 | 4.65 (4.3–5.32) | 0.175 | <0.001 |
| TT (s) | 10–18 | 12.6 (12.1–13.1) | –0.113 | 0.020 |
| D-dimer (mg/L) | 0–0.3 | 0.70 (0.52–0.95) | 0.136 | 0.005 |
| FDP (mg/L) | 0–5 | 7.23 (5.19–10.54) | 0.114 | 0.018 |

*ALT, alanine aminotransferase; AST, aspartate transaminase; PT, prothrombin time; APTT, activated partial thromboplastin time; TT, thrombin time; Fib, fibrinogen; FDP, fibrinogen degradation products.*

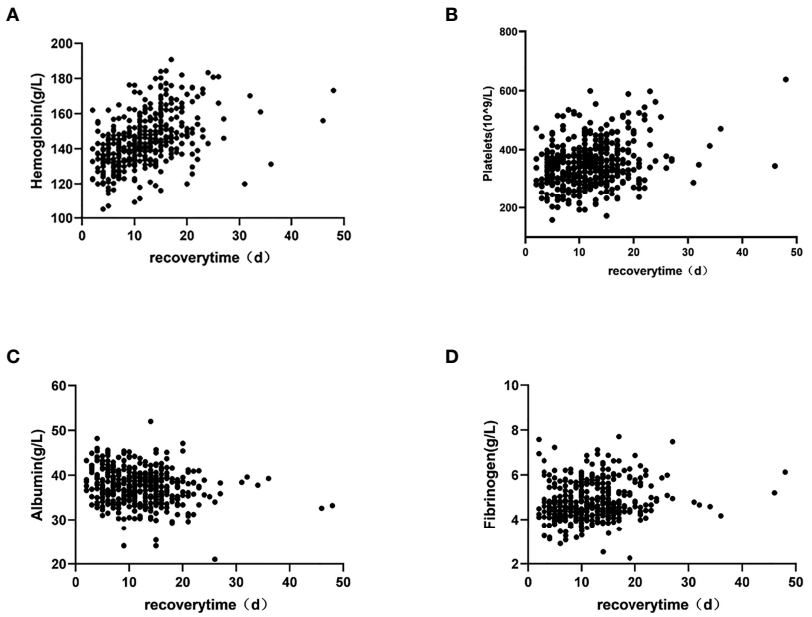



**FIGURE 2** | Relationship between the recovery time and hemoglobin, platelets, albumin and Fib.

positively correlated with the recovery time, and the difference was statistically significant. There are two reasons for the changes in these blood-related parameters: stress and hemoconcentration (27). OHSS is a potentially lethal disease, the pathophysiological hallmark of which is massive extravascular exudate accumulation combined with profound intravascular volume depletion and hemoconcentration (28). The degree of hemoconcentration seems to have the best correlation with the severity of OHSS (29). The levels of hemoglobin, platelets, and Fib are the earliest and most sensitive indicators of changes in the blood and are also ideal predictors for recovery time from OHSS.

It seems that an elevation in circulating estrogens during ovulation induction causes a shift in the hemostatic balance in the direction of a procoagulable state. Fib is a conventional

**TABLE 4** | Multiple linear regression analysis of factors affecting recovery time.

| Indexes | Unstandardized coefficients | | Standardized coefficients | *t* value | *p*-value | Collinearity statistics | |
|---|---|---|---|---|---|---|---|
| | *B* | SE | *β* | | | Tolerance | VIF |
| Age (years) | –0.006 | 0.089 | –0.003 | –0.066 | 0.947 | 0.939 | 1.065 |
| BMI (kg/m$^2$) | –0.130 | 0.104 | –0.055 | –1.244 | 0.214 | 0.901 | 1.110 |
| AMH (μg/L) | –0.086 | 0.082 | –0.049 | –1.051 | 0.294 | 0.805 | 1.242 |
| PCOS | –1.988 | 0.871 | –0.105 | –2.281 | 0.023 | 0.821 | 1.218 |
| Serum E2 level on hCG day (pg/ml) | 0.000 | 0.000 | 0.005 | 0.102 | 0.919 | 0.877 | 1.141 |
| No. of oocytes retrieved | 0.073 | 0.059 | 0.061 | 1.245 | 0.214 | 0.733 | 1.365 |
| Cleavage embryo or blastocyst | –0.314 | 0.582 | –0.028 | –0.540 | 0.589 | 0.669 | 1.495 |
| Clinical pregnancy (singleton or multiple) | –0.190 | 0.607 | –0.015 | –0.312 | 0.755 | 0.779 | 1.284 |
| Leukocyte (10$^9$/L) | 0.077 | 0.079 | 0.057 | 0.981 | 0.327 | 0.523 | 1.910 |
| Hemoglobin (g/L) | 0.146 | 0.024 | 0.349 | 6.213 | <0.001 | 0.552 | 1.811 |
| Platelets (10$^9$/L) | 0.010 | 0.004 | 0.129 | 2.690 | 0.007 | 0.752 | 1.329 |
| Hematocrit (L/L) | 0.039 | 0.069 | 0.025 | 0.570 | 0.569 | 0.939 | 1.065 |
| Creatinine (mmol/L) | 0.015 | 0.026 | 0.029 | 0.562 | 0.575 | 0.656 | 1.523 |
| Albumin (g/L) | –0.239 | 0.067 | –0.154 | –3.587 | <0.001 | 0.944 | 1.060 |
| PT (s) | 0.046 | 0.052 | 0.037 | 0.880 | 0.379 | 0.981 | 1.019 |
| Fib (g/L) | 0.835 | 0.356 | 0.113 | 2.348 | 0.019 | 0.755 | 1.324 |
| TT (s) | –0.227 | 0.269 | –0.043 | –0.843 | 0.400 | 0.662 | 1.510 |
| D-dimer (mg/L) | 0.537 | 0.553 | 0.056 | 0.969 | 0.333 | 0.517 | 1.934 |
| FDP (g/L) | –0.032 | 0.071 | –0.024 | –0.442 | 0.659 | 0.587 | 1.705 |

*AMH, anti-müllerian hormone; PCOS, polycystic ovary syndrome; hCG, human chorionic gonadotropin; PT, prothrombin time; Fib, fibrinogen; TT, thrombin time; FDP, fibrinogen degradation product.*

coagulation indicator that indicates the activation of the coagulation system. Coagulation causes an increased Fib consumption and promotes Fib synthesis in the body, resulting in increased plasma Fib levels. Thrombosis is the most serious complication of OHSS, leading to dysfunction of coagulation and fibrinolysis *in vivo*. Thrombosis causes secondary hyperfibrinolysis; as plasma D-dimer and FDP are degradation products of fibrinogen, their levels will therefore increase rapidly. Therefore, monitoring the levels of plasma Fib, D-dimer, and FDP can prompt clinical correction of hypercoagulable blood concentration. Heparin ameliorates the risk of thrombotic complications associated with OHSS and has become the recommended treatment protocol (30). VEGF plays a leading role in increasing vascular permeability, and a 5-kDa heparin fragment can inhibit VEGF-A-mediated angiogenesis (31). Tissue factor (TF) is also important in angiogenesis because it enhances the expression of VEGF-A, and heparin reduces this by inhibiting the release of TF (32).

This is the first study to comprehensively assess the coagulation function and recovery time of conceived women with moderate to severe OHSS. As a result of OHSS-specific symptoms and the costs of the treatment program, decreased quality of life and economic losses are inevitable. Therefore, recovery time should be a clinically important parameter, and our study is of great value. Simultaneously, this study has some limitations. We conducted this retrospective study without considering all confounding factors. Only patients receiving a particular ovulation induction program were included in our study. Moreover, differences in the dietary habits of patients during hospitalization have a certain impact on the recovery time from OHSS.

In general, the existence of PCOS and the levels of hemoglobin, platelets, albumin, and Fib may contribute to the prognostic evaluation of OHSS. In consideration of the particular study population and limitations, large-scale, multicenter, prospective studies are necessary to confirm our results.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The First Affiliated Hospital of Zhengzhou University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

## AUTHOR CONTRIBUTIONS

KH and JZ designed the research and guided the writing. YS collected and analyzed the data. KH and YS drafted the manuscript. G-ZC helped to collect and analyze data. HS contributed to the data analysis. All authors contributed to the article and approved the submitted version.

## FUNDING

This study was supported by the National Natural Science Foundation of China (nos. 81701448 and 82071649).

## ACKNOWLEDGMENTS

We would like to thank the medical staff and patients in The First Affiliated Hospital of Zhengzhou University for recording the data and cooperating with the treatment.

## REFERENCES

1. ASRM. Prevention and Treatment of Moderate and Severe Ovarian Hyperstimulation Syndrome: A Guideline. *Fertil Steril* (2016) 106(7):1634–47. doi: 10.1016/j.fertnstert.2016.08.048
2. Luke B, Brown MB, Morbeck DE, Hudson SB. Factors Associated With Ovarian Hyperstimulation Syndrome (OHSS) and its Effect on Assisted Reproductive Technology (ART) Treatment and Outcome. *Fertil Steril* (2010) 94(4):1399–404. doi: 10.1016/j.fertnstert.2009.05.092
3. Sousa M, Cunha M, Teixeira da Silva J, Oliveira C, Silva J, Viana P, et al. Ovarian Hyperstimulation Syndrome: A Clinical Report on 4894 Consecutive ART Treatment Cycles. *Reprod Biol Endocrinol* (2015) 13:66. doi: 10.1186/s12958-015-0067-3
4. Fauser BC, Diedrich K, Devroey P. Evian Annual Reproduction Workshop Group 2007. Predictors of Ovarian Response: Progress Towards Individualized Treatment in Ovulation Induction and Ovarian Stimulation. *Hum Reprod Update* (2008) 14(1):1–14. doi: 10.1093/humupd/dmm034
5. Courbiere B, Oborski V, Braunstein D, Desparoir A, Noizet A, Gamerre M. Obstetric Outcome of Women With *In Vitro* Fertilization Pregnancies Hospitalized for Ovarian Hyperstimulation Syndrome: A Case-Control Study. *Fertil Steril* (2011) 95(5):1629–32. doi: 10.1016/j.fertnstert.2010.12.015
6. Delvigne A, Rozenberg S. Epidemiology and Prevention of Ovarian Hyperstimulation Syndrome (OHSS): A Review. *Hum Reprod Update* (2002) 8(6):559–77. doi: 10.1093/humupd/8.6.559
7. Nakhuda GS, Chu MC, Wang JG, Sauer MV, Lobo RA. Elevated Serum Müllerian-Inhibiting Substance may be a Marker for Ovarian Hyperstimulation Syndrome in Normal Women Undergoing *In Vitro* Fertilization. *Fertil steril* (2006) 85(5):1541–3. doi: 10.1016/j.fertnstert.2005.10.052
8. Ocal P, Sahmay S, Cetin M, Irez T, Guralp O, Cepni I. Serum Anti-Mullerian Hormone and Antral Follicle Count as Predictive Markers of OHSS in ART Cycles. *J Assist Reprod Genet* (2011) 28(12):1197–203. doi: 10.1007/s10815-011-9627-4
9. Ashrafi M, Bahmanabadi A, Akhond MR, Arabipoor A. Predictive Factors of Early Moderate/Severe Ovarian Hyperstimulation Syndrome in non-Polycystic Ovarian Syndrome Patients: A Statistical Model. *Arch Gynecol Obstet* (2015) 292(5):1145–52. doi: 10.1007/s00404-015-3723-0
10. Mathur RS, Akande AV, Keay SD, Hunt LP, Jenkins JM. Distinction Between Early and Late Ovarian Hyperstimulation Syndrome. *Fertil Steril* (2000) 73(5):901–7. doi: 10.1016/s0015-0282(00)00492-1
11. Steward RG, Lan L, Shah AA, Yeh JS, Price TM, Goldfarb JM, et al. Oocyte Number as a Predictor for Ovarian Hyperstimulation Syndrome and Live Birth: An Analysis of 256,381 *In Vitro* Fertilization Cycles. *Fertil Steril* (2014) 101(4):967–73. doi: 10.1016/j.fertnstert.2013.12.026
12. Enskog A, Henriksson M, Unander M, Nilsson L, Brannstrom M. Prospective Study of the Clinical and Laboratory Parameters of Patients in Whom Ovarian Hyperstimulation Syndrome Developed During Controlled Ovarian Hyperstimulation for *In Vitro* Fertilization. *Fertil Steril* (1999) 71(5):808–14. doi: 10.1016/s0015-0282(99)00090-4

13. Delvigne A, Dubois M, Battheu B, Bassil S, Meuleman C, De Sutter P, et al. The Ovarian Hyperstimulation Syndrome in in-Vitro Fertilization: A Belgian Multicentric Study. II. Multiple Discriminant Analysis for Risk Prediction. *Hum Reprod* (1993) 8(9):1361–6. doi: 10.1093/oxfordjournals.humrep.a138261
14. Aboulghar MA, Mansour RT. Ovarian Hyperstimulation Syndrome: Classifications and Critical Analysis of Preventive Measures. *Hum Reprod Update* (2003) 9(3):275–89. doi: 10.1093/humupd/dmg018
15. McClure N, Healy DL, Rogers PA, Sullivan J, Beaton L, Haning RVJr, et al. Vascular Endothelial Growth Factor as Capillary Permeability Agent in Ovarian Hyperstimulation Syndrome. *Lancet* (1994) 344(8917):235–6. doi: 10.1016/s0140-6736(94)93001-5
16. Soares SR, Gómez R, Simón C, García-Velasco JA, Pellicer A. Targeting the Vascular Endothelial Growth Factor System to Prevent Ovarian Hyperstimulation Syndrome. *Hum Reprod Update* (2008) 14(4):321–33. doi: 10.1093/humupd/dmn008
17. Geva E, Jaffe RB. Role of Vascular Endothelial Growth Factor in Ovarian Physiology and Pathology. *Fertil Steril* (2000) 74(3):429–38. doi: 10.1016/s0015-0282(00)00670-1
18. Choux C, Barberet J, Ginod P, Cottenet J, Bruno C, Benzénine E, et al. Severe Ovarian Hyperstimulation Syndrome Modifies Early Maternal Serum Beta-Human Chorionic Gonadotropin Kinetics, But Obstetrical and Neonatal Outcomes are Not Impacted. *Fertil Steril* (2017) 108(4):650–8. doi: 10.1016/j.fertnstert.2017.07.1160
19. Nouri K, Tempfer CB, Lenart C, Windischbauer L, Walch K, Promberger R, et al. Predictive Factors for Recovery Time in Patients Suffering From Severe OHSS. *Reprod Biol Endocrinol* (2014) 5(12):59. doi: 10.1186/1477-7827-12-59
20. Wiser A, Levron J, Kreizer D, Achiron R, Shrim A, Schiff E, et al. Outcome of Pregnancies Complicated by Severe Ovarian Hyperstimulation Syndrome (OHSS): A Follow-Up Beyond the Second Trimester. *Hum Reprod* (2005) 20(4):910–4. doi: 10.1093/humrep/deh713
21. Salama KM, Abo Ragab HM, El Sherbiny MF, Morsi AA, Souidan II. Sequential E2 Levels Not Ovarian Maximal Diameter Estimates Were Correlated With Outcome of Cetrotide Therapy for Management of Women at High-Risk of Ovarian Hyperstimulation Syndrome: A Randomized Controlled Study. *BMC Womens Health* (2017) 17(1):108. doi: 10.1186/s12905-017-0466-z
22. Fiedler K, Ezcurra D. Predicting and Preventing Ovarian Hyperstimulation Syndrome (OHSS): The Need for Individualized Not Standardized Treatment. *Reprod Biol Endocrinol* (2012) 10(1):32. doi: 10.1186/1477-7827-10-32
23. Kamat BR, Brown LF, Manseau EJ, Senger DR, Dvorak HF. Expression of Vascular Permeability Factor/Vascular Endothelial Growth Factor by Human Granulosa and Theca Lutein Cells. Role in Corpus Luteum Development. *Am J Pathol* (1995) 146(1):157–65. doi: 10.1097/00002093-199509020-00009
24. Youssef MA, Al-Inany HG, Evers JL, Aboulghar M. Intra-Venous Fluids for the Prevention of Severe Ovarian Hyperstimulation Syndrome. *Cochrane Database Syst Rev* (2011) 2(2):CD001302. doi: 10.1002/14651858.CD001302.pub2
25. Nastri CO, Teixeira DM, Moroni RM, Leitão VM, Martins WP. Ovarian Hyperstimulation Syndrome: Pathophysiology, Staging, Prediction and Prevention. *Ultrasound Obstet Gynecol* (2015) 45(4):377–93. doi: 10.1002/uog.14684
26. Levin I, Gamzu R, Hasson Y, Lessing JB, Amit A, Shapira I, et al. Increased Erythrocyte Aggregation in Ovarian Hyperstimulation Syndrome: A Possible Contributing Factor in the Pathophysiology of This Disease. *Hum Reprod* (2004) 19(5):1076–80. doi: 10.1093/humrep/deh210
27. Navot D, Bergh PA, Laufer N. Ovarian Hyperstimulation Syndrome in Novel Reproductive Technologies: Prevention and Treatment. *Fertil Steril* (1992) 58(2):249–61. doi: 10.1016/s0015-0282(16)55188-7
28. Schenker JG, Weinstein D. Ovarian Hyperstimulation Syndrome: A Current Survey. *Fertil Steril* (1978) 30(3):255–68. doi: 10.1016/s0015-0282(16)43508-9
29. Borenstein R, Elhalah U, Lunenfeld B, Schwartz ZS. Severe Ovarian Hyperstimulation Syndrome: A Reevaluated Therapeutic Approach. *Fertil Steril* (1989) 51(5):791–5. doi: 10.1016/s0015-0282(16)60668-4
30. Dourron NE, Williams DB. Prevention and Treatment of Ovarian Hyperstimulation Syndrome. *Semin Reprod Endocrinol* (1996) 14(4):355–65. doi: 10.1055/s-2008-1067980
31. Norrby K. 2.5 kDa and 5.0 kDa Heparin Fragments Specifically Inhibit Microvessel Sprouting and Network Formation in VEGF165-Mediated Mammalian Angiogenesis. *Int J Exp Pathol* (2000) 81(3):191–8. doi: 10.1046/j.1365-2613.2000.00150.x
32. Bobek V, Kovaŕik J. Antitumor and Antimetastatic Effect of Warfarin and Heparins. *BioMed Pharmacother* (2004) 58(4):213–9. doi: 10.1016/j.biopha.2003.11.007

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Endocrinology

ORIGINAL RESEARCH
published: 21 June 2022
doi: 10.3389/fendo.2022.884972

# Effects of Anticoagulants and Immune Agents on Pregnancy Outcomes and Offspring Safety in Frozen-Thawed Embryo Transfer Cycles—A Retrospective Cohort Study

*Yanli Fan†, Yizhuo Wang†, Zhuoye Luo, Yueming Xu, Jie Zhang, Wei Wang, Na Cui and Guimin Hao**

*Hebei Key Laboratory of Infertility and Genetics, Hebei Clinical Research Center for Birth Defects, Department of Reproductive Medicine, Second Hospital of Hebei Medical University, Shijiazhuang, China*

OPEN ACCESS

**Edited by:**
*Yimin Zhu,*
*Zhejiang University, China*

**Reviewed by:**
*Shuo Li,*
*Shandong University, China*
*Rong Li,*
*Peking University Third Hospital, China*
*Lixue Chen,*
*Peking University Third Hospital, China*
*Fan Jin,*
*Zhejiang University, China*

***Correspondence:**
*Guimin Hao*
*haoguimin@163.com*

*†These authors have contributed equally to this work*

**Specialty section:**
*This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology*

**Received:** *27 February 2022*
**Accepted:** *09 May 2022*
**Published:** *21 June 2022*

**Citation:**
*Fan Y, Wang Y, Luo Z, Xu Y, Zhang J, Wang W, Cui N and Hao G (2022) Effects of Anticoagulants and Immune Agents on Pregnancy Outcomes and Offspring Safety in Frozen-Thawed Embryo Transfer Cycles—A Retrospective Cohort Study. Front. Endocrinol. 13:884972. doi: 10.3389/fendo.2022.884972*

The application of anticoagulants and immune agents in assisted reproduction technology has been in a chaotic state, and no clear conclusion has been reached regarding the effectiveness and safety of this treatment. We aimed to explore the potential association between adjuvant medication and pregnancy outcomes and offspring safety in a retrospective cohort study including 8,873 frozen-thawed embryo transfer cycles. The included cycles were divided into three groups according to the drugs used, namely, the routine treatment group (without anticoagulant agents and immune agents), the anticoagulant agent group, and the immunotherapy group. Among normal ovulatory patients, those who used immune agents had a 1.4-fold increased risk of miscarriage (≤13 weeks), but a 0.8-fold decreased chance of birth (≥28 weeks) compared with the routine treatment group. Among patients with more than 1 embryo transferred, those who used anticoagulant agents showed a 1.2-fold higher risk of multiple birth than those undergoing routine treatment. Among patients without pregnancy complications, anticoagulant treatment was associated with a 2.1-fold increased risk of congenital anomalies. Among young patients (<26 years) with a singleton pregnancy, the neonatal birth weight of the immunotherapy group and the anticoagulant treatment group was 305.4 g and 175.9 g heavier than the routine treatment group, respectively. In conclusion, adjuvant anticoagulants or immune agent treatment in assisted reproductive technology should be used under strict supervision, and the principle of individualized treatment should be followed.

**Keywords: anticoagulation, immunotherapy, frozen-thawed embryo transfer, congenital anomalies, recurrent miscarriage**

# 1 INTRODUCTION

With the rapid and dramatic development of assisted reproductive technology (ART) (1, 2), the demand of infertile couples for ART has expanded from helping to obtain pregnancy to improving the ongoing pregnancy rate and live birth rate and reducing the miscarriage rate per embryo transfer cycle, reflecting people's enthusiastic expectation of a high pregnancy rate with a low risk of adverse events. In particular, the etiologies of recurrent miscarriage, repeated implantation failure, and long-term infertility with unknown reasons remain unclear, resulting in the lack of standardized investigation and management. Therefore, numerous adjuvant therapies have been introduced, such as the application of anticoagulants, immunosuppressants, and immunomodulators (3, 4). Due to the lack of strict supervision, the clinical application of such medications lacks standardization, bringing a potential risk of drug abuse. On this issue, some experts suggested that overtreatment should be avoided when prescribing individualized therapy according to couples' preferences (5). The application of anticoagulants and immune agents in ART has been in a chaotic state (5–7), and no clear conclusion has been reached. Frozen-thawed embryo transfer (FET) cycles are ideal models for investigating the independent effect of adjuvant drugs since the confounding effect of ovarian stimulation is removed. Thus, we aimed to explore the effectiveness and safety of the adjuvant use of anticoagulants and immune agents in this retrospective cohort study on FET cycles.

# 2 MATERIALS AND METHODS

This retrospective cohort study was conducted in the Reproductive Medicine Center of the Second Hospital of Hebei Medical University, a tertiary hospital. A total of 12,053 FET cycles from January 1, 2017 to May 1, 2021 were reviewed for eligibility. Women aged 20–49 years who underwent FET were included in this study. Subjects who met any of the following criteria were excluded: (a) thin endometrium (<7 mm, measured at least three times) (8); (b) uterine malformation; (c) preimplantation genetic testing (PGT); (d) missing essential data and information; and (e) chromosome polymorphism. After excluding 3,180 subjects, a total of 8,873 cycles (resulting in 9,918 newborns) with complete data were included in the study (**Figure 1**). The study protocol was approved by the Ethics Committee of the Second Hospital of Hebei Medical University. The Second Hospital of Hebei Medical University provided administrative permission for the research team to access and use the data included in this research.

**Abbreviations:** FET, frozen-thawed embryo transfer; PGT, preimplantation genetic testing; HRT, hormone replacement therapy; PIO, progesterone in oil injection; NC, natural cycle; OI, Ovulation Induction; GnRHa-HRT, gonadotropin-releasing hormone agonist downregulation combined with hormone replacement therapy; aPL, anti-phospholipid; ART, assisted reproduction technology; HDP, hypertensive disorder of pregnancy; GDM, gestational diabetes mellitus.

## 2.1 Cycle Regimens

### 2.1.1 Hormone Replacement Therapy

The transfer of thawed embryos was carried out when the endometrial thickness reached 8 mm after a step-up regimen for endometrial preparation. Estradiol valerate (Progynova®, Bayer) was administered orally at 6–8 mg/day on day 2 of the menstrual cycle, which was followed by vaginal administration of micronized progesterone (Uterogestan, Besins International, France) 400 mg BID or combined administration of oral dydrogesterone (Duphaston®; Abbott Biologicals, Netherlands) 10 mg BID and progesterone/oil injection (Progesterone Injection 20 mg/ml, Zhejiang Xianju Pharmaceutical Co., Ltd., China) 40–60 mg QD.

### 2.1.2 Natural Cycle

A serial ultrasound scan was performed every 2 days from menstrual cycle day 10–12. Once the dominant follicle reached 16–20 mm in diameter, HCG was injected for the trigger of ovulation, and progesterone/oil injection or oral dydrogesterone was prescribed at 40 mg QD or 10 mg BID, respectively, as luteal phase support.

### 2.1.3 Ovulation Induction Cycle

Letrozole 2.5–5 mg QD was started from menstrual cycle day 2–3, followed by human menopausal gonadotropin (HMG) injections for ovulation induction. The starting dose of HMG (37 or 75 IU) was determined by follicular development. When the dominant follicle reached 18 mm in diameter and the endometrial thickness reached 8 mm, HCG was administered at 10,000 IU for the trigger of ovulation. The transfer of frozen-thawed cleavage-stage embryo was performed 4 days later and luteal phase support was given as described above.

### 2.1.4 Gonadotropin-Releasing Hormone Agonist Downregulation Combined With Hormone Replacement Therapy

Patients received a single injection of 3.75 mg of long-acting triptorelin acetate on menstrual cycle day 2 after an ultrasound scan confirmed ovarian quiescence and the presence of a thin endometrium (<5 mm). After 28 to 30 days, sequential estrogen and progesterone were prescribed as in the HRT cycles.

## 2.2 Adjuvant Medication

Patients who used aspirin or low-molecular-weight heparin were allocated into the anticoagulant group, while those who used prednisone, hydroxychloroquine, or cyclosporine (whether in combination with anticoagulants or not) were allocated into the immunotherapy group. The remaining patients without anticoagulant and immune agent treatment were allocated into the routine treatment group.

Anticoagulants

Aspirin: Aspirin Enteric-Coated Tablets (Bayer Health Care Manufacturing S.r.l.), 50-75 mg, QD was started from the day of estradiol valerate tablets or progesterone initiation until 10–12 weeks of pregnancy.

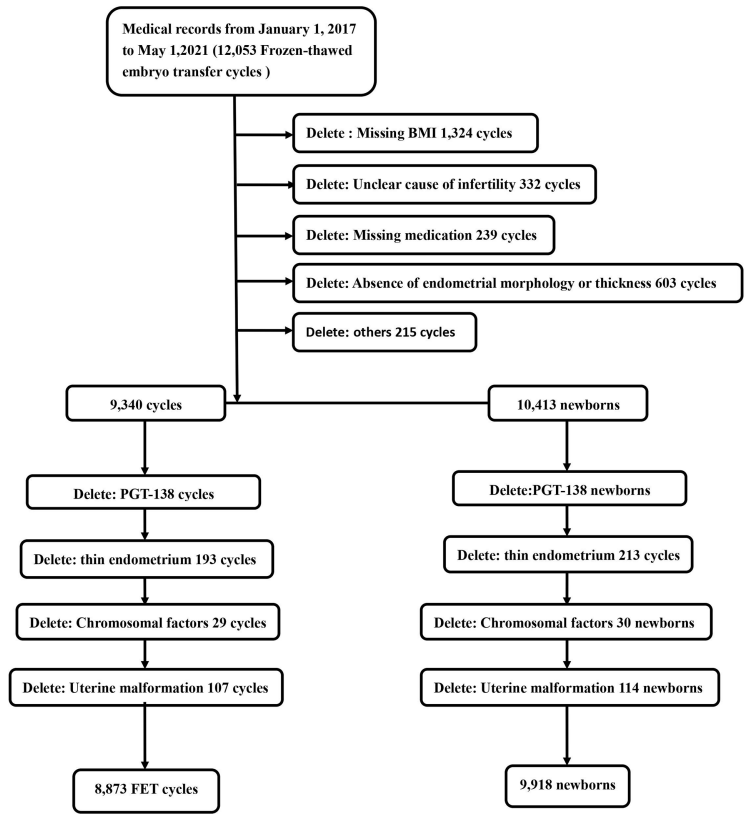



**FIGURE 1** | Study flowchart.

Low-molecular-weight heparins: Enoxaparin Sodium Injection (Clexane, Sanofi-Aventis SA, Paris, France) 0.6 ml: 6000AxaIU or Nadroparin Calcium Injection (Fraxiparine, Glaxo Smith Kline, Brentford, UK) 0.4 ml: 4100AxaIU or Low-molecular-weight heparin calcium injection (Aosida, Hebei Changshan, Shijiazhuang, China) 0.4 ml: 4100AxaIU was injected subcutaneously QD from the day of embryo transfer until 6–8 weeks after transfer.

Immune agents

Prednisone (Prednisone Acetate Tables, Xianju, Taizhou, China), 5 mg, QD or Hydroxychloroquine Sulfate tablets (Fenle, SPH zhongxi, Shanghai, China) 0.1 g*14/ Hydroxychloroquine Sulfate tablets (Plaquenil, Sanofi-Aventis SA, Paris, France) 0.2 g*10, 0.2 g, BID or Cyclosporine Soft Capsules (New sespin, Zhongmeihuadong Pharmaceutical Co. Hangzhou, China) 50 mg*50, 50 mg, BID were prescribed from the day of embryo transfer until 6–8 weeks after transfer.

## 2.3 Clinical and Birth Outcomes

Two authors independently extracted and reexamined the clinical and birth outcome data from medical records. The diagnosis of clinical outcomes or diseases was made by professional physicians using standardized criteria. Miscarriage was defined as pregnancy loss before 13 weeks of gestation; birth referred to both live birth and stillbirth after 28 weeks of gestation. Preterm delivery was defined as birth between 28 and 37 gestational weeks. Pregnancy complications included hypertensive disorder of pregnancy (HDP) (329 cycles) (9, 10), gestational diabetes mellitus (GDM) (83 cycles) (11), HDP and GDM (13 cycles), premature rupture of membranes (296 cycles) (12), cervical insufficiency (20 cycles) (13), oligohydramnios (28 cycles) (14), polyhydramnios (6 cycles) (15), placenta previa (36 cycles) (16), abruptio placentae (7 cycles) (17), postpartum hemorrhage (6 cycles) (18), disseminated hematogenous tuberculosis (1 cycle) (19), intrahepatic cholestasis of pregnancy (2 cycles) (20), postpartum thrombotic disease (3 cycles) (21), chronic nephritis (1 cycle), and unclear diagnosis (45 cycles). The categorization of pregnancy complications is shown in **Table S2** as **Supplemental Material**. The diagnosis criteria for the above-mentioned pregnancy complications were reported previously.

There were 85 cases of congenital anomalies involving six major systems; 7 cases were found to have chromosomal abnormalities or abnormal nuchal translucency and 4 cases were without clear description. Detailed information is shown in **Table S1** as **Supplemental Material**.

## 2.4 Statistical Analysis

Continuous variables were presented as median (interquartile range) or mean ± standard deviation (SD) according to the

normality of distribution. Kruskal–Wallis test was used to compare the continuous variables among the three groups. Categorical variables were presented as count (percentage) and compared using the chi-square test. Logistic regression analysis and stratified analysis were used to explore the associations between adjuvant medication and miscarriage, birth, multiple birth, congenital anomaly, and birth weight with the generalized estimation equation (GEE) model to deal with the repeat cycles and data of twins. Two regression models were applied: baseline characteristics of study subjects in model 1, while other confounding factors were adjusted in model 2. Confounders on the basis of their associations with the outcomes of interest or a change in effect estimate of more than 10% were selected. All the analyses were performed using R 3.6.3 (http://www.r-project.org) and EmpowerStats (www.empowerstats.net, X&Y Solutions Inc., Boston, MA, USA), and a two-sided $p$-value of <0.05 was considered to indicate statistical significance.

# 3 RESULTS

## 3.1 Baseline Characteristics, Laboratory Data, Pregnancy, and Neonatal Outcomes of Study Subjects

As shown in **Table 1**, there were 8,873 FET cycles included in this retrospective study, among which 4,253 cycles were allocated into the routine treatment group, 3,698 cycles were allocated into the anticoagulant group, and 922 were allocated into the immunotherapy group.

As for the baseline characteristics of the study population, the age of couples in the immunotherapy group was greater than those in the routine treatment group (female: 31.4 ± 4.3 vs. 30.6 ± 4.3, $p < 0.001$; male: 32.2 ± 5.0 vs. 31.4 ± 4.8, $p < 0.001$). Patients in the immunotherapy group had longer infertility duration (4.6 ± 2.8 vs. 4.4 ± 3.0, $p = 0.055$), higher proportion of patients with 1–2 previous miscarriages [313 (33.9%) vs. 1,126 (26.5%), $p < 0.001$], and more repetition cycles (≥3) [233 (25.3%) vs. 417 (9.8%), $p < 0.001$], but the proportion of infertility type, etiology of infertility, and body mass index (BMI) was comparable between the two groups. Compared with the routine treatment group, the anticoagulant treatment group had a higher proportion of patients with 1 cycle [2,570 (69.5%) vs. 2,838 (66.7%), $p = 0.027$] and more patients with ovulation disorders [400 (10.8%) vs. 396 (9.3%), $p = 0.026$], while the age of couples, infertility duration, BMI, infertility type, and previous miscarriages were comparable between the two groups.

In terms of laboratory variables, the routine treatment group had a higher proportion of cleavage-stage embryo transfer [3,148 (74.0%) vs. 2,653 (71.7%) vs. 448 (48.6%)], while the immunotherapy group had a higher proportion of blastocyst transfer [473 (51.3%) vs. 1,085 (25.5%) vs. 1,034 (28.0%)]. As for pregnancy outcomes, the immunotherapy group demonstrated a higher miscarriage rate [104 (21.4%) vs. 370 (16.3%) vs. 358 (17.5%)] but a lower multiple birth rate [74 (19.8%) vs. 488 (26.4%) vs. 455 (27.6%)] and birth rate [373(76.7%) vs.1,849 (81.3%)vs.1,648 (80.5%)). However, there was no significant difference in the gestational weeks at birth, pregnancy location, and pregnancy complications among the three groups.

Neonatal outcomes are shown in **Table 2**, including 9,918 newborns. There were 4,758 newborns in the routine medication group, 4,164 newborns in the anticoagulant treatment group, and 996 newborns in the immunotherapy group. No significant differences were found in congenital anomaly and gender, while the immunotherapy group had greater neonatal weight (3,031.3 ± 684.2 vs. 2,960.9 ± 692.7 vs. 2,965.5 ± 678.4, $p = 0.048$).

## 3.2 Adjuvant Medications Were Associated With Inferior Pregnancy Outcomes by Multivariate Regression Analysis With Stratification

Multivariate regression analysis with stratification was used to investigate the effectiveness of adjuvant medication on improving pregnancy outcomes. After adjusting for age of the couples, BMI, infertility duration, the number of transferred embryos, endometrial echogenicity, FET protocol, number of previous miscarriage, and cycle number, normal ovulatory patients undergoing immunotherapy demonstrated a 40% (OR = 1.4, 95% CI: 1.0, 1.8) higher risk of miscarriage (**Table 3**) and a 20% (OR = 0.8, 95% CI: 0.6, 1.0) lower probability of birth (**Table 3**) compared with those without adjuvant medication. Moreover, patients with more than 1 embryo transferred and anticoagulant treatment showed an increased risk of multiple birth (OR = 1.2, 95% CI: 1.0, 1.4) after controlling for confounding factors including age of the couples, BMI, infertility duration, endometrial thickness, cycle number, and the number of previous miscarriage (**Table 4**).

## 3.3 Adjuvant Medications Significantly Impact Offspring Safety by Multivariate Regression Analysis With Stratification

After controlling for gestational weeks at birth, multiple birth, age of the couples, BMI, developmental stage of transferred embryos, infertility type, the number of embryos transferred, and cycle number, neonates of patients without pregnancy complications but undergoing anticoagulant therapy showed an increased risk of congenital anomalies (adjusted OR = 2.1, 95% CI: 1.0, 4.5) (**Table 5**). However, in patients with pregnancy complications, the risk of congenital anomaly was comparable among the three groups.

Given the significant influence of maternal age on neonatal birth weight, stratification by female age was performed when investigating the association between adjuvant medication use and neonatal birth weight. The results showed that, among young patients (<26 years) (23) with a singleton pregnancy, the neonatal birth weight of the immunotherapy group was 305.4 g heavier than the routine treatment group (adjusted $\beta = 305.4$; 95% CI: 55.2, 555.5), while that of the anticoagulant treatment group was 175.9 g heavier than the routine treatment group (adjusted $\beta = 175.9$, 95% CI, 68.1, 283.7) after adjusting for gestational weeks at birth, male age, BMI, developmental stage of transferred embryos, cycle number, infertility type, and the number of embryos transferred (**Table 6**). In other age strata

**TABLE 1** | Patient characteristics (8,873 FET cycles).

| | Routine treatment | Anticoagulant treatment | | Immunotherapy | |
|---|---|---|---|---|---|
| ***N* (cycles)** | **4,253** | **3,698** | | **922** | |
| | **Mean ± SD/*N* (%)** | **Mean ± SD/*N* (%)** | ***p*** | **Mean ± SD/*N* (%)** | ***p*** |
| **Age—Female (years)** | 30.6 ± 4.3 | 30.7 ± 4.3 | 0.248 | 31.4 ± 4.3 | <0.001 |
| **Age—Male (years)** | 31.4 ± 4.8 | 31.5 ± 4.7 | 0.407 | 32.2 ± 5.0 | <0.001 |
| **Infertility Duration (years)** | 4.4 ± 3.0 | 4.5 ± 3.0 | 0.192 | 4.6 ± 2.8 | 0.055 |
| **BMI** | 23.4 ± 3.6 | 23.5 ± 3.6 | 0.301 | 23.3 ± 3.4 | 0.519 |
| **Cycle Number** | 1.5 ± 0.8 | 1.4 ± 0.8 | 0.014 | 2.0 ± 1.1 | <0.001 |
| **Endometrial Thickness (mm)** | 9.7 ± 1.5 | 9.6 ± 1.5 | 0.008 | 9.6 ± 1.5 | 0.033 |
| **No. of Transferred Embryos** | 1.8 ± 0.4 | 1.8 ± 0.4 | / | 1.6 ± 0.5 | <0.001 |
| **Infertility (type)** | | | 0.549 | | 0.049 |
| Primary infertility | 2,200 (51.7%) | 1,888 (51.1%) | | 444 (48.2%) | |
| Secondary infertility | 2,053 (48.3%) | 1,810 (48.9%) | | 478 (51.8%) | |
| **Etiology of Infertility** | | | 0.026 | | 0.125 |
| Ovulation disorders★ | 396 (9.3%) | 400 (10.8%) | | 101 (11.0%) | |
| Other | 3,857 (90.7%) | 3,298 (89.2%) | | 821 (89.0%) | |
| **Previous Miscarriage** | | | 0.441 | | <0.001 |
| 0 | 3,002 (70.6%) | 2,563 (69.3%) | | 582 (63.1%) | |
| 1–2 | 1,126 (26.5%) | 1,026 (27.7%) | | 313 (33.9%) | |
| ≥3 | 125 (2.9%) | 109 (2.9%) | | 27 (2.9%) | |
| **FET Protocol** | | | <0.001 | | <0.001 |
| NC | 643 (15.1%) | 74 (2.0%) | | 43 (4.7%) | |
| OI | 180 (4.2%) | 42 (1.1%) | | 45 (4.9%) | |
| HRT | 2,980 (70.1%) | 3,039 (82.2%) | | 674 (73.1%) | |
| GnRHa-HRT | 450 (10.6%) | 543 (14.7%) | | 160 (17.4%) | |
| **Endometrial Echogenicity** | | | 0.812 | | <0.001 |
| A | 2,791 (65.6%) | 2,433 (65.8%) | | 453 (49.1%) | |
| B | 1,442 (33.9%) | 1,244 (33.6%) | | 462 (50.1%) | |
| C | 20 (0.5%) | 21 (0.6%) | | 7 (0.8%) | |
| **Developmental Stage of the Transferred Embryo** | | | 0.025 | | <0.001 |
| Cleavage-stage embryo | 3,148 (74.0%) | 2,653 (71.7%) | | 448 (48.6%) | |
| Blastocyst | 1,085 (25.5%) | 1,034 (28.0%) | | 473 (51.3%) | |
| Sequential transfer of cleavage stage Embryo and blastocyst | 20 (0.5%) | 11 (0.3%) | | 1 (0.1%) | |
| **Pregnancy Location** | | | 0.341 | | 0.837 |
| No | 1,980 (46.6%) | 1,652 (44.7%) | | 436 (47.3%) | |
| Intrauterine pregnancy | 2,213 (52.0%) | 1,999 (54.1%) | | 476 (51.6%) | |
| Ectopic pregnancy | 51 (1.2%) | 40 (1.1%) | | 9 (1.0%) | |
| Heterotopic pregnancy | 9 (0.2%) | 7 (0.2%) | | 1 (0.1%) | |
| **Early Miscarriage (≤13 weeks)** | | | 0.285 | | 0.007 |
| No | 1,903 (83.7%) | 1,688 (82.5%) | | 382 (78.6%) | |
| Yes | 370 (16.3%) | 358 (17.5%) | | 104 (21.4%) | |
| **Birth (≥28 weeks)** | | | 0.504 | | 0.020 |
| No | 424 (18.7%) | 398 (19.5%) | | 113 (23.3%) | |
| Yes | 1,849 (81.3%) | 1,648 (80.5%) | | 373 (76.7%) | |
| **Multiple Birth** | | | 0.418 | | 0.008 |
| No | 1,361 (73.6%) | 1,193 (72.4%) | | 299 (80.2%) | |
| Yes | 488 (26.4%) | 455 (27.6%) | | 74 (19.8%) | |
| **Gestational Weeks at Birth** | | | 0.891 | | 0.708 |
| Preterm | 406 (22.0%) | 363 (22.0%) | | 79 (21.2%) | |
| Term | 1,441 (77.9%) | 1,284 (77.9%) | | 293 (78.6%) | |
| Postterm | 2 (0.1%) | 1 (0.1%) | | 1 (0.3%) | |
| **Pregnancy Complications** | | | 0.662 | | 0.934 |
| No | 3,817 (90.2%) | 3,294 (89.9%) | | 825 (90.3%) | |
| Yes | 416 (9.8%) | 371 (10.1%) | | 89 (9.7%) | |
| **Cycles (categorized)** | | | 0.027 | | <0.001 |
| 1 | 2,838 (66.7%) | 2,570 (69.5%) | | 359 (38.9%) | |
| 2 | 998 (23.5%) | 785 (21.2%) | | 330 (35.8%) | |
| ≥3 | 417 (9.8%) | 343 (9.3%) | | 233 (25.3%) | |

★*Ovulation disorders refers to the patients with ovulation disorder of group II and III as defined by WHO (22).*

*FET, frozen-thawed embryo transfer; BMI, body mass index; NC, natural cycle; OI, ovulation induction; HRT, hormone replacement therapy; GnRHa-HRT, gonadotropin-releasing hormone (GnRH) agonist downregulation combined with hormone replacement therapy.*

**TABLE 2 |** Neonatal characteristics (9,918 neonates).

| | Routine treatment | Anticoagulant treatment | | Immunotherapy | |
|---|---|---|---|---|---|
| **N** | **4,758** | **4,164** | | **996** | |
| | **Mean ± SD/*N* (%)** | **Mean ± SD/*N* (%)** | ***p*** | **Mean ± SD/*N* (%)** | ***p*** |
| **Neonatal weight (g)** | 2,960.9 ± 692.7 | 2,965.5 ± 678.4 | 0.824 | 3,031.3 ± 684.2 | 0.048 |
| **Gender** | | | 0.355 | | 0.302 |
| Female | 1,221 (51.6%) | 1,125 (53.0%) | | 243 (54.2%) | |
| Male | 1,146 (48.4%) | 999 (47.0%) | | 205 (45.8%) | |
| **Congenital anomaly** | | | 0.396 | | 0.319 |
| No | 4,714 (99.1%) | 4,118 (98.9%) | | 990 (99.4%) | |
| Yes | 44 (0.9%) | 46 (1.1%) | | 6 (0.6%) | |

**TABLE 3 |** Multivariate logistic regression of miscarriage and birth (≥28 weeks) stratified by the etiology of infertility among patients undergoing routine treatment, anticoagulant treatment, and immunotherapy.

| | N | Routine treatment | Anticoagulant treatment | | Immunotherapy | |
|---|---|---|---|---|---|---|
| | | **Reference** | **OR (95% CI)** | **P** | **OR (95%CI)** | **p** |
| **Y=miscarriage** | | | | | | |
| Etiology of Infertility | | | | | | |
| OD | 529 | 1.0 | 0.8 (0.5, 1.4) | 0.414 | 0.6 (0.3, 1.5) | 0.302 |
| Other | 4,276 | 1.0 | 1.1 (0.9, 1.3) | 0.441 | 1.4 (1.0, 1.8) | 0.021 |
| **Y = birth (≥28 weeks)** | | | | | | |
| Etiology of Infertility | | | | | | |
| OD | 529 | 1.0 | 1.3 (0.8, 2.1) | 0.385 | 1.7 (0.7, 3.9) | 0.230 |
| Other | 4,276 | 1.0 | 1.0 (0.8, 1.1) | 0.710 | 0.8 (0.6, 1.0) | 0.032 |

*OD, ovulation disorder.*

*Adjusted for Age—female, Age—male, BMI, Infertility duration, No. of transferred embryos, Endometrial echogenicity, FET protocol, Previous miscarriage, and Cycle number.*

**TABLE 4 |** Multivariate logistic regression of multiple birth stratified by the number of transferred embryos among patients undergoing routine treatment, anticoagulant treatment, and immunotherapy.

| **Y = Multiple birth** | | **Routine treatment** | **Anticoagulant treatment** | | **Immunotherapy** | |
|---|---|---|---|---|---|---|
| **No. of transferred embryos** | ***n*** | **Reference** | **OR (95% CI)** | ***P*** | **OR (95% CI)** | ***p*** |
| 1 | 785 | 1.0 | 0.6 (0.2, 1.8) | 0.286 | 1.0 (0.3, 3.5) | 0.971 |
| >1 | 3,085 | 1.0 | 1.2 (1.0, 1.4) | 0.021 | 1.0 (0.7, 1.3) | 0.880 |

*Adjusted for Age—female, Age—male, BMI, Infertility duration, Endometrial thickness, Previous miscarriage, and Cycle number.*

and among patients with multiple pregnancy, the neonatal birth weight was comparable among the three groups.

## DISCUSSION

In this retrospective cohort study of 8,873 FET cycles (9,918 newborns), we observed that anticoagulation and immunotherapy had a significant influence on pregnancy outcomes and offspring safety.

Compared with the routine treatment group, using immune agents was associated with an increased risk of miscarriage and a decreased rate of birth in normal ovulatory patients. A fetus has antigens of maternal and paternal origins (5). The physiological mechanisms of the immunotolerance of paternal antigens during pregnancy are poorly understood. However, a dysfunction in immune modulation has been hypothesized to be one of the causes of infertility or miscarriage. Several systematic reviews (24–26) have evaluated the effectiveness and safety of immunological interventions for recurrent miscarriage, and none of such interventions were associated with a reduction in miscarriages or an increase in live births. Thus, there was insufficient evidence to recommend immunotherapy in the management of recurrent miscarriage. In this study, we found that the adjuvant immunotherapy during FET cycles significantly increased the risk of miscarriage, but markedly decreased the probability of birth among normal ovulatory patients only. In contrast, an increase in birth and a decrease in miscarriage were witnessed among non-ovulatory patients undergoing immunotherapy, although both were without statistical significance. This suggested that patients with ovulation disorder may have underlying defects in immunomodulation during embryo

**TABLE 5 |** Univariate and multivariate logistic regression of congenital anomaly stratified by pregnancy complications among patients undergoing routine treatment, anticoagulant treatment, and immunotherapy.

| Y = Congenital Anomalies | Without pregnancy complications | | With pregnancy complications | | Total | |
|---|---|---|---|---|---|---|
| | OR (95% CI) | *p* | OR (95% CI) | *p* | OR (95% CI) | *p* |
| **Non-adjusted** | | | | | | |
| Routine treatment[a] | 1.0 | | 1.0 | | 1.0 | |
| Anticoagulant treatment | 1.9 (1.1, 3.4) | 0.022 | 0.6 (0.3, 1.2) | 0.121 | 1.2 (0.8, 1.8) | 0.470 |
| Immunotherapy | 1.0 (0.3, 3.0) | 0.992 | 0.4 (0.1, 1.8) | 0.228 | 0.7 (0.3, 1.6) | 0.372 |
| **Model I** | | | | | | |
| Routine treatment | 1.0 | | 1.0 | | 1.0 | |
| Anticoagulant treatment | 1.9 (1.1, 3.4) | 0.021 | 0.6 (0.3, 1.2) | 0.132 | 1.2 (0.8, 1.8) | 0.493 |
| Immunotherapy | 0.9 (0.3, 2.6) | 0.881 | 0.5 (0.1,2.0) | 0.320 | 0.7 (0.3, 1.6) | 0.358 |
| **Model II** | | | | | | |
| Routine treatment | 1.0 | | 1.0 | | 1.0 | |
| Anticoagulant treatment | 2.1 (1.0, 4.5) | 0.046 | 0.6 (0.3, 1.4) | 0.234 | 1.1 (0.7, 1.9) | 0.629 |
| Immunotherapy | 0.9 (0.2, 4.8) | 0.946 | 0.6 (0.1, 2.5) | 0.498 | 0.7(0.2, 2.1) | 0.528 |

*Non-adjusted model adjust for: None.*

*Model I adjusted for Age—female, Age—male, BMI, and Cycle Number.*

*Model II adjusted for Age—female, Age—male, BMI, Gestational weeks at birth, No. of transferred embryos, Developmental stage of transferred embryos, Multiple birth, and Cycle Number.*

[a]*Routine treatment group served as the reference.*

**TABLE 6 |** Univariate and multivariate linear regression of birth weight stratified by maternal age among singletons and non-singletons.

| Y = Birth Weight (g) | Singleton | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | <26 years | | ≥26, <38 years | | ≥38 years | | Total | |
| | β (95% CI) | *p* | β (95% CI) | *p* | β (95% CI) | *p* | β (95% CI) | *p* |
| **Non-adjusted** | | | | | | | | |
| Routine treatment[a] | 0 | | 0 | | 0 | | 0 | |
| Anticoagulant treatment | 200.3 (50.2, 350.5) | 0.009 | −11.6 (−69.8, 46.6) | 0.696 | −172.8 (−435.6, 89.9) | 0.197 | 2.2 (−51.2, 55.6) | 0.936 |
| Immunotherapy | 382.9 (119.3, 646.4) | 0.004 | −26.7 (−106.3, 52.9) | 0.511 | −37.4 (−293.2, 218.4) | 0.775 | 9.2 (−65.2, 83.7) | 0.808 |
| **Model I** | | | | | | | | |
| Routine treatment | 0 | | 0 | | 0 | | 0 | |
| Anticoagulant treatment | 210.1 (55.3, 365.0) | 0.008 | −10.7 (−68.9, 47.5) | 0.718 | −235.9 (−491.8, 20.0) | 0.071 | 3.0 (−50.4, 56.5) | 0.911 |
| Immunotherapy | 351.1(77.1, 625.0) | 0.012 | −29.6 (−110.6, 51.5) | 0.475 | −90.8 (−339.1, 157.4) | 0.473 | 4.0 (−72.0, 80.1) | 0.917 |
| **Model II** | | | | | | | | |
| Routine treatment | 0 | | 0 | | 0 | | 0 | |
| Anticoagulant treatment | 175.9 (68.1, 283.7) | 0.001 | −14.0 (−53.2, 25.2) | 0.484 | −175.4 (−354.5, 3.8) | 0.055 | −1.1 (−37.2, 35.0) | 0.951 |
| Immunotherapy | 305.4 (55.2, 555.5) | 0.017 | −48.6 (−110.2, 13.1) | 0.123 | −159.9 (−406.6, 86.8) | 0.204 | −21.6 (−80.8, 37.5) | 0.473 |
| | Non-singleton | | | | | | | |
| **Non-adjusted** | | | | | | | | |
| Routine treatment[a] | 0 | | 0 | | 0 | | 0 | |
| Anticoagulant treatment | −8.8 (−174.0, 156.3) | 0.916 | 39.7 (−22.5, 101.8) | 0.211 | 40.9 (−384.1, 465.9) | 0.851 | 33.6 (−24.0, 91.2) | 0.253 |
| Immunotherapy | 30.5 (−151.7, 212.8) | 0.743 | −8.7 (−152.8, 135.4) | 0.906 | 110.5 (−259.6, 480.6) | 0.558 | −6.8 (−143.1, 129.5) | 0.922 |
| **Model I** | | | | | | | | |
| Routine treatment | 0 | | 0 | | 0 | | 0 | |
| Anticoagulant treatment | −13.0 (−178.4, 152.4) | 0.878 | 43.6 (−19.6, 106.7) | 0.177 | −36.8 (−461.8, 388.2) | 0.865 | 36.8 (−21.5, 95.1) | 0.216 |
| Immunotherapy | 63.8 (−174.3, 301.8) | 0.600 | −6.3 (−145.3, 132.7) | 0.929 | 169.6 (−171.2, 510.5) | 0.329 | −9.1 (−140.3, 122.2) | 0.892 |
| **Model II** | | | | | | | | |
| Routine treatment | 0 | | 0 | | | | 0 | |
| Anticoagulant treatment | −40.5 (−182.8, 101.7) | 0.576 | 33.4 (−20.3, 87.2) | 0.223 | | | 23.7 (−25.3, 72.8) | 0.344 |
| Immunotherapy | 17.2 (−189.9, 224.3) | 0.870 | 20.0 (−104.8, 144.8) | 0.753 | | | 2.3 (−115.5, 120.1) | 0.970 |

*Non-adjusted model adjusted for: None*

*Model I adjusted for Age—male, BMI, and Cycle Number.*

*Model II adjusted for Age—male, BMI, Gestational weeks at birth, No. of transferred embryos, Developmental stage of transferred embryos, and Cycle Number.*

[a]*Routine treatment group served as the reference.*

implantation, so that they can benefit from immunotherapy. However, among patients with normal ovulation, the administration of exogenous immune agents may in turn disturb their immunotolerance to fetal antigen, resulting in an increased miscarriage rate and a decreased birth rate.

Using anticoagulant agents was associated with a higher risk of multiple deliveries and an increased risk of congenital anomalies. In terms of anticoagulant therapy, several systematic reviews and meta-analyses (4, 27–29) have shown that low-dose aspirin and low-molecular-weight heparin could effectively reduce the miscarriage rate and increase the live birth rate in women with antiphospholipid syndrome or a history of recurrent miscarriage. The combination of low-molecular-weight heparin and aspirin during pregnancy may increase the live birth rate in women with persistent anti-phospholipid (aPL) when compared with aspirin treatment alone. In this study, anticoagulant therapy significantly increased twin birth rate in patients with more than 1 embryo transferred. Our finding is consistent with the published studies suggesting the improvement in live birth rate by using anticoagulation therapy. However, there were few articles focusing on the relationship between anticoagulation therapy and congenital malformations. A randomized controlled trial reported few cases of congenital anomalies, but this may be underestimated given the small sample size (30). Our study collected the clinical data of 9,918 neonates, among which 96 cases of congenital anomalies were observed. We classified fetal congenital anomalies according to the human body system (**Table S1**). There were 44 cases in the routine treatment group, 46 cases in the anticoagulant group, and 6 cases in the immunotherapy group. In the anticoagulant group, 44 cases were exposed to aspirin and 2 cases were exposed to both aspirin and low-molecular-weight heparin, suggesting that aspirin was associated with congenital anomalies. Aspirin can inhibit prostaglandin synthesis and subsequent reduction of platelet aggregation by inactivating cyclooxygenase (31). According to the latest guideline on low-dose aspirin use during pregnancy by the American College of Obstetricians and Gynecologists (ACOG), low-dose aspirin use during the first and the second trimester was considered to be effective and safe (32). In this study, the incidence of congenital anomalies was comparable among the three groups in patients with pregnancy complications, which was consistent with the ACOG guideline. However, in patients without comorbidities during pregnancy, the risk of fetal malformation increased when adjuvant anticoagulants were prescribed. The relationship between aspirin and genitourinary abnormalities and gastroschisis has been reported (33–36). In our study, there were four cases of genitourinary abnormalities: two cases of cryptorchidism (one exposed to aspirin, while the other was from the routine treatment group), one case of hypospadias (from the routine treatment group), and one case of gastroschisis (from the routine treatment group). In addition, after excluding cases with parental chromosomal abnormalities, malformations of systems other than the genitourinary system and gastrointestinal systems were also reported, which is interesting and unexpected. Furthermore, high-quality prospective studies and comprehensive neonatal physical examination are warranted to evaluate the safety of aspirin in the field of reproduction.

In terms of the effect of adjuvant medication on neonatal birth weight, previous systematic reviews only focused on fetal growth restriction and no firm conclusions were drawn (4, 7). In this study, the neonatal birth weight of each group was quantitatively analyzed, and multivariate linear regression was performed to adjust for confounding factors. The results showed that there was a statistically significant increase in neonatal birth weight after the adjuvant use of either anticoagulants or immune agents among patients under the age of 26. This is different from previous reports that fetal weight increases with maternal age (37, 38), suggesting that anticoagulation combined with or without immunotherapy has a positive impact on birth weight.

To summarize, using immune agents was associated with an increased risk of miscarriage and a decrease in birth among normal ovulatory patients. Using anticoagulant agents was associated with a higher risk of multiple birth and an increased risk of congenital anomalies. Young mothers had heavier newborns after either anticoagulant agent or immune agent treatment during FET cycles. Therefore, adjuvant anticoagulant or immune agent treatment in ART should be used under strict supervision, and the principle of individualized treatment should be followed.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Second Hospital of Hebei Medical University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

## AUTHOR CONTRIBUTIONS

YF, YW, and GH devised the idea and designed the study. ZL and JZ contributed to the primary data collection. YX and JZ reexamined the data and analyzed the data. YF wrote the original draft, which was revised by GH. WW and NC supervised the study and administered the project. All authors contributed to the article and approved the submitted version.

## FUNDING

This study was supported by the Natural Science Foundation of Hebei Province (H2021206377), S&T Program of Hebei (21377760D), Clinical Medicine Outstanding Talents Program

of Government Funds, and Tracking Project of Hebei Province Medical Applicable Technology (GZ2022019). The funding sponsors had no role in the study design; in the collection, analyses, and interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.884972/full#supplementary-material

## REFERENCES


1. Bai F, Wang DY, Fan YJ, Qiu J, Wang L, Dai Y, et al. Assisted Reproductive Technology Service Availability, Efficacy and Safety in Mainland China: 2016 [Published Correction Appears in Hum Reprod]. *Hum Reprod* (2020) 35 (2):446–52. doi: 10.1093/humrep/dez245
2. Kushnir VA, Barad DH, Albertini DF, Darmon SK, Gleicher N. Systematic Review of Worldwide Trends in Assisted Reproductive Technology 2004-2013. *Reprod Biol Endocrinol* (2017) 15(1):6. doi: 10.1186/s12958-016-0225-2
3. Carp H. Immunotherapy for Recurrent Pregnancy Loss. *Best Pract Res Clin Obstet Gynaecol.* (2019) 60:77–86. doi: 10.1016/j.bpobgyn.2019.07.005
4. Hamulyák EN, Scheres LJ, Marijnen MC, Goddijn M, Middeldorp S. Aspirin or Heparin or Both for Improving Pregnancy Outcomes in Women With Persistent Antiphospholipid Antibodies and Recurrent Pregnancy Loss. *Cochrane Database Syst Rev* (2020) 5(5):CD012852. doi: 10.1002/14651858CD012852.pub2
5. Lee SY, Cabral HJ, Aschengrau A, Pearce EN. Associations Between Maternal Thyroid Function in Pregnancy and Obstetric and Perinatal Outcomes. *J Clin Endocrinol Metab* (2020) 105(5):e2015–23. doi: 10.1210/clinem/dgz275
6. Makino A, Sugiura-Ogasawara M. Anticoagulant Therapy and Pregnancy. *Reprod Med Biol* (2008) 7(1):1–10. doi: 10.1111/j.1447-0578.2007.00195.x
7. Andrikopoulou A, Korakiti AM, Apostolidou K, Dimopoulos MA, Zagouri F. Immune Checkpoint Inhibitor Administration During Pregnancy: A Case Series. *ESMO Open* (2021) 6(5):100262. doi: 10.1016/j.esmoop.2021.100262
8. Jing Z, Guo-ning H, Hai-xiang S, Liqing F, Yun F, Huan S, et al. Chineseexpert Consensus on Diagnosis and Management of Abnormal Endometrium in Assisted Reproductive Technology. *J Reprod Med* (2018) 27(11):1057–64. doi: 10.3969/j.issn.1004-3845.2018.11.003
9. ACOG. Gestational Hypertension and Preeclampsia: ACOG Practice Bulletin Summary, Number 222. *Obstet Gynecol.* (2020) 135(6):1492–5. doi: 10.1097/AOG.0000000000003892
10. American College of Obstetricians and Gynecologists' Committee on Practice Bulletins—Obstetrics. ACOG Practice Bulletin No. 203: Chronic Hypertension in Pregnancy. *Obstet Gynecol* (2019) 133(1):e26–50. doi: 10.1097/AOG.0000000000003020
11. Wu Y, Liu B, Sun Y, Du Y, Santillan MK, Santillan DA, et al. Association of Maternal Prepregnancy Diabetes and Gestational Diabetes Mellitus With Congenital Anomalies of the Newborn. *Diabetes Care* (2020) 43(12):2983–90. doi: 10.2337/dc20-0261
12. ACOG. American College of Obstetricians and Gynecologists' Committee on Practice Bulletins—Obstetrics. Practice Bulletin No. 172: Premature Rupture of Membranes. *Obstet Gynecol* (2016) 128(4):e165–77. doi: 10.1097/AOG.0000000000001712
13. Brown R, Gagnon R, Delisle MF. No. 373-Cervical Insufficiency and Cervical Cerclage. *J Obstet Gynaecol Can* (2019) 41(2):233–47. doi: 10.1016/j.jogc.2018.08.009
14. Rabie N, Magann E, Steelman S, Ounpraseuth S. Oligohydramnios in Complicated and Uncomplicated Pregnancy: A Systematic Review and Meta-Analysis. *Ultrasound Obstet Gynecol* (2017) 49(4):442–9. doi: 10.1002/uog.15929
15. Society for Maternal-Fetal Medicine (SMFM). Electronic address: pubs@smfm.org, Dashe JS, Pressman EK, Hibbard JU. SMFM Consult Series #46: Evaluation and Management of Polyhydramnios. *Am J Obstet Gynecol* (2018) 219(4):B2–8. doi: 10.1016/j.ajog.2018.07.016
16. Jain V, Bos H, Bujold E. Guideline No. 402: Diagnosis and Management of Placenta Previa. *J Obstet Gynaecol Can* (2020) 42(7):906–917.e1. doi: 10.1016/j.jogc.2019.07.019
17. Hall DR. Abruptio Placentae and Disseminated Intravascular Coagulopathy. *Semin Perinatol* (2009) 33(3):189–95. doi: 10.1053/j.semperi.2009.02.005
18. Committee on Practice Bulletins-Obstetrics. Practice Bulletin No. 183: Postpartum Hemorrhage. *Obstet Gynecol* (2017) 130(4):e168–86. doi: 10.1097/AOG.0000000000002351
19. Barbosa OA, Teles FM, Maia ACC, Pessoa GMF, de Alencar IM, de Sousa ET, et al. Disseminated Hematogenous Tuberculosis in Puerperium-Case Report. *Oxf Med Case Rep* (2019) 2019(11):479–81. doi: 10.1093/omcr/omz116
20. Williamson C, Geenes V. Intrahepatic Cholestasis of Pregnancy. *Obstet Gynecol* (2014) 124(1):120–33. doi: 10.1097/AOG.0000000000000346
21. James AH, Tapson VF, Goldhaber SZ. Thrombosis During Pregnancy and the Postpartum Period. *Am J Obstet Gynecol* (2005) 193(1):216–9. doi: 10.1016/j.ajog.2004.11.037
22. WHO. Agents Stimulating Gonadal Function in the Human. Report of a WHO Scientific Group. *World Health Organ Tech Rep Ser* (1973) 514:1–30.
23. Franasiak JM, EJ F, KH H, Werner MD, Upham KM, Treff NR, et al. The Nature of Aneuploidy With Increasing Age of the Female Partner: A Review of 15,169 Consecutive Trophectoderm Biopsies Evaluated With Comprehensive Chromosomal Screening. *Fertil Steril* (2014) 101(3):656–663.e1. doi: 10.1016/j.fertnstert.2013.11.004
24. Egerup P, Lindschou J, Gluud C, Christiansen OBImmuReM IPD Study Group. The Effects of Intravenous Immunoglobulins in Women With Recurrent Miscarriages: A Systematic Review of Randomised Trials With Meta-Analyses and Trial Sequential Analyses Including Individual Patient Data. *PloS One* (2015) 10(10):e0141588. doi: 10.1371/journal.pone.0141588
25. Porter TF, LaCoursiere Y, Scott JR. Immunotherapy for Recurrent Miscarriage. *Cochrane Database Syst Rev* (2006) 2):CD000112. doi: 10.1002/14651858.CD000112.pub2
26. Achilli C, Duran-Retamal M, Saab W, Serhal P, Seshadri S. The Role of Immunotherapy in *In Vitro* Fertilization and Recurrent Pregnancy Loss: A Systematic Review and Meta-Analysis. *Fertil Steril* (2018) 110(6):1089–100. doi: 10.1016/j.fertnstert.2018.07.004
27. de Jong PG, Kaandorp S, Di Nisio M, Goddijn M, Middeldorp S. Aspirin and/or Heparin for Women With Unexplained Recurrent Miscarriage With or Without Inherited Thrombophilia. *Cochrane Database Syst Rev* (2014) 2014 (7):CD004734. doi: 10.1002/14651858.CD004734.pub4
28. Areia AL, Fonseca E, Areia M, Moura P. Low-Molecular-Weight Heparin Plus Aspirin Versus Aspirin Alone in Pregnant Women With Hereditary Thrombophilia to Improve Live Birth Rate: Meta-Analysis of Randomized Controlled Trials. *Arch Gynecol Obstet* (2016) 293(1):81–6. doi: 10.1007/s00404-015-3782-2
29. Zhang T, Ye X, Zhu T, Xiao X, Liu Y, Wei X, et al. Antithrombotic Treatment for Recurrent Miscarriage: Bayesian Network Meta-Analysis and Systematic Review. *Med (Baltimore).* (2015) 94(45):e1732. doi: 10.1097/MD.0000000000001732
30. Fouda UM, Sayed AM, Ramadan DI, Fouda IM. Efficacy and Safety of Two Doses of Low Molecular Weight Heparin (Enoxaparin) in Pregnant Women With a History of Recurrent Abortion Secondary to Antiphospholipid Syndrome. *J Obstet Gynaecol* (2010) 30(8):842–6. doi: 10.3109/01443615.2010.518651
31. Vane JR, Botting RM. The Mechanism of Action of Aspirin. *Thromb Res* (2003) 110(5-6):255–8. doi: 10.1016/s0049-3848(03)00379-7
32. Committee Opinion No ACOG. 743: Low-Dose Aspirin Use During Pregnancy. *Obstet Gynecol* (2018) 132(1):e44–52. doi: 10.1097/AOG.0000000000002708
33. Werler MM, Mitchell AA, Shapiro S. First Trimester Maternal Medication Use in Relation to Gastroschisis. *Teratology* (1992) 45:361–7. doi: 10.1002/tera.1420450407

34. Werler MM, Sheehan JE, Mitchell AA. Maternal Medication Use and Risks of Gastroschisis and Small Intestinal Atresia. *Am J Epidemiol* (2002) 155:26–31. doi: 10.1093/aje/155.1.26
35. Martinez-Frias ML, Rodriguez-Pinilla E, Prieto L. Prenatal Exposure to Salicylates and Gastroschisis: A Case-Control Study. *Teratology* (1997) 56:241–3. doi: 10.1002/(SICI)1096-9926(199710)56:4<241::AID-TERA2>3.0.CO;2-1
36. Kristensen DM, Hass U, Lesné L, Lottrup G, Jacobsen PR, Lethimonier CD, et al. Intrauterine Exposure to Mild Analgesics Is a Risk Factor for Development of Male Reproductive Disorders in Human and Rat. *Hum Reprod* (2011) 26(1):235–44. doi: 10.1093/humrep/deq323
37. Ezemagu UK, Uzomba GC, Chuwkuemeka U, Rachel O, EC F, Onuora O. Maternal and Neonatal Anthropometric Analyses: Determining Birth Outcomes in Low-Risk Pregnancies at Alex Ekwueme Federal University Teaching Hospital, Abakaliki. *Int J Gynaecol Obstet.* (2021) 154(2):324–30. doi: 10.1002/ijgo.13527
38. Li YM, Chang TK. Maternal Demographic and Psyhosocial Factors Associated With Low Birth Weight in Eastern Taiwan. *Kaohsiung J Med Sci* (2005) 21(11):502–10. doi: 10.1016/s1607-551x(09)70158-5

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers | Frontiers in Endocrinology

ORIGINAL RESEARCH
published: 23 June 2022
doi: 10.3389/fendo.2022.796199

# Which Factors Are Associated With Reproductive Outcomes of DOR Patients in ART Cycles: An Eight-Year Retrospective Study

*Lu Li*[1,2,3,4†], *Bo Sun*[1,2,3,4†], *Fang Wang*[1,2,3,4], *Yile Zhang*[1,2,3,4] *and Yingpu Sun*[1,2,3,4*]

[1] Center for Reproductive Medicine, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [2] Henan Key Laboratory of Reproduction and Genetics, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [3] Henan Provincial Obstetrical and Gynecological Diseases (Reproductive Medicine) Clinical Research Center, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [4] Henan Engineering Laboratory of Preimplantation Genetic Diagnosis and Screening, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China

OPEN ACCESS

**Edited by:**
Yiping Shen,
Harvard Medical School,
United States

**Reviewed by:**
Yugui Cui,
Nanjing Medical University, China
Wenzhu Yu,
Henan Provincial People's Hospital, China

***Correspondence:**
Yingpu Sun
syp2008@vip.sina.com

[†]These authors have contributed equally to this work

**Specialty section:**
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

**Received:** 16 October 2021
**Accepted:** 25 April 2022
**Published:** 23 June 2022

**Citation:**
Li L, Sun B, Wang F, Zhang Y and Sun Y (2022) Which Factors Are Associated With Reproductive Outcomes of DOR Patients in ART Cycles: An Eight-Year Retrospective Study. Front. Endocrinol. 13:796199. doi: 10.3389/fendo.2022.796199

**Introduction:** Women with diminished ovarian reserve (DOR) have a lower pregnancy rate and higher cancellation rate compared to those without DOR when seeking assisted reproductive technology. However, which factors are associated with reproductive outcomes and whether AMH is a predictor of clinical pregnancy remain unclear.

**Objective:** This retrospective study was designed to find factors associated with reproductive outcomes in DOR patients and then discuss the role of AMH in predicting cycle results among this population.

**Method:** A total of 900 women were included in the study. They were diagnosed with DOR with the following criteria: (i) FSH > 10 IU/L; (ii)AMH < 1.1 ng/ml; and (iii) AFC <7. They were divided into different groups: firstly, based on whether they were clinically pregnant or not, pregnant group vs. non-pregnant group (comparison 1); secondly, if patients had transferrable embryos (TE) or not, TE vs. no TE group (comparison 2); thirdly, patients undergoing embryo transfer (ET) cycles were divided into pregnant I and non-pregnant I group (comparison 3). The baseline and ovarian stimulation characteristics of these women in their first IVF/ICSI cycles were analyzed. Logistic regression was performed to find factors associated with clinical pregnancy.

**Results:** Of the 900 DOR patients, 138 women got pregnant in their first IVF/ICSI cycles while the rest did not. AMH was an independent predictor of TE after adjusting for confounding factors (adjusted OR:11.848, 95% CI: 6.21-22.62, P< 0.001). Further ROC (receiver operating characteristic) analysis was performed and the corresponding AUC (the area under the curve) was 0.679 (95% CI: 0.639-0.72, P< 0.001). Notably, an AMH level of 0.355 had a sensitivity of 62.6% and specificity of 65.6%. However, there was no statistical difference in AMH level in comparison 3, and multivariate logistic regression showed female age was associated with clinical pregnancy in ET cycles and women who

were under 35 years old were more likely to be pregnant compared to those older than 40 years old (adjusted OR:4.755, 95% CI: 2.81-8.04, P< 0.001).

**Conclusion:** AMH is highly related to oocyte collection rate and TE rate,and 0.355 ng/ml was a cutoff value for the prediction of TE. For DOR patients who had an embryo transferred, AMH is not associated with clinical pregnancy while female age is an independent risk factor for it.

**Keywords: IVF *in vitro* fertilization, AMH (anti-Müllerian hormone), DOR (diminished ovarian reserve), reproductive outcomes, antral follicle count (AFC)**

## BACKGROUND

Diminished ovarian reserve (DOR) refers to the reduction of the quantity of oocytes in the ovary, which is one of the major causes of infertility in women of child-bearing age (1). Ovarian surgery and gene mutation may be associated with DOR while most patients with DOR cannot find an identified etiology (2, 3). Patients with DOR have a lower number of oocytes acquired and a rate of high-quality embryos compared to those with normal ovarian reserve (NOR), and the rate of a clinical pregnancy is lower while the early miscarriage rate is higher (4–6). Although various treatments are made to assist them to improve the outcome of pregnancy, it remains a big challenge for clinicians.

Based on the Bologna criteria (7), follicle-stimulating hormone (FSH), anti-mullerian hormone (AMH), and antra follicular count (AFC) are the most frequently used biomarkers to access the ovarian reserve, and the latter two have gained widespread attention in recent years. Generally, AMH levels and AFC decline while the incidence of DOR increases, however, discordance between AFC and AMH levels is not rare in clinical work. Measuring by ultrasound, AFC is highly affected by different machines and operating doctors. Studies have demonstrated that priority should be given to AMH compared to AFC in predicting ovarian marker and fertility (8–10).

Controversy has existed on whether AMH is associated with reproductive outcomes in assisted reproductive technology (ART) cycles. A meta-analysis reviewed 19 articles including unspecified ovarian reserve, DOR, and polycystic ovary syndrome (PCOS) patients. Results showed that AMH had weak association with clinical pregnancy but could be a predictor in DOR women (11). A study analyzed 85,062 fresh and embryo-thawed (ET) cycles and demonstrated AMH cannot be a reliable independent predictor of live birth rate (12).

In this retrospective study, we collected data from the first IVF/ICSI cycles of patients with DOR and analyzed baseline and controlled ovarian stimulation (COS) characteristics to find out factors associated with reproductive outcomes and then discuss the role of AMH in predicting cycle results among this population.

## METHODS AND MATERIALS

### Patient Selection

Patients who came to the First Affiliated Hospital of Zhengzhou University for autologous IVF/ICSI cycles were enrolled into this study during January 2011 to December 2019. Patients who have fulfilled the following criteria were included: (i) FSH > 10 IU/L and AMH < 1.1 ng/ml and AFC <7; (ii) the first fresh IVF/ICSI cycle in our center. While the participants who had (i) endometriosis, polycystic ovarian syndrome (PCOS); (ii) chromosomal abnormalities; (iii) hypertension, diabetes, or other chronic diseases; (iv) immune system diseases, such as hypothyroidism; (v) multiple uterine fibroids or a history of ovarian surgery or chemotherapy or radiation exposure; (vi) experienced IVF/ICSI cycles at other hospitals; (vii) premature ovarian insufficiency were excluded (**Figure 1**) as our purpose was to characterize women with idiopathic decrease in ovarian reserve and eliminate other possible confounding factors that had influence on reproductive outcomes. This study was performed under institutional review board approval.

### Grouping Method

A total of 900 patients with DOR were included (**Figure 1**). To find out factors associated with reproductive outcomes in DOR patients at their first ART cycles, firstly, patients were divided into two groups based on whether they were clinically pregnant or not: pregnant group vs. non-pregnant group (comparison 1); secondly, patients who had transferrable embryos (TE) were compared with those did not: TE vs. no TE group (comparison 2); thirdly, patients undergoing embryo transfer (ET) cycles were divided into two groups: pregnant I and non-pregnant I group (comparison 3). Clinical pregnancy was defined as the following: 35 days after transplantation, transvaginal ultrasound examination showed that there was at least one gestational sac in the uterus, including ectopic pregnancy (13).

### AMH Level Detection

Two ml blood samples were aseptically collected from the subjects on days 2 – 4 of the menstrual cycle to assess basal AMH. After centrifugation, serum was analyzed by an electrochemical luminescence analyzer (Roche, Cobas e601, Canada) to detect AMH levels (ng/ml). The theoretical sensitivity of the method was 0.006 ng/ml. Within batches and between batches coefficient of variations were ≤10% and ≤15%, respectively.

### Controlled Ovarian Stimulation Protocol

The ovarian stimulation protocol was determined by the ovarian reserve testing (AMH, AFC and basal FSH) of each patient.

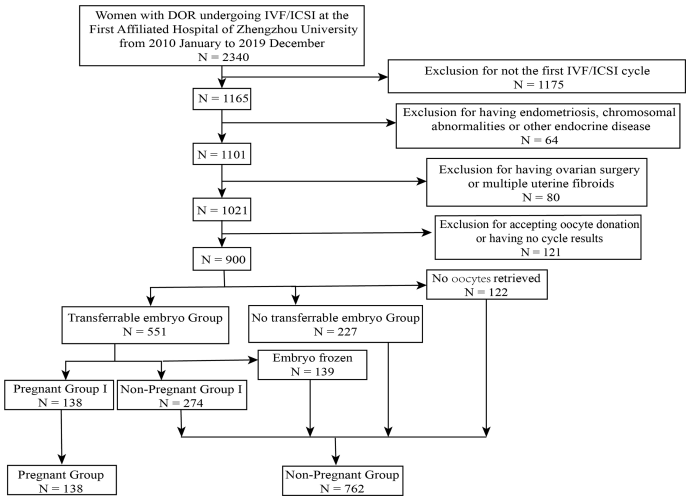



**FIGURE 1** | Flow chart of patient selection.

### Progestin-Primed Ovarian Stimulation Protocol (PPOS)

Medroxyprogesterone acetate and human menopausal gonadotropin (HMG) were used on the third day of menstruation, human chorionic gonadotropin (HCG) was used at the time of triggering.

### Follicular Phase Long-Acting Protocol

Patients were given a starting dose of 3.75 mg GnRH agonist (GnRH-a) on the second day of menstruation. Gonadotropins (Gn) were used to induce ovulation, and we adjusted the dose according to the number, size, and growth of the follicles.

### Mild Stimulation Protocol

Letrozole was given to patients at a dose of 2.5 mg per day on the third day of menstruation, and HMG was added on the fifth day. Once the diameter of a primary follicle was > 18 mm, HCG and Gn were used.

### Natural Cycle

The number, size, and growth of follicles and hormone levels, especially LH, E2, and P4, were observed during menstruation to determine the time of triggering.

### Luteal Phase Short-Acting Long Protocol

GnRH-a was used on the 21st day of menstruation, and ultrasound and hormone levels were used to observe the growth of the follicles. Ovulation was induced using HCG according to the size of the follicles.

### GnRH Antagonist Protocol

FSH was given to patients on their second day of menstruation, and Gn and HCG were injected when the diameter of the primary follicle was > 18 mm.

In the above protocols, HCG was used for 36-37 h before oocyte retrieval. IVF or ICSI was used according to the semen quality of the husband.

## Statistical Methods

The baseline and ovarian stimulation characteristics of patients were compared between each two groups (the grouping method was as described above). Continuous variables were compared by Mann–Whitney U since they were not normally distributed. Chi-squared tests were used to compare categorical variables. The numerical data are presented as the mean with standard deviation (SD), while categorical variables are shown as % (n/N). We performed the univariate and multivariate logistic regression analyses to examine factors that were associated with reproductive outcomes. IBM SPSS version 26.0 (IBM Corp., Armonk, NY, USA) was used, and a P value < 0.05 was considered statistically significant.

# RESULTS

Of the 900 patients who met the criteria in the study, 778 (86.44%) women had oocytes retrieved at their first cycle, 551 (61.22%) women had transferrable embryos after egg collection, 139 (15.44%) patients had embryos frozen, and 138 (15.33%) women got pregnant after implantation.

## Baseline Characteristics

As shown in **Table 1**, in comparison 1, both women and their husbands in the pregnant group were younger than those in the non-pregnant group (34.64 ± 0.43 vs. 38.16 ± 0.22, P < 0.001; 35.13 ± 0.51 vs. 38.80 ± 0.24, P < 0.001). Compared to the non-pregnant group, patients in the pregnant group had shorter years of infertility (4.14 ± 0.31 vs. 5.02 ± 0.16, P=0.045) and experienced fewer times of delivery (0.47 ± 0.05 vs. 0.63 ± 0.02, P =0.010); their basal follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels were much

**TABLE 1** | Baseline characteristics and hormonal profiles between groups of women with DOR undergoing IVF/ICSI.

| | Comparison 1 | | | Comparison 2 | | | Comparison 3 | | |
|---|---|---|---|---|---|---|---|---|---|
| | Pregnant | Non-pregnant | p value | TE | No TE | p value | Pregnant I | Non-pregnant I | p value |
| Number | 138 | 762 | | 551 | 227 | | 138 | 274 | |
| Female age | 34.64 ± 0.43 | 38.16 ± 0.22 | <0.001 | 37.30 ± 0.24 | 38.09 ± 0.42 | 0.021 | 34.64 ± 0.43 | 38.13 ± 0.29 | <0.001 |
| Male age | 35.13 ± 0.51 | 38.80 ± 0.24 | <0.001 | 37.99 ± 0.27 | 38.97 ± 0.46 | 0.055 | 35.13 ± 0.51 | 39.07 ± 0.36 | <0.001 |
| Type of infertility | | | 0.100 | | | 0.612 | | | 0.020 |
| Primary infertility | 51 (36.96) | 228 (29.92) | | 167 (30.31) | 73 (32.16) | | 51 (36.96) | 71 (25.91) | |
| Secondary infertility | 87 (63.04) | 534 (70.08) | | 384 (69.69) | 154 (67.84) | | 87 (63.04) | 203 (74.09) | |
| Years of infertility | 4.14 ± 0.31 | 5.02 ± 0.16 | 0.045 | 4.80 ± 0.19 | 5.04 ± 0.30 | 0.350 | 4.14 ± 0.31 | 5.13 ± 0.29 | 0.086 |
| No. of previous pregnancy | 1.23 ± 0.10 | 1.40 ± 0.04 | 0.126 | 1.36 ± 0.49 | 1.36 ± 0.08 | 0.875 | 1.23 ± 0.10 | 1.49 ± 0.07 | 0.024 |
| No. of previous deliveries | 0.47 ± 0.05 | 0.63 ± 0.02 | 0.010 | 0.59 ± 0.03 | 0.62 ± 0.04 | 0.573 | 0.47 ± 0.05 | 0.66 ± 0.04 | 0.005 |
| No. of previous abortion | 0.49 ± 0.06 | 0.59 ± 0.03 | 0.149 | 0.56 ± 0.03 | 0.59 ± 0.05 | 0.723 | 0.49 ± 0.06 | 0.62 ± 0.04 | 0.083 |
| BMI | 22.86 ± 0.24 | 23.07 ± 0.10 | 0.506 | 23.01 ± 0.12 | 23.24 ± 0.20 | 0.644 | 22.86 ± 0.24 | 23.04 ± 0.17 | 0.544 |
| Basal FSH (mIU/mL) | 15.23 ± 0.58 | 17.46 ± 0.36 | 0.006 | 15.79 ± 0.33 | 18.00 ± 0.72 | 0.001 | 15.23 ± 0.58 | 14.25 ± 0.29 | 0.155 |
| Basal E2 (pg/mL) | 38.09 ± 3.76 | 80.48 ± 9.80 | 0.177 | 73.10 ± 12.60 | 77.19 ± 11.52 | 0.682 | 38.09 ± 3.76 | 93.98 ± 23.76 | 0.219 |
| Basal P4 (ng/mL) | 0.45 ± 0.03 | 0.46 ± 0.01 | 0.215 | 0.45 ± 0.01 | 0.46 ± 0.02 | 0.213 | 0.45 ± 0.03 | 0.45 ± 0.02 | 0.227 |
| Basal LH (mIU/mL) | 6.17 ± 0.33 | 8.10 ± 0.28 | 0.003 | 7.19 ± 0.28 | 7.67 ± 0.47 | 0.295 | 6.17 ± 0.33 | 6.55 ± 0.39 | 0.980 |
| Basal T (ng/mL) | 0.20 ± 0.01 | 0.19 ± 0.00 | 0.927 | 0.20 ± 0.01 | 0.19 ± 0.01 | 0.345 | 0.20 ± 0.01 | 0.19 ± 0.01 | 0.968 |
| AMH (ng/mL) | 0.54 ± 0.02 | 0.37 ± 0.01 | <0.001 | 0.48 ± 0.01 | 0.30 ± 0.02 | <0.001 | 0.54 ± 0.02 | 0.53 ± 0.02 | 0.677 |
| Antral follicular count | 3.70 ± 0.14 | 2.79 ± 0.07 | <0.001 | 3.18 ± 0.08 | 2.72 ± 0.12 | 0.002 | 3.70 ± 0.14 | 3.33 ± 0.11 | 0.049 |
| Basal endometrial thickness | 6.21 ± 0.331 | 6.24 ± 0.13 | 0.764 | 6.32 ± 0.15 | 6.07 ± 0.23 | 0.591 | 6.21 ± 0.33 | 6.65 ± 0.21 | 0.205 |
| Live birth* | 96 (10.67) | / | | | | | | | |
| Abortion* | 38 (4.22) | / | | | | | | | |
| Ectopic pregnancy* | 4 (0.44) | / | | | | | | | |
| Transferrable embryos* | 138 (15.33) | 227 (25.22) | | | | | | | |
| Embryo frozen* | 0 (0.00) | 139 (15.44) | | | | | | | |

*Data are mean ± standard deviation or N (% of response group * % of all participants). FSH, follicle-stimulating hormone; E2, estradiol; P4, progesterone; LH, luteinizing hormone; T, testosterone; BMI, body mass index; AMH, anti-Mullerian hormone; TE, transferrable embryo.*

lower (15.23 ± 0.58 vs. 17.46 ± 0.36, P =0.006; 6.17 ± 0.33 vs. 8.10 ± 0.28, P = 0.003) while basal AMH levels and AFC were much higher (0.54 ± 0.02 vs. 0.37 ± 0.01, P< 0.001; 3.70 ± 0.14 VS 2.79 ± 0.07, P< 0.001). There was no significant difference in the type of infertility, number of pregnancy or abortion, body mass index (BMI), basal estradiol (E2), progesterone (P4), testosterone (T) levels, or basal endometrial thickness between two groups.

In terms of transferrable embryo, only age of female (37.30 ± 0.24 vs. 38.09 ± 0.42, P = 0.021), basal FSH levels (15.79 ± 0.33 vs. 18.00 ± 0.72, P = 0.001), basal AMH levels (0.48 ± 0.01 vs. 0.30 ± 0.02, P < 0.001), and AFC (3.18 ± 0.08 vs. 2.72 ± 0.12, P = 0.002) differed between TE and no TE group. Others were not significantly different.

In comparison 3, FSH (15.23 ± 0.58 vs. 14.25 ± 0.29, P = 0.155) and AMH (0.54 ± 0.02 vs. 0.53 ± 0.02, P = 0.68) had no statistical difference between pregnant I and non-pregnant I group, while AFC (3.70 ± 0.14 vs. 3.33 ± 0.11, P = 0.049) was at the threshold value of P < 0.05. Both maternal (34.64 ± 0.43 vs. 38.13 ± 0.29, P < 0.001) and paternal (35.13 ± 0.24 vs. 39.07 ± 0.36, P< 0.001) age differed significantly between the two groups.

## Ovarian Stimulation Characteristics

As shown in **Table 2**, patients in the pregnant group used more gonadotropin (Gn) and the length of stimulation was much longer than those in the non-pregnant group. In addition, their E2 levels on the day of HCG administration was much higher (1673.67 ± 92.12 vs. 1036.99 ± 32.80, P< 0.001) and the endometrial thickness on that day was much thicker (11.57 ± 0.23 vs. 10.05 ± 0.11, P< 0.001). Not surprisingly, pregnant women had more oocytes retrieved (5.11 ± 0.26 vs. 3.11 ± 0.10, P< 0.001) and had more embryos to implant (2.70 ± 0.14 vs. 1.82 ± 0.07, P< 0.001) compared to women who were not pregnant. Women who were pregnant were more likely to be treated with follicular phase long-acting protocol (74/138 53.62%) and luteal phase ultra-long protocol (51/138 36.96%), however, there was no significant difference in the embryo stage when transferring between two groups.

In comparison 2, the usage of Gn, hormone levels on the day of HCG administration, type of ART, and the choice of stimulation protocol were different between two groups. Due to the difference of protocol choice, more dosage and days of Gn were used in TE group (P < 0.001).

In ET cycles, all patients underwent embryos implantation, number of oocytes (retrieved and MII oocytes, P = 0.004 and 0.001, respectively) and embryos (P < 0.001) differed significantly between pregnant and non-pregnant groups; hormone levels, Gn usage, embryo stage, and type of ART had no significant difference.

## Multivariate Logistic Regression and ROC Curve

To find which factors were associated with reproductive outcomes in women with DOR, univariate and multivariate logistic regression analyses were performed (**Table 3**).

Model 1 included factors associated with TE, and results showed that AMH was an independent predictor of TE after adjusting for confounding factors (adjusted OR:11.848, 95% CI: 6.21-22.62, P< 0.001). Further ROC (receiver operating characteristic) analysis was performed and corresponding AUC (the area under the curve) was 0.679 (95% CI: 0.639-0.72, P< 0.001). Notably, AMH level of 0.355 had sensitivity of 62.6% and specificity of 65.6%. (**Figure 2**)

**TABLE 2 |** Ovarian stimulation characteristics between groups of women with DOR undergoing IVF/ICSI.

| | Comparison 1 | | | Comparison 2 | | | Comparison 3 | | |
|---|---|---|---|---|---|---|---|---|---|
| | Pregnant | Non-pregnant | p value | TE | No TE | p value | Pregnant I | None-pregnant I | p value |
| Number | 138 | 762 | | 551 | 227 | | 138 | 274 | |
| Total amount of Gn (IU) | 3823.46 ± 82.49 | 3039.97 ± 50.26 | <0.001 | 3524.21 ± 50.20 | 2958.22 ± 89.92 | <0.001 | 3823.46 ± 82.49 | 3828.10 ± 59.99 | 0.767 |
| Duration of stimulation (d) | 13.23 ± 0.24 | 10.95 ± 0.15 | <0.001 | 12.30 ± 0.15 | 10.68 ± 0.27 | <0.001 | 13.23 ± 0.24 | 13.09 ± 0.19 | 0.453 |
| Endometrial thickness on HCG (mm) | 11.53 ± 0.23 | 10.23 ± 0.13 | <0.001 | 10.96 ± 0.12 | 9.93 ± 0.20 | <0.001 | 11.57 ± 0.23 | 11.48 ± 0.15 | 0.904 |
| Hormone levels on HCG | | | | | | | | | |
| E2 (pg/mL) | 1673.67 ± 92.12 | 1036.99 ± 32.80 | <0.001 | 1435.10 ± 44.88 | 768.02 ± 37.21 | <0.001 | 1673.67 ± 92.12 | 1579.76 ± 64.24 | 0.326 |
| LH (mIU/mL) | 5.85 ± 0.33 | 8.21 ± 0.69 | <0.001 | 3.36 ± 0.18 | 7.47 ± 0.57 | <0.001 | 1.95 ± 0.19 | 2.32 ± 0.14 | 0.070 |
| P4 (ng/mL) | 0.63 ± 0.04 | 0.61 ± 0.03 | 0.293 | 0.67 ± 0.04 | 0.53 ± 0.05 | 0.001 | 0.63 ± 0.04 | 0.75 ± 0.07 | 0.483 |
| No. of ≧14mm oocytes | 4.32 ± 0.31 | 3.20 ± 0.17 | <0.001 | 3.42 ± 0.10 | 1.81 ± 0.09 | <0.001 | 4.11 ± 0.20 | 3.73 ± 0.13 | 0.098 |
| Total oocytes retrieved | 5.86 ± 0.48 | 3.90 ± 0.23 | <0.001 | 4.06 ± 2.91 | 2.02 ± 1.84 | <0.001 | 5.11 ± 0.26 | 4.26 ± 0.16 | 0.004 |
| Rate of MII oocytes | 5.02 ± 0.41 | 3.29 ± 0.20 | <0.001 | 3.41 ± 2.55 | 1.34 ± 1.31 | <0.001 | 4.38 ± 0.23 | 3.54 ± 0.14 | 0.001 |
| Rate of 2PN embryos | 3.86 ± 0.32 | 2.54 ± 0.15 | <0.001 | 2.72 ± 1.94 | 0.66 ± 1.02 | <0.001 | 3.55 ± 0.19 | 2.78 ± 0.11 | <0.001 |
| No. of transferrable embryo | 2.79 ± 0.18 | 1.87 ± 0.09 | <0.001 | 2.04 ± 1.24 | 0 | | 2.68 ± 0.12 | 2.05 ± 0.07 | <0.001 |
| No. of good-quality embryos | 2.54 ± 0.17 | 1.72 ± 0.09 | <0.001 | / | / | | 2.39 ± 0.12 | 1.80 ± 0.07 | <0.001 |
| No. of embryo transferred | 1.68 ± 0.07 | 0.95 ± 0.06 | <0.001 | / | / | | 1.76 ± 0.04 | 1.59 ± 0.03 | 0.001 |
| Stimulation protocol | | | <0.001 | | | <0.001 | | | 0.010 |
| PPOS | 0 (0) | 75 (9.84) | | 25 (4.54) | 18 (7.93) | | 0 (0) | 0 (0) | |
| Follicular phase long-acting protocol | 74 (53.62) | 188 (24.67) | | 214 (38.84) | 45 (19.82) | | 74 (53.62) | 124 (45.26) | |
| GnRH antagonist protocol | 12 (8.70) | 233 (30.58) | | 133 (24.14) | 79 (34.80) | | 12 (8.70) | 54 (19.71) | |
| Mild stimulation protocol | 1 (0.72) | 81 (10.63) | | 20 (3.63) | 30 (13.22) | | 1 (0.72) | 0 (0) | |
| Luteal phase short-acting long protocol | 51 (36.96) | 169 (22.18) | | 157 (28.49) | 50 (22.03) | | 51 (36.96) | 96 (35.04) | |
| Natural cycle | 0 (0) | 16 (2.10) | | 2 (0.36) | 5 (2.20) | | 0 (0) | 0 (0) | |
| Type of ART | | | 0.005 | | | 0.005 | | | 0.881 |
| IVF | 113 (81.88) | 686 (90.03) | | 469 (85.12) | 210 (92.51) | | 113 (81.88) | 226 (82.48) | |
| ICSI | 25 (15.22) | 76 (9.97) | | 82 (14.88) | 17 (7.49) | | 25 (18.12) | 48 (17.52) | |
| Embryo stage | | | 0.587 | | | | | | 0.587 |
| D2 | 3 (2.17) | 11 (1.44) | | / | / | | 3 (2.17) | 11 (4.01) | |
| D3 | 132 (95.65) | 258 (33.86) | | / | / | | 132 (95.65) | 258 (94.16) | |
| D5 | 3 (2.17) | 5 (0.66) | | / | / | | 3 (2.17) | 5 (1.82) | |

*Data are mean ± standard deviation or N (% of response group). TE, transferrable embryos; Gn, gonadotropin; E2, estradiol; P4, progesterone; LH, luteinizing hormone; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; on HCG, on the day of human chorionic gonadotropin used.*

Model 2 included factors associated with clinical pregnancy in ET cycles. Female age was the only factor associated with clinical pregnancy in ET cycles and women under 35 years old were more likely to be pregnant compared to those older than 40 years old (adjusted OR:4.755, 95% CI: 2.81-8.04, P< 0.001).

## DISCUSSION

In a woman's life, the development of follicle pools begin when *in utero*. However, it begins to decline before the time of birth and continues to decline throughout the fertile years (14). Generally, the ovarian reserve drops sharply in the mid-40s, which is a normal physiological phenomenon. Some women, however, experience DOR long before the usual time, which causes infertility in their child-bearing years (15). DOR has multiple adverse implications for a woman's health due to the change of ovarian hormones. A previous study demonstrated that it impairs renal function, increases the risk of cardiovascular disease and decreases bone mineral density (4). The most disastrous impact of DOR for a woman, however, may be infertility. With the development of ART, the number of patient visits is increasing rapidly, and about 31%of patients who go to reproductive centers for help have reduced ovarian reserve, and the incidence rises significantly with age (16). However, due to the long period and high cost of IVF/ICSI cycles, patients and families may have heavy burdens after failure, not only economically but also psychologically.

Therefore, we designed this study to find factors that affect fecundity in DOR women. Like previous reports (17, 18), patients were divided into groups based on if they were clinically pregnant in their first IVF/ICSI cycle, and age, AMH, and AFC were highly different between the two groups. Yet many patients (n = 139) had embryoes frozen because of elevated progesterone levels, uterus factor, or self-factors, and they were divided into the non-pregnant group. Biases may exist in the results above. It's believed that women with higher AMH could have more eggs collect after COS (19). Next, to demonstrate AMH is also associated with the rate of embryo formation after retrieving oocytes, comparison 2 was constructed. Results showed AMH is an independent predictor of TE rate, and 0.355 ng/ml was a cutoff value for the prediction of TE. Last, we

**TABLE 3** | Factors associated with reproductive outcomes in women with DOR.

| | Univariate | | | Multivariate | | |
|---|---|---|---|---|---|---|
| | **crude OR** | **95% CI** | **P value** | **adjusted OR** | **95% CI** | **P value** |
| **Model 1*** | | | | | | |
| Female age | 0.096 | 0.95-1.00 | 0.978 | 0.981 | 0.95-1.01 | 0.191 |
| Basal FSH (mIU/mL) | 0.975 | 0.96-0.99 | 0.003 | 0.996 | 0.98-1.02 | 0.696 |
| AFC | 1.147 | 1.05-1.25 | 0.002 | 0.989 | 0.90-1.09 | 0.833 |
| AMH (ng/mL) | 11.848 | 6.21-22.62 | <0.001 | 11.848 | 6.21-22.62 | <0.001 |
| **Model 2#** | | | | | | |
| Type of infertility | 1.676 | 1.08-2.60 | 0.021 | 0.995 | 0.54-1.83 | 0.988 |
| No. of previous pregnancy | 0.820 | 0.68-0.98 | 0.033 | 0.996 | 0.75-1.32 | 0.979 |
| No. of previous deliveries | 0.602 | 0.43-0.85 | 0.004 | 0.95 | 0.58-1.57 | 0.84 |
| AFC | 1.134 | 1.01-1.28 | 0.041 | 1.076 | 0.95-1.22 | 0.266 |
| Female age | | | | | | |
| <35 | 4.755 | 2.81-8.04 | <0.001 | 4.755 | 2.81-8.04 | <0.001 |
| 35-40 | 2.160 | 1.21-3.85 | 0.009 | 2.16 | 1.21-3.85 | 0.009 |
| >40 | reference | | | reference | | |
| Male age | | | | | | |
| <35 | 4.124 | 2.52-6.76 | <0.001 | 2.067 | 0.93-4.62 | 0.077 |
| 35-40 | 1.504 | 0.83-2.73 | 0.181 | 1.049 | 0.52-2.10 | 0.893 |
| >40 | reference | | | reference | | |

*OR, odds ratio; CI, confidence interval; AMH, anti-Müllerian hormone; FSH, follicle-stimulating hormone; AFC, antral follicle count.*
**Model 1 included factors associated with TE; #Model 2 included factors associated with clinical pregnancy in ET cycles.*

wondered if AMH can affect clinical pregnancy after embryo implantation, comparison 3 was made. Not surprisingly, female age is the only factor related to success in ET cycles.

## There Is No Best Protocol for DOR Patients Due to the Existence of Huge Individual Differences

Of the 900 women included, 762 women did not conceive. These patients were older and had much lower ovarian reserve according to FSH, AFC, and AMH compared to those who are pregnant. Considering baseline characteristics and poor ovarian response (POR) of these women, appropriate stimulation protocol was selected to avoid the adverse reactions of high-dose exogenous hormones and reduce the economic burden on patients. In COS cycles, patients in the non-pregnant group tended to use GnRH antagonist protocol and Follicular phase long-acting protocol. Natural protocol and mild stimulation protocol were in the non-pregnant group only. Correspondingly, women in non-pregnant group used much lower Gn and got fewer oocytes and embryos. Published data compared various protocols in DOR patients, GnRH agonist protocol and modified natural cycle were thought to be more effective than other protocols, since they could improve the

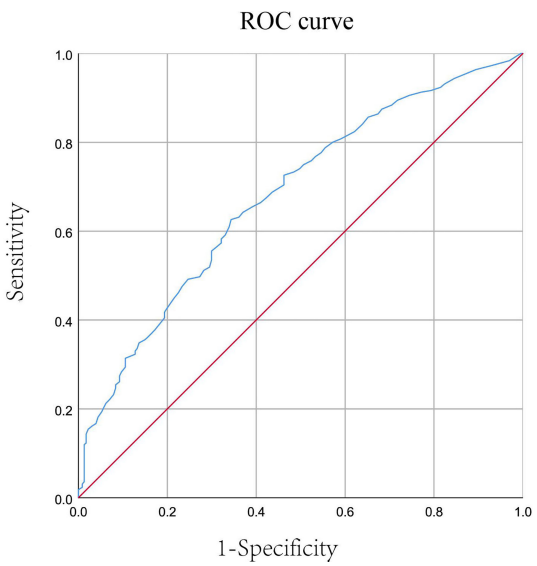



**FIGURE 2** | Receiver operating characteristics curve of the predictive utility of AMH for TE among women with DOR (area under the curve (AUC) = 0.679, 95% CI: 0.639-0.72, P < 0.001).

quality of oocytes and probability of live birth of women with DOR (20, 21). The latest meta-analysis does not promote GnRH antagonist protocol for DOR patients because it correlates with higher cancellation rates and less pregnancies compared to agonist protocols (22). Ovarian stimulation protocol choice should be based on physical condition of DOR patients, which may be different each cycle. It is our opinion that there is no best protocol for this population because of the existence of huge individual differences.

### AMH Is an Independent Predictor of TE But Not of Clinical Pregnancy in ET Cycles

Produced by developing antral follicles in the ovaries and involved in the regulatory process of maturation of primordial follicles, AMH is considered an accurate biomarker to access ovarian reserve and ovarian response (15, 23). Recent studies tried to find out whether AMH had an association with reproductive outcomes, including rate of oocyte collected, clinical pregnancy, live birth, and miscarriage. A retrospective study reviewed 34,540 cycles with AMH<1 ng/ml and demonstrated serum AMH is highly correlated with cumulative live birth rates (CLBR) in women with DOR independent of age (24). Similarly, AMH was statistically differed between TE and no TE group in our study, and we identified 0.355 ng/ml as a cutoff value for the prediction of TE. Yet it had no correlation with clinical pregnancy in ET cycles, which means the AMH level was not associated with pregnancy rate in patients with implanted embryos. This result is consistent with a previous report (25). The ability of AMH on predicting the likelihood of IVF/ICSI success continues to be a subject of debate. Our study demonstrated that AMH is highly related to the oocyte acquired rate and TE rate but not to the clinical pregnancy rate in ET cycles. Large cohort studies are needed to discuss the relationship between them.

### Female Age Is a Risk Factor of Clinical Pregnancy in ET Cycles

Follicles in female ovarian apoptosis and the decrease with increasing of age means the capacity of fertility is dropping over time, therefore, age can largely determine whether conception can be successful. Studies have shown that in DOR patients, younger women have higher pregnancy rate and lower miscarriage rate compared to their older peers (8, 26, 27). Similar results were found in our study. Patients 35-40 years of age were 2.16 times more likely to getpregnant compared to those > 40 years old, and the number increased to 4.755 in patients < 35 years old. Researchers believe that DOR not only has adverse implications on oocyte quantity but also on quality (28). Moreover, data from our center investigated by Zhang et al. (29) showed that the aberration-related miscarriages among women with DOR were more frequent in patients older than 32 years old, and they demonstrated age is an independent risk factor for chromosomal abnormality after adjustment. For those who are diagnosed with DOR, younger women can have better reproductive outcomes with ART compared to older ones. This should be explained to patients so they can get a better understanding of their situation and get anxiety and stress released.

### Strength and Limitation

This study has some strengths. First, due to the rigorous definition of DOR, the homogeneity of the patients included was high, and possible confounding factors were removed. Second, we divided the patients into different groups step by step, and deeply explored the relevant factors related to the fecundity of DOR patients. This grouping method is conducive to controlling the influence of confounding factors.

There were also several limitations in our study. One is the nature of the retrospective study. Data from a single center also weakened the reliability. In addition, we only included the first IVF/ICSI cycle of these patients; CLBR were not analyzed. Conception, not live birth, was our main outcome, while live birth is crucial for accessing fecundity. Therefore, the conclusions from this study are not definitive but indicative, and these findings need to be confirmed by more prospective and multi-center studies.

## CONCLUSION

AMH is highly related to oocyte collection rate and TE rateand 0.355 ng/ml was a cutoff value for the prediction of TE. For DOR patients who had embryo transferred, AMH is not associated with clinical pregnancy while female age is an independent risk factor for it.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

This study was performed under institutional review board approval of the First Affiliated Hospital of Zhengzhou University

## AUTHOR CONTRIBUTIONS

LL and BS: designed study, analyzed data, and drafted the manuscript. FW and YZ: reviewed the manuscript. YS: Study conceptualization and review. All authors approved the final manuscript.

## FUNDING

This study was supported by Henan Province Medical Science and Technology Research (Key) Project (FDN-SBGJ202002049); Henan Province Medical Scienceand Technology Research Project (FDN-LHGJ20210353).

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.796199/full#supplementary-material

## REFERENCES


1. Christodoulaki A, Boel A, Tang M, De Roo C, Stoop D, Heindryckx B. Prospects of Germline Nuclear Transfer in Women With Diminished Ovarian Reserve. *Front Endocrinol (Lausanne)* (2021) 12:635370. doi: 10.3389/fendo.2021.635370
2. Kitajima M, Khan KN, Harada A, Taniguchi K, Inoue T, Kaneuchi M, et al. Association Between Ovarian Endometrioma and Ovarian Reserve. *Front Biosci (Elite Ed)* (2018) 10:92–102. doi: 10.2741/e810
3. Jaillard S, Sreenivasan R, Beaumont M, Robevska G, Dubourg C, Knarston IM, et al. Analysis of NR5A1 in 142 Patients With Premature Ovarian Insufficiency, Diminished Ovarian Reserve, or Unexplained Infertility. *Maturitas* (2020) 131:78–86. doi: 10.1016/j.maturitas.2019.10.011
4. Pal L, Bevilacqua K, Zeitlian G, Shu J, Santoro N. Implications of Diminished Ovarian Reserve (DOR) Extend Well Beyond Reproductive Concerns. *Menopause* (2008) 15(6):1086–94. doi: 10.1097/gme.0b013e3181728467
5. Morin SJ, Patounakis G, Juneau CR, Neal SA, Scott RT, Seli E. Diminished Ovarian Reserve and Poor Response to Stimulation in Patients <38 Years Old: A Quantitative But Not Qualitative Reduction in Performance. *Hum Reprod* (2018) 33(8):1489–98. doi: 10.1093/humrep/dey238
6. Fan Y, Chang Y, Wei L, Chen J, Li J, Goldsmith S, et al. Apoptosis of Mural Granulosa Cells Is Increased in Women With Diminished Ovarian Reserve. *J Assist Reprod Genet* (2019) 36(6):1225–35. doi: 10.1007/s10815-019-01446-5
7. Ferraretti AP, La Marca A, Fauser BC, Tarlatzis B, Nargund G, Gianaroli L. ESHRE Consensus on the Definition of 'Poor Response' to Ovarian Stimulation for *In Vitro* Fertilization: The Bologna Criteria. *Hum Reprod* (2011) 26(7):1616–24. doi: 10.1093/humrep/der092
8. Bishop LA, Richter KS, Patounakis G, Andriani L, Moon K, Devine K. Diminished Ovarian Reserve as Measured by Means of Baseline Follicle-Stimulating Hormone and Antral Follicle Count Is Not Associated With Pregnancy Loss in Younger *In Vitro* Fertilization Patients. *Fertil Steril* (2017) 108(6):980–7. doi: 10.1016/j.fertnstert.2017.09.011
9. Fleming R, Seifer DB, Frattarelli JL, Ruman J. Assessing Ovarian Response: Antral Follicle Count Versus Anti-Müllerian Hormone. *Reprod BioMed Online* (2015) 31(4):486–96. doi: 10.1016/j.rbmo.2015.06.015
10. Ligon S, Lustik M, Levy G, Pier B. Low Antimüllerian Hormone (AMH) Is Associated With Decreased Live Birth After *In Vitro* Fertilization When Follicle-Stimulating Hormone and AMH are Discordant. *Fertil Steril* (2019) 112(1):73–81.e1. doi: 10.1016/j.fertnstert.2019.03.022
11. Tal R, Tal O, Seifer BJ, Seifer DB. Antimüllerian Hormone as Predictor of Implantation and Clinical Pregnancy After Assisted Conception: A Systematic Review and Meta-Analysis. *Fertil Steril* (2015) 103(1):119–30.e3. doi: 10.1016/j.fertnstert.2014.09.041
12. Tal R, Seifer DB, Wantman E, Baker V, Tal O. Antimüllerian Hormone as a Predictor of Live Birth Following Assisted Reproduction: An Analysis of 85,062 Fresh and Thawed Cycles From the Society for Assisted Reproductive Technology Clinic Outcome Reporting System Database for 2012-2013. *Fertil Steril* (2018) 109(2):258–65. doi: 10.1016/j.fertnstert.2017.10.021
13. Zegers-Hochschild F, Adamson GD, de Mouzon J, Ishihara O, Mansour R, Nygren K, et al. International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) Revised Glossary of ART Terminology, 2009. *Fertil Steril* (2009) 92(5):1520–4. doi: 10.1016/j.fertnstert.2009.09.009
14. Janas P, Kucybała I, Radoń-Pokracka M, Huras H. Telocytes in the Female Reproductive System: An Overview of Up-to-Date Knowledge. *Adv Clin Exp Med* (2018) 27(4):559–65. doi: 10.17219/acem/68845
15. Pastore LM, Christianson MS, Stelling J, Kearns WG, Segars JH. Reproductive Ovarian Testing and the Alphabet Soup of Diagnoses: DOR, POI, POF, POR, and FOR. *J Assist Reprod Genet* (2018) 35(1):17–23. doi: 10.1007/s10815-017-1058-4
16. Ma R, Song J, Si J, Liu Y, Li X, Cheng R, et al. Acupuncture for Diminished Ovarian Reserve: Protocol for a Systematic Review and Meta-Analysis. *Med (Baltimore)* (2019) 98(34):e16852. doi: 10.1097/MD.0000000000016852
17. Huang Y, Li J, Zhang F, Liu Y, Xu G, Guo J, et al. Factors Affecting the Live-Birth Rate in Women With Diminished Ovarian Reserve Undergoing IVF-ET. *Arch Gynecol Obstet* (2018) 298(5):1017–27. doi: 10.1007/s00404-018-4884-4
18. Huang R, Wang NN, Li TT, Li MC, Yang X, Liang XY. Predictors of Live Birth Rate in Women With Diminished Ovarian Reserve. *Int J Gynaecol Obstet* (2020) 150(2):222–7. doi: 10.1002/ijgo.13174
19. Dai X, Wang Y, Yang H, Gao T, Yu C, Cao F, et al. AMH has No Role in Predicting Oocyte Quality in Women With Advanced Age Undergoing IVF/ICSI Cycles. *Sci Rep* (2020) 10(1):19750. doi: 10.1038/s41598-020-76543-y
20. Yu CM, Dai XL, Wang YF, Gao TT, Cao F, Xia XY, et al. Progestin-Primed Ovarian Stimulation Improves the Outcomes of IVF/ICSI Cycles in Infertile Women With Diminished Ovarian Reserve. *J Chin Med Assoc* (2019) 82(11):845–8. doi: 10.1097/JCMA.0000000000000177
21. Huang MC, Tzeng SL, Lee CI, Chen HH, Huang CC, Lee TH, et al. GnRH Agonist Long Protocol Versus GnRH Antagonist Protocol for Various Aged Patients With Diminished Ovarian Reserve: A Retrospective Study. *PloS One* (2018) 13(11):e0207081. doi: 10.1371/journal.pone.0207081
22. Papamentzelopoulou M, Stavros S, Mavrogianni D, Kalantzis C, Loutradis D, Drakakis P. Meta-Analysis of GnRH-Antagonists Versus GnRH-Agonists in Poor Responder Protocols. *Arch Gynecol Obstet* (2021) 304(2):547–557. doi: 10.1007/s00404-020-05954-z
23. Panay N, Anderson RA, Nappi RE, Vincent AJ, Vujovic S, Webber L, et al. Premature Ovarian Insufficiency: An International Menopause Society White Paper. *Climacteric* (2020) 23(5):426–46. doi: 10.1080/13697137.2020.1804547
24. Tal R, Seifer DB, Tal R, Granger E, Wantman E, Tal O. AMH Highly Correlates With Cumulative Live Birth Rate in Women With Diminished Ovarian Reserve Independent of Age. *J Clin Endocrinol Metab* (2021) 106(9):2754–2766. doi: 10.1210/clinem/dgab168
25. Pereira N, Setton R, Petrini AC, Lekovich JP, Elias RT, Spandorfer SD. Is Anti-Müllerian Hormone Associated With IVF Outcomes in Young Patients With Diminished Ovarian Reserve? *Womens Health (Lond)* (2016) 12(2):185–92. doi: 10.2217/whe.15.102
26. Chang Y, Li J, Li X, Liu H, Liang X. Egg Quality and Pregnancy Outcome in Young Infertile Women With Diminished Ovarian Reserve. *Med Sci Monit* (2018) 24:7279–84. doi: 10.12659/MSM.910410
27. Kushnir VA, Safdie M, Darmon SK, Albertini DF, Barad DH, Gleicher N. Age-Specific IVF Outcomes in Infertile Women With Baseline FSH Levels ≥20 mIU/mL. *Reprod Sci* (2018) 25(6):893–8. doi: 10.1177/1933719117697130
28. Jaswa EG, McCulloch CE, Simbulan R, Cedars MI, Rosen MP. Diminished Ovarian Reserve Is Associated With Reduced Euploid Rates *via* Preimplantation Genetic Testing for Aneuploidy Independently From Age: Evidence for Concomitant Reduction in Oocyte Quality With Quantity. *Fertil Steril* (2021) 115(4):966–973. doi: 10.1016/j.fertnstert.2020.10.051
29. Zhang W, Zhang L, Liu Y, Li J, Xu X, Niu W, et al. Higher Chromosomal Aberration Frequency in Products of Conception From Women Older Than 32 Years Old With Diminished Ovarian Reserve Undergoing IVF/ICSI. *Aging (Albany NY)* (2021) 13(7):10128–40. doi: 10.18632/aging.202772


**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers | Frontiers in Endocrinology

ORIGINAL RESEARCH
published: 24 June 2022
doi: 10.3389/fendo.2022.851167

# Fetal Reduction Could Improve but Not Completely Reverse the Pregnancy Outcomes of Multiple Pregnancies: Experience From a Single Center

*Zhu Yimin*†, Tang Minyue†, Fu Yanling, Yan Huanmiao, Sun Saijun, Li Qingfang, Hu Xiaoling and Xing Lanfeng*

*Department of Reproductive Endocrinology, Women's Hospital, Zhejiang University School of Medicine, Hangzhou, China*

**Objective:** To investigate the effectiveness and limitations of multifetal pregnancy reduction (MFPR) on the improvement of pregnancy outcomes of triplet or twin pregnancies conceived by *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI).

**Methods:** We performed a cohort study of women undergoing IVF or ICSI from 2002–2016 in reproductive center, women's hospital, Zhejiang University School of Medicine. The cohort included 502 women who underwent MFPR and 9641 non-reduced women. Pregnancy outcomes were gestational age (GA) at delivery, pregnancy loss, preterm delivery, low birth weight (LBW), very low birth weight (VLBW), and small for gestational age (SGA). Multiple linear regression and logistic regression models were used to compare pregnancy outcomes between groups.

**Results:** Triplets reduced to singletons had a longer median GA (39.07 vs 37.00, P<0.001), and lower rates of LBW (8.9% vs 53.2%, P<0.001) and SGA (17.8% vs 44.7%, P=0.001) than triplets reduced to twins, with a similar pregnancy loss rate (6.7% vs 6.6%, P=0.701). Twins reduced to singletons had a comparable pregnancy loss rate (4.8% vs. 6.5%, P=0.40), a longer median GA (38.79 vs. 37.00, P<0.001), and lower rates of LBW (13.5% vs. 47.0%, P<0.001) and SGA (13.5% vs. 39.6%, P<0.001) than primary twins. Triplets reduced to twins had higher rates of LBW (53.2% vs. 47.0%, P=0.028) and SGA (44.7% vs. 39.6%, P=0.040) than primary twins, with a similar pregnancy loss rate (6.6% vs. 6.5%, P=0.877). Singletons reduced from triplets/twins had higher rates of preterm delivery (15.8% vs. 7.3%, P<0.001), LBW (12.3% vs. 4.32%, P<0.001), VLBW (2.3% vs. 0.4%, P=0.002), and SGA (14.6% vs.6.6%, P<0.001) than primary singletons, with a comparable pregnancy loss rate (5.3% vs. 5.4%, P=0.671).

OPEN ACCESS

**Edited by:**
*MaryEllen Pavone, Northwestern Medicine, United States*

**Reviewed by:**
*Dana Kimelman, Centro de Esterilidad Montevideo, Uruguay*
*Liang Hu, Central South University, China*
*Xinru Xia, First Affiliated Hospital of Nanjing Medical University, China*

***Correspondence:**
*Zhu Yimin*
*zhuyim@zju.edu.cn*

†*These authors have contributed equally to this work*

**Specialty section:**
*This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology*

**Received:** *09 January 2022*
**Accepted:** *13 May 2022*
**Published:** *24 June 2022*

**Citation:**
*Yimin Z, Minyue T, Yanling F, Huanmiao Y, Saijun S, Qingfang L, Xiaoling H and Lanfeng X (2022) Fetal Reduction Could Improve but Not Completely Reverse the Pregnancy Outcomes of Multiple Pregnancies: Experience From a Single Center. Front. Endocrinol. 13:851167. doi: 10.3389/fendo.2022.851167*

**Conclusions:** This study suggests that the pregnancy loss rate is similar between reduction and non-reduction groups. MFPR improves pregnancy outcomes, including the risk of preterm delivery, LBW, and SGA, but still could not completely reverse the adverse pregnancy outcomes of multiple pregnancies.

**Keywords: multifetal pregnancy reduction, assisted reproductive technology, pregnancy outcome, twins, triplets**

## INTRODUCTION

There has been a growing trend for the increasing use of assisted reproductive technology (ART) to combat infertility in recent years. However, ART constitutes a major risk factor for the prevalence of multiple pregnancies (1, 2). Multiple pregnancies are associated with an increasing risk for mothers and fetuses, including maternal complications, as well as low birth weight (LBW) and small for gestational age (SGA) (3, 4).

As the risks of multiple pregnancies have gradually been recognized, several countries have legally mandated a decrease in the number of embryos transferred and advocated for elective single embryo transfer (SET) (5–8). However, transfer of more than one embryo is still common in many countries (9, 10). Multifetal pregnancy reduction (MFPR) is a secondary preventive measure for managing multiple pregnancies that have occurred. MFPR began in the 1980s to salvage pregnancies with too many fetuses by ART (11). Because MFPR is an interventional operation, a major difficulty is the lack of clarity regarding the explicit benefits and limitations of MFPR when counselling for triplet or twin pregnancies conceived by IVF or ICSI (12). It can always be difficult for couples with triplet or twin pregnancies conceived by IVF or ICSI to weigh the pros and cons to decide whether to reduce fetus since the fetuses are hard-won for them. In recent years, with a growing awareness of the adverse outcomes of multiple pregnancies and accumulating data supporting the safety of MFPR, reduction of triplets is a widely accepted option (13, 14). However, for triplet pregnancies, reducing to singles or twins is still a tough decision. Moreover, the effectiveness of reduction from twins to singletons is controversial (15–17). Previous studies regarding pregnancy outcomes after MFPR were based on limited and conflicting data, which require further investigation.

In this study, we aimed to address this inconsistency and to further investigate whether MFPR get equal benefit as primary singleton/twin pregnancies using a large dataset from a single center during 15 years. Pregnancy outcomes of triplets and twins who underwent MFPR were recorded, and the benefits and limitations from reduction were evaluated to provide a comprehensive understanding of MFPR.

## MATERIALS AND METHODS

### Study Design

This is a cohort study performed in reproductive center, women's hospital, Zhejiang University School of Medicine (**Figure 1**). The reduction group included a cohort of multiple-pregnant women conceived by IVF or ICSI who underwent MFPR and continued follow-up in the reproductive center from 2002 to 2016. Exclusion criteria for the reduction group: 1) initial fetuses >3;2) ectopic pregnancy;3) heterotopic pregnancy; 4) ART or reduction data missed in database; 5) data on pregnancy outcomes were not available. After exclusion, the reduction group included a cohort of 502 women conceived after ART with triplet or twin pregnancies and reduced to twins or singletons at 6-16 weeks of gestation. In this cohort, there were 331 women with twins reduced from triplets at 6-13 weeks, 45

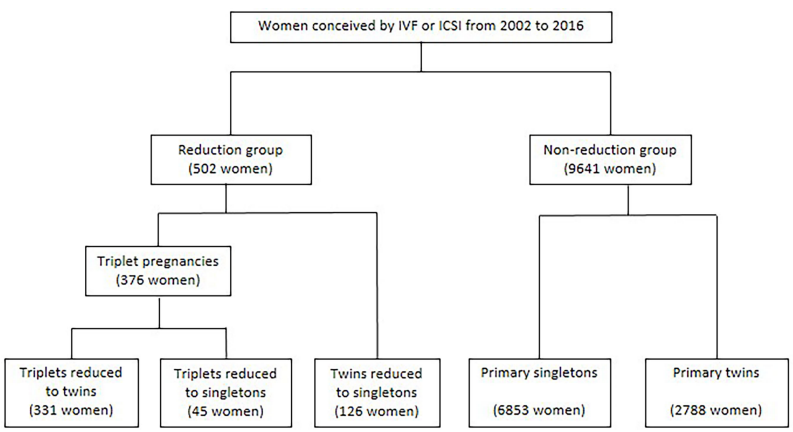



**FIGURE 1** | Study design.

women with singletons reduced from triplets at 7-12 weeks, 126 women with singletons reduced from twins at 7-16 weeks. This study was approved by the Women's hospital, Zhejiang University School of Medicine, and written informed consents were obtained from all participants.

All women conceived by IVF or ICSI at our center during the same period without undergoing MFPR who met the inclusion criteria were enrolled in the non-reduction group. Inclusion criteria for non-reduction group: 1) with integrated ART data in database; 2) underwent ultrasound exam at our center confirmed singletons or twins intrauterine live pregnancies between 6-8 weeks of gestation; 3) continued follow-up in this reproductive center from 2002 to 2016. The non-reduction group included 2788 women with primary twins and 6853 women with primary singletons.

All MFPR procedures were carried out by highly skilled physicians. All patients underwent counseling regarding the risks and benefits of MFPR, and were advised to reduce the number of embryos to one or two, depending on previous obstetric history, religious beliefs, and patient preference. The reason for reduction could be either a genetic abnormality or structural abnormality in one or two of the fetuses diagnosed by ultrasound or an invasive diagnostic test, the prevention of preterm birth or completely elective. Fetal reduction procedures were performed transvaginal between 6 to 16 weeks gestation. The patient with an empty bladder was in the lithotomy position. After cleaning the vagina with povidone iodine, the fetuses were visualized using a transvaginal ultrasound transducer to verify the number, position, size, and heart activity of each fetuses. The smallest or abnormal fetuses and/or the fetuses that was located in a position with the easiest access route was selected for reduction. An appropriate size needle was inserted into the fetal heart to aspirate the fluid and fetus from the sac or inject potassium chloride solution. Prophylactic antibiotic therapy was used for 3 days in all cases. Women were discharged from the clinic after bed rest and an average observation period of 120 minutes. A follow-up ultrasound was carried out within 1 week.

Maternal and ART characteristics were prospectively recorded in inpatient database of reproductive center, women's hospital, Zhejiang University. All patients underwent subsequent follow-up by telephone. Delivery and offspring characteristics were collected through telephone interview and review of medical records. GA was calculated based on the embryo transfer (ET) time and was correlated to a first trimester ultrasound exam. Reduction weeks was defined as the GA at the MFPR. Parental characteristics included maternal age at conception, height, weight and BMI before pregnancy. ART characteristics included type of infertility, ART methods, embryo transplantation, and source of semen. Primary infertility was defined as the inability to ever become pregnant after at least one year of having sex and not using birth control methods. Secondary infertility was defined by the inability of a couple that already has conceived and delivered a newborn to conceive again. ART methods were defined as IVF or ICSI, ET was defined as fresh-ET or Frozen-ET, source of semen was defined as ejaculated semen, sperm aspiration and donor semen.

## Outcomes

Pregnancy outcomes assessed in this study included GA at delivery, the rates of preterm delivery before <32 weeks, <34 weeks, and <37 weeks of gestation, pregnancy loss < 24 weeks, abortion of one fetus and caesarean section as well as neonatal outcomes such as neonatal birth weight, the rates of at least one fetus LBW, at least one fetus very low birth weight (VLBW) and SGA. LBW was defined as birth weight below 2500g, and VLBW was defined as birth weight below 1500g. SGA was defined as birth weight below the 10th percentile for the gestational age at delivery (18).

## Statistical Analysis

Comparison of continuous variables was analyzed using Mann–Whitney U tests. Categorical variables were compared by using Chi-square tests or Fisher's exact test. Logistic regression and linear regression were used for adjusting certain confounders. Significance was accepted at P<0.05. All reported P values were two-sided. Statistical analyses were conducted using the IBM SPSS 23.0 (IBM, USA).

# RESULTS

The study cohort and groups are shown in **Figure 1**. The demographics and ART characteristics for five groups are given in **Table 1**.

## Triplets Reduced to Singletons Versus Triplets Reduced to Twins

Pregnancy outcomes of the two groups are shown in **Table 2**. For triplets reduced to singletons, the median GA at delivery was more than 2 weeks longer than that for triplets reduced to twins (39.07 vs 37.00 weeks; P<0.001). Triplets reduced to singletons had significantly lower rates of preterm delivery at <37 weeks (13.3% vs 45.3%; adjusted OR, 0.58; 95% CI, 0.43–0.79; P<0.001) and cesarean section compared with triplets reduced to twins (66.7% vs 93.9%; adjusted OR, 0.51; 95% CI, 0.38–0.67; P<0.001). There was no difference observed in rate of preterm delivery at <32 weeks (2.2% vs 5.1%, P=0.412) or <34 weeks (4.4% vs 11.5%, P=0.206). Similarly, no significant differences were found in the rates of pregnancy loss at <24 weeks (6.7% vs 6.6%) and at least one VLBW (0% vs 3.3%) between the two groups. Singletons reduced from triplets had a significantly higher rate of all surviving (93.3% vs 79.5%, adjusted OR, 1.62; 95% CI, 1.07-2.44; P=0.023), and higher median birth weight than twins reduced from triplets (3050 g vs 2500 g, P <0.001, **Figure S1**). Women with singletons reduced from triplets had a significantly lower risk of having at least one LBW neonate compared with women with twins reduced from triplets (8.9% vs 53.2%; adjusted OR, 0.44; 95% CI, 0.31–0.63; P<0.001). Additionally, we analyzed the incidence of SGA to exclude the effect of different gestational ages. Women with singletons reduced from triplets had a significantly lower risk of having at least one SGA neonate than women with twins reduced from triplets (17.8% vs 44.7%; adjusted OR, 0.62; relative risk, 0.48–0.82, P=0.001).

**TABLE 1** | Demographic and ART characteristics.

| | Primary singletons (n=6853) | Primary twins (n=2788) | Twins reduced to singletons (n=126) | Triplets reduced to singletons (n=45) | Triplets reduced to twins (n=331) | *P* value 1 | *P* value 2 | *P* value 3 | *P* value 4 | *P* value 5 |
|---|---|---|---|---|---|---|---|---|---|---|
| **Maternal age at conception** | 30 (28-33) | 30 (28-33) | 33 (29-35) | 30 (28-35) | 31 (28-34) | <0.001 | 0.616 | 0.474 | <0.001 | <0.001 |
| **Maternal height before pregnancy** | 160.0 (157.0-163.0) | 160.0 (157.0-163.0) | 159.0 (155.0-162.3) | 158.0 (156.0-160.0) | 160.0 (157.0-163.0) | 0.786 | 0.123 | 0.118 | 0.056 | 0.073 |
| **Maternal weight before pregnancy** | 55.0 (50.4-60.0) | 55.0 (51.0-61.0) | 56.0 (50.9-60.1) | 53.0 (48.0-58.8) | 55.0 (51.0-60.0) | 0.426 | 0.090 | 0.087 | 0.778 | 0.558 |
| **Maternal BMI before pregnancy** | 21.6 (20.0-23.6) | 21.7 (20.0-23.9) | 22.2 (20.3-23.9) | 20.9 (19.5-23.4) | 21.6 (20.0-23.7) | 0.407 | 0.242 | 0.203 | 0.346 | 0.070 |
| **Type of infertility** | | | | | | 0.005 | 0.004 | 0.070 | 0.026 | 0.048 |
| Primary infertility | 3054 (44.6) | 1278 (45.8) | 45 (35.7) | 14 (31.1) | 179 (54.1) | | | | | |
| Secondary infertility | 3799 (55.4) | 1510 (54.2) | 81 (64.3) | 31 (68.9) | 152 (45.9) | | | | | |
| **ART methods** | | | | | | <0.001 | 0.073 | 0.435 | 0.125 | 0.212 |
| IVF | 5774 (84.3) | 2374 (85.2) | 101 (80.2) | 36 (80.0) | 221 (66.8) | | | | | |
| ICSI | 1079 (15.7) | 414 (14.8) | 25 (19.8) | 9 (20.0) | 110 (33.2) | | | | | |
| **Embryo transplantation** | | | | | | 0.070 | 0.094 | 0.934 | 0.134 | 0.775 |
| Fresh-ET | 3460 (50.5) | 1561 (56.0) | 62 (49.2) | 23 (51.1) | 168 (50.8) | | | | | |
| Frozen-ET | 3393 (49.5) | 1227 (44.0) | 64 (50.8) | 22 (48.9) | 163 (49.2) | | | | | |
| **Source of semen** | | | | | | 0.731 | 0.178 | 0.019 | 0.191 | 0.254 |
| Ejaculated semen | 6623 (96.6) | 2668 (95.7) | 125 (99.2) | 41 (91.1) | 316 (95.5) | | | | | |
| Sperm aspiration | 154 (2.2) | 76 (2.7) | 1 (0.8) | 1 (2.2) | 8 (2.4) | | | | | |
| Donor Semen | 76 (1.1) | 44 (1.6) | 0 | 3 (6.7) | 7 (2.1) | | | | | |
| **Reduction weeks** | – | – | 8.3 (7.7-9.0) | 8.1 (7.7-8.6) | 8.0 (7.7-8.4) | – | 0.161 | – | – | – |

*Data are presented as median (IQR) or number (%). Comparison of continuous variables was analyzed using Mann–Whitney U tests. Categorical variables were compared by using Chi-square tests or Fisher's exact test. P value 1 represents for triplets reduced to twins versus primary twins; P value 2 represents for triplets reduced to twins versus triplets reduced to singleton; P value 3 represents for triplets reduced to singleton versus primary singleton; P value 4 represents for twins reduced to singleton versus primary twins; P value 5 represents for twins reduced to singleton versus primary singleton;*

*BMI, body mass index (kg/m$^2$); ART, assisted reproduction technique; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; ET, embryo transplantation; TESA, testicular sperm aspiration; PESA, percutaneous epididymal sperm aspiration; MESA, microsurgical epididymal sperm aspiration. Sperm aspiration includes TESA, PESA, and MESA.*

## Twins Reduced to Singletons Versus Primary Twins

Pregnancy outcomes of twins reduced to singletons and primary twins are shown in **Table 3**. No significant differences were found in the rates of pregnancy loss at <24 weeks (4.8% vs 6.5%), preterm delivery at <32 weeks (4.0% vs 4.5%) or <34 weeks (5.6% vs 10.1%), and at least one VLBW (3.2% vs 3.8%) between the two groups. The rate of all surviving was significantly higher in twins reduced to singletons than primary twins (78.5% vs 95.2%; adjusted OR, 1,44; 95% CI, 1.22-1.70; P<0.001). For twins reduced to singletons, the median GA at delivery was 38.79 weeks, which was significantly longer than 37.00 weeks for primary twins (P<0.001). Twins reduced

**TABLE 2** | Pregnancy outcomes of triplets reduced to singletons versus triplets reduced to twins.

| | Triplets reduced to singleton (n=45) | Triplets reduced to twins (n=331) | Unadjusted *P* Value | Unadjusted OR (95%CI) | Adjusted *P* Value | Adjusted OR (95% CI) |
|---|---|---|---|---|---|---|
| **GA at delivery** | 39.07 (38.25-40.04) | 37.00 (35.71-37.86) | <0.001 | – | <0.001 | – |
| **Delivery<32weeks** | 1 (2.2) | 17 (5.1) | 0.626 | 0.42 (0.06-3.23) | 0.412 | 0.75 (0.37-1.50) |
| **Delivery<34weeks** | 2 (4.4) | 38 (11.5) | 0.239 | 0.36 (0.08-1.54) | 0.206 | 0.73 (0.44-1.19) |
| **Delivery<37weeks** | 6 (13.3) | 150 (45.3) | <0.001 | 0.19 (0.08-0.45) | <0.001 | 0.58 (0.43-0.79) |
| **Pregnancy loss <24weeks** | 3 (6.7) | 22 (6.6) | 1.000 | 1.00 (0.19-3.50) | 0.701 | 0.92 (0.59-1.42) |
| **All surviving** | 42 (93.3) | 263 (79.5) | 0.026 | 3.62 (1.09-12.03) | 0.023 | 1.62 (1.07-2.44) |
| **Caesarean section** | 28/42 (66.7) | 290/309 (93.9) | <0.001 | 0.13 (0.06-0.29) | <0.001 | 0.51 (0.38-0.67) |
| **Birth weight (g)** | 3050 (2775-3300) | 2500 (2200-2800) | <0.001 | – | <0.001 | – |
| **At least one LBW** | 4 (8.9) | 176 (53.2) | <0.001 | 0.09 (0.03-0.25) | <0.001 | 0.44 (0.31-0.63) |
| **At least one VLBW** | 0 | 11 (3.3) | 0.441 | – | 0.997 | – |
| **At least one SGA** | 8 (17.8) | 148 (44.7) | 0.001 | 0.27 (0.12-0.59) | 0.001 | 0.62 (0.48-0.82) |

*Data are presented as median (IQR) or number (%). Mann–Whitney U tests and Chi-square tests or Fisher's exact test were used for unadjusted analysis. Logistic regression and linear regression were used for adjusting certain confounders, including maternal age at conception, maternal BMI before pregnancy, type of infertility, ART methods, embryo transplantation, source of semen, and weeks of reduction.*

*GA, gestational age; LBW, low birth weight; VLBW, very low birth weight; SGA, small for gestational age; OR, odds ratio; CI, confidence interval.*

**TABLE 3** | Pregnancy outcomes of twins reduced to singletons versus primary twins.

| | Primary twins (n=2788) | Twins reduced to singleton (n=126) | Unadjusted *P* Value | Unadjusted OR (95% CI) | Adjusted *P* Value | Adjusted OR (95% CI) |
|---|---|---|---|---|---|---|
| **GA at delivery** | 37.00 (35.71-38.00) | 38.79 (37.46-39.43) | <0.001 | – | <0.001 | – |
| **Delivery<32weeks** | 126 (4.5) | 5 (4.0) | 0.770 | 0.87 (0.35-2.17) | 0.743 | 0.97 (0.81-1.17) |
| **Delivery<34weeks** | 281 (10.1) | 7 (5.6) | 0.096 | 0.53 (0.24-1.14) | 0.116 | 0.88 (0.76-1.03) |
| **Delivery<37weeks** | 1240 (44.5) | 21 (16.7) | <0.001 | 0.25 (0.16-0.40) | <0.001 | 0.76 (0.69-0.83) |
| **Pregnancy loss <24weeks** | 182 (6.5) | 6 (4.8) | 0.430 | 0.72 (0.31-1.65) | 0.400 | 0.93 (0.79-1.10) |
| **All surviving** | 2188(78.5) | 120 (95.2) | <0.001 | 5.48 (2.40-12.52) | <0.001 | 1.44(1.22-1.70) |
| **Caesarean section** | 2401/2591 (92.7) | 92/120 (76.7) | <0.001 | 0.26 (0.17-0.41) | <0.001 | 0.76 (0.69-0.83) |
| **Birth weight (g)** | 2550 (2225-2850) | 3080 (2750-3350) | <0.001 | – | <0.001 | – |
| **At least one LBW** | 1311 (47.0) | 17 (13.5) | <0.001 | 0.18 (0.11-0.30) | <0.001 | 0.71 (0.64-0.79) |
| **At least one VLBW** | 106 (3.8) | 4 (3.2) | 0.903 | 0.83 (0.30-2.29) | 0.703 | 0.96 (0.78-1.18) |
| **At least one SGA** | 1103 (39.6) | 17 (13.5) | <0.001 | 0.24 (0.14-0.40) | <0.001 | 0.75 (0.68-0.85) |

*Data are presented as median (IQR) or number (%). Mann–Whitney U tests and Chi-square tests or Fisher's exact test were used for unadjusted analysis. Logistic regression and linear regression were used for adjusting certain confounders, including maternal age at conception, maternal BMI before pregnancy, type of infertility, ART methods, embryo transplantation, source of semen.*

*GA, gestational age; LBW, low birth weight; VLBW, very low birth weight; SGA, small for gestational age; OR, odds ratio; CI, confidence interval.*

to singletons had significantly lower rates of preterm delivery at <37 weeks (16.7% vs 44.5%; adjusted OR, 0.76; 95% CI, 0.69–0.83; P<0.001) and cesarean section (76.7% vs 92.7%; adjusted OR, 0.76; 95% CI, 0.69–0.83; P<0.001) compared with primary twins. Singletons reduced from twins had a significantly higher birth weight (3080g vs 2550 g, P<0.001, **Figure S1**) and significantly lower rate of at least one LBW (13.5% vs 47.0%, adjusted OR, 0.71; 95% CI, 0.64-0.79; P<0.001) compared with primary twins. The incidence of at least one SGA in singletons reduced from twins was significantly lower than that in primary twins (13.5% vs 39.6%; adjusted OR, 0.75; 95% CI, 0.68–0.85; P<0.001).

## Triplets Reduced to Twins Versus Primary Twins

Pregnancy outcomes of triplets reduced to twins versus primary twins are given in **Table 4**. No significant differences were found in the rates of preterm delivery at <32 weeks (5.1% vs 4.5%), <34 weeks (11.5% vs 10.5%), and <37 weeks (45.3% vs 44.5%), pregnancy loss at <24 weeks (6.6% vs 6.5%), abortion of one fetus (13.9% vs 14.1%), all surviving (78.5% vs 79.5%), cesarean section (93.9% vs 92.7%) between twins reduced from triplets and primary twins. Likewise, median birth weight (2500 vs 2550 g, P=0.195, **Figure S1**) and the rate of at least one VLBW (3.3% vs 3.8%, P=0.708) were also comparable between two groups. Twins reduced from triplets had a significantly higher rate of at least one LBW (53.2% vs 47.0%; adjusted OR, 1.07; 95% CI, 1.01–1.13; P=0.028) compared with primary twins. Additionally, the incidence of at least one SGA in triplets reduced to twins was significantly higher than that in primary twins (44.7% vs 39.6%; adjusted OR, 1.06; 95% CI, 1.00–1.13; P=0.040).

## Triplets/Twins Reduced to Singletons Versus Primary Singletons

Pregnancy outcomes of triplets/twins reduced to singletons and primary singletons are shown in **Table 5**. Triplet/twin pregnancies reduced to singletons included 126 singletons

**TABLE 4** | Pregnancy outcomes of triplets reduced to twins versus primary twins.

| | Primary twins (n=2788) | Triplets reduced to twins (n=331) | Unadjusted *P* Value | Unadjusted OR (95% CI) | Adjusted *P* Value | Adjusted OR (95% CI) |
|---|---|---|---|---|---|---|
| **GA at delivery** | 37.00 (35.71-38.00) | 37.00 (35.71-37.86) | 0.992 | – | 0.957 | – |
| **Delivery<32weeks** | 126 (4.5) | 17 (5.1) | 0.612 | 1.14 (0.68-1.92) | 0.590 | 1.04 (0.91-1.19) |
| **Delivery<34weeks** | 281 (10.1) | 38 (11.5) | 0.426 | 1.16 (0.81-1.66) | 0.284 | 1.05 (0.96-1.15) |
| **Delivery<37weeks** | 1240 (44.5) | 150 (45.3) | 0.771 | 1.04 (0.82-1.30) | 0.764 | 1.01 (0.95-1.07) |
| **Pregnancy loss <24weeks** | 182 (6.5) | 22 (6.6) | 0.934 | 1.02 (0.65-1.61) | 0.877 | 1.01 (0.90-1.14) |
| **Abortion of one fetus** | 392 (14.1) | 46 (13.9) | 0.936 | 0.99 (0.71-1.37) | 0.549 | 0.98 (0.90-1.06) |
| **All surviving** | 2188(78.5) | 263(79.5) | 0.682 | 1.06 (0.80-1.41) | 0.397 | 1.03(0.96-1.11) |
| **Caesarean section** | 2401/2591 (92.7) | 290/309 (93.9) | 0.447 | 1.21 (0.74-1.97) | 0.371 | 1.06 (0.94-1.20) |
| **Birth weight (g)** | 2550 (2225-2850) | 2500 (2200-2800) | 0.130 | – | 0.195 | – |
| **At least one LBW** | 1311 (47.0) | 176 (53.2) | 0.034 | 1.28 (1.02-1.61) | 0.028 | 1.07 (1.01-1.13) |
| **At least one VLBW** | 106 (3.8) | 11 (3.3) | 0.665 | 0.87 (0.46-1.64) | 0.708 | 0.97 (0.83-1.14) |
| **At least one SGA** | 1103 (39.6) | 148 (44.7) | 0.071 | 1.24 (0.98-1.55) | 0.040 | 1.06 (1.00-1.13) |

*Data are presented as median (IQR) or number (%). Mann–Whitney U tests and Chi-square tests or Fisher's exact test were used for unadjusted analysis. Logistic regression and linear regression were used for adjusting certain confounders, including maternal age at conception, maternal BMI before pregnancy, type of infertility, ART methods, embryo transplantation, source of semen.*

*Birth weight, at least one LBW, and at least one VLBW were additionally adjusted for GA at delivery.*

*GA, gestational age; LBW, low birth weight; VLBW, very low birth weight; SGA, small for gestational age; OR, odds ratio; CI, confidence interval.*

**TABLE 5 |** Pregnancy outcomes of triplets or twins reduced to singletons versus primary singletons.

| | Primary singletons (n=6853) | Triplets/twins reduced to singletons (n=171) | Unadjusted *P* Value | Unadjusted OR (95%CI) | Adjusted *P* Value | Adjusted OR (95%CI) |
|---|---|---|---|---|---|---|
| **GA at delivery** | 39.00 (38.0-40.0) | 38.93 (37.71-39.57) | 0.830 | – | 0.155 | – |
| **Delivery<32weeks** | 65 (0.9) | 6 (3.5) | 0.004 | 3.80 (1.62-8.89) | 0.002 | 1.25 (1.08-1.44) |
| **Delivery<34weeks** | 119 (1.7) | 9 (5.3) | 0.002 | 3.14 (1.57-6.30) | 0.001 | 1.22 (1.09-1.37) |
| **Delivery<37weeks** | 500 (7.3) | 27 (15.8) | <0.001 | 2.38 (1.56-3.63) | <0.001 | 1.51 (1.07-1.24) |
| **Pregnancy loss <24weeks** | 371 (5.4) | 9 (5.3) | 0.932 | 0.97 (0.49-1.92) | 0.671 | 0.98 (0.87-1.09) |
| **Live birth** | 6450 (94.1) | 162 (94.7) | 0.734 | 1.13 (0.57-2.22) | 0.496 | 1.04 (0.93-1.17) |
| **Caesarean section** | 4871/6463 (75.4) | 120/162 (74.1) | 0.706 | 0.93 (0.65-1.33) | 0.325 | 0.97 (0.91-1.03) |
| **Birth weight (g)** | 3340 (3038.73-3650) | 3055 (2750-3312.50) | <0.001 | – | <0.001 | – |
| **LBW** | 295 (4.3) | 21 (12.3) | <0.001 | 3.11 (1.94-4.99) | <0.001 | 1.21 (1.11-1.30) |
| **VLBW** | 29 (0.4) | 4 (2.3) | 0.008 | 5.64 (1.96-16.21) | 0.002 | 1.32 (1.10-1.58) |
| **SGA** | 451 (6.6) | 25 (14.6) | <0.001 | 2.43 (1.57-3.76) | <0.001 | 1.17 (1.09-1.26) |

*Data are presented as median (IQR) or number (%). Mann–Whitney U tests and Chi-square tests or Fisher's exact test were used for unadjusted analysis. Logistic regression and linear regression were used for adjusting certain confounders, including maternal age at conception, maternal BMI before pregnancy, type of infertility, ART methods, embryo transplantation, source of semen.*

*GA, gestational age; LBW, low birth weight; VLBW, very low birth weight; SGA, small for gestational age; OR, odds ratio; CI, confidence interval.*

reduced from twins and 45 singletons reduced from triplets. No significant differences were found in the rates of pregnancy loss at <24 weeks (5.4% vs 5.3%) and live birth (94.1% vs 94.7%) between the groups. Although GA at delivery was comparable between the two groups, analysis across different GA cut–offs showed a significant disadvantage for triplet/twin pregnancies reduced to singletons, with higher rates of preterm delivery either at <37 weeks (15.8% vs 7.3%; adjusted OR, 1.51; 95% CI, 1.07–1.24; P<0.001), <34 weeks (5.3% vs 1.7%; adjusted OR, 1.22; 95% CI 1.09–1.37; P=0.001), or <32 weeks (3.5% vs 0.9%; adjusted OR, 1.25; 95% CI, 1.08–1.44; P=0.002). Newborns in triplet/twin pregnancies reduced to singletons had significantly lower median birth weights (3055 vs 3340 g, P<0.001, **Figure S1**) and higher rates of LBW (12.3% vs 4.3%; adjusted OR, 1.21; 95% CI, 1.11–1.30; P<0.001) and VLBW (2.3% vs 0.4%; adjusted OR, 1.32; 95% CI, 1.10–1.58; P=0.002) compared with primary singletons. Additionally, the incidence of SGA in triplets/twins reduced to singletons was significantly higher than that in primary twins (14.6% vs 6.6%; adjusted OR, 1.17; 95% CI, 1.09–1.26; P<0.001). Additionally, the comparison between triplets reduced to singletons and primary singletons, twins reduced to singletons and primary singletons are given in **Table S1**.

## DISCUSSION

This cohort study showed that MFPR improved pregnancy outcomes, including preterm delivery, LBW, and SGA, but still could not completely reverse the adverse pregnancy outcomes of multiple pregnancies. Additionally, MFPR was a relatively safe operation that did not increase pregnancy loss at <24 weeks. To the best of our knowledge, this is the largest study to compare the pregnancy outcomes of transvaginal MFPR in women with triplet or twin pregnancies, which provides a systematic and comprehensive interpretation to the benefits and limitations of MFPR.

Multiple pregnancies are an inevitable consequence of more than one embryo transfer in ART, which is responsible for increasing risks in prematurity (19). Numerous studies have shown that twins reduced from triplets have better pregnancy outcomes than ongoing triplets (13, 14, 20). Therefore, the benefits of MFPR for triplet pregnancies have been recognized. However, the decision of whether to reduce to twins or a singleton is still difficult. Some previous small size studies compared triplets reduced to twins and to singletons as follows. Haas et al. compared 55 twins and 19 singletons reduced from triplets and showed that reduction to a singleton resulted in a longer GA at delivery and higher birth weight (21). However, some researchers still believe that MFPR from triplets to singletons is associated with a higher risk of pregnancy loss (22). In our study, triplets reduced to singletons did not increase pregnancy loss at <24 weeks compared with triplets reduced to twins. Triplets reduced to singletons had better outcomes in almost every aspect compared with primary twins, including a longer GA, lower preterm delivery rate, lower cesarean section rate, higher birth weight, and lower frequency of LBW or SGA newborns.

For twin pregnancies, there is still controversy regarding whether to perform MFPR. A previous study (23) showed that in the twins reduced to singletons group, the percentage of women without any surviving child was significantly higher compared with the ongoing twin. Gupta et al. (24) reported that reduction of twin pregnancies decreased the risk of preterm delivery at <37 weeks and birth weight below the 10th percentile, but not the risk of preterm birth at <34 weeks or birth weight below the 5th percentile. There is no doubt that an increased risk of adverse pregnancy outcomes is associated with twin pregnancies (3, 4, 25). In our study, we found that twins reduced to singletons had better outcomes in almost every aspect compared with primary twins, including a longer GA, lower preterm delivery rate, lower cesarean section rate, higher birth weight, and lower frequency of LBW or SGA newborns. Importantly, twins reduced to singletons did not increase pregnancy loss at <24 weeks. These findings are consistent with previous studies (26) (27), which suggest that MFPR from twins to singletons has a clear advantage for twin pregnancies.

The conclusion can be drawn from previous studies and the present study that MFPR improves the outcomes of triplet or twin pregnancies. However, there is still controversy whether reduced singletons or twins after MFPR have the same pregnancy outcomes as non–reduced singletons or twins.

To date, the findings of studies have been inconsistent with the pregnancy outcomes of reduced twins and primary twins. In some studies, reduced twins have similar outcomes compared with primary twins. Hershko–Klement et al. (28) evaluated the pregnancy outcomes of 70 twins after reduction, and found that the mean GA at delivery and birth weight were comparable between the reduced and non–reduced twins. Lipitz et al. (29) also showed that the mean GA at delivery was similar in reduced and non–reduced twins, as well as the risk of LBW. Our study included 331 women with twin pregnancies who underwent MFPR. This is the largest cohort described to date and it provides a more precise estimation of preterm delivery and birth weight. In the current study, most of the pregnancy outcomes were comparable between twins reduced from triplets and primary twins, including the rates of preterm delivery at <32, <34, and <37 weeks, pregnancy loss at <24 weeks, abortion of one fetus, and cesarean section. However, the probability for women who had twins reduced from triplets to have a LBW or SGA neonate was higher than that for those who had primary twins. The findings of our study are consistent with those presented by Cheang et al. (30) and Hwang et al. (16), which suggested the higher risk of prematurity in reduced twins.

Due to the sample size, we combined the triplets reduced to singletons group and twins reduced to singletons group for statistics analyze. Triplets/twins reduced to singletons were more likely to have preterm delivery at <32, <34, and <37 weeks. Birth weight of reduced singletons was 285g lighter than that of primary singletons. Women who had triplets/twins reduced to singletons were more likely to have a LBW, VLBW or SGA neonate compared with women who had primary singletons. Moreover, the rates of pregnancy loss at <24 weeks and cesarean section were comparable between the two groups in our study. Consistent with our study, van de Mheen al (23). found that reduced singletons had a shorter GA at delivery and lower birth weight than primary singletons.

The major strength of this study is that it is the largest study to analyze the pregnancy outcomes of twin or triplet pregnancies undergoing MFPR to date. In this single–center study, all of the experienced operators followed a unified operating standard, reducing the interference caused by operating variability. Moreover, we included patients over a long timeframe, which increased the validity of the study. However, this study has some limitations. Although this is the largest study to date, the numbers of some subgroups were small, which might have restricted our ability to detect differences in some pregnancy outcomes of low probability, such as extreme preterm delivery and VLBW. Data regarding pregnancy complications and perinatal mortality were not available. Our study has a large sample size over a long period. Over this time, the outcomes of IVF/ICSI pregnancies in our center were relatively stable, and all MFPR procedures were performed by the same five highly skilled physicians, thus ensuring the reliability of this study. Thus, the year of conception or birth was not put in regression model in our study, sine time-changes might not significantly contribute to apparent group differences. In addition, some baseline characteristics were different between groups, because this was not a randomized trial owing to the fact that randomization of patients was not applicable. To reduce interference of confounding factors, we used multiple regressions to verify our results.

In conclusion, MFPR is a relatively safe and efficacious procedure based on our findings, but the objective of our study was not to advocate MFPR. MFPR could improve but still cannot completely reverse adverse pregnancy outcomes of multiple pregnancies. The best way to prevent multiple pregnancies and all related risks is limiting the number of transferred embryos and the advocating of SET. For those infertile couples seeking for ART, we must attach particular importance to inform them the risk of multiple pregnancies and benefits of SET. We should be aware that it is SET, not MFPR, the optimal choice for reducing the risk of multiple pregnancies from the beginning (31). All of this information should be considered when counselling couples about the number of embryos transferred or women with multiple pregnancies who are considering MFPR. The long-term impact of MFPR on the health of the offspring should also been further investigated in the future.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Women's hospital, Zhejiang University School of Medicine. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

ZY is the principal investigator for the study. ZY and TM conceived the present study and carried out most of the research for this study. All authors contributed to the analysis and interpretation of the data. TM interpreted the results and wrote the manuscript, which was critically revised by all authors. All the authors approved the submitted version.

## FUNDING

This work was supported by grants from National Key R&D Program of China (2021YFC2700603), the Zhejiang Natural Science Foundation of China (NO. LQ22H040006 and

Q22H049995), the National Natural Science Foundation of China (No. 82101759 and NO.81803245), the Program for Key Subjects of Zhejiang Province in Medicine & Hygiene.

## ACKNOWLEDGMENTS

The authors acknowledge the statistics assistance of Professor Yu Yunxian.

## REFERENCES


1. Calhaz-Jorge C, de Geyter C, Kupka MS, de Mouzon J, Erb K, Mocanu E, et al. Assisted Reproductive Technology in Europe, 2012: Results Generated From European Registers by ESHRE. *Hum Reprod (Oxford England)* (2016) 31 (8):1638–52. doi: 10.1093/humrep/dew151
2. Osterman MJ, Kochanek KD, MacDorman MF, Strobino DM, Guyer B. Annual Summary of Vital Statistics: 2012-2013. *Pediatrics* (2015) 135 (6):1115–25. doi: 10.1542/peds.2015-0434
3. Conde-Agudelo A, Belizan JM, Lindmark G. Maternal Morbidity and Mortality Associated With Multiple Gestations. *Obstet Gynecol* (2000) 95(6 Pt 1):899–904. doi: 10.1097/00006250-200006000-00022
4. Luke B, Brown MB. Contemporary Risks of Maternal Morbidity and Adverse Outcomes With Increasing Maternal Age and Plurality. *Fertil Steril* (2007) 88 (2):283–93. doi: 10.1016/j.fertnstert.2006.11.008
5. Thurin A, Hausken J, Hillensjö T, Jablonowska B, Pinborg A, Strandell A, et al. Elective Single-Embryo Transfer Versus Double-Embryo Transfer in *In Vitro* Fertilization. *New Engl J Med* (2004) 351(23):2392–402. doi: 10.1056/NEJMoa041032
6. McLernon DJ, Harrild K, Bergh C, Davies MJ, de Neubourg D, Dumoulin JC, et al. Clinical Effectiveness of Elective Single Versus Double Embryo Transfer: Meta-Analysis of Individual Patient Data From Randomised Trials. *BMJ (Clin Res ed)* (2010) 341:c6945. doi: 10.1136/bmj.c6945
7. Practice Committee of Society for Assisted Reproductive Technology; Practice Committee of American Society for Reproductive MedicineElective Single-Embryo Transfer. *Fertil Steril* (2012) 97(4):835–42. doi: 10.1016/j.fertnstert.2011.11.050
8. Harbottle S, Hughes C, Cutting R, Roberts S, Brison D. Elective Single Embryo Transfer: An Update to UK Best Practice Guidelines. *Hum Fertility (Cambridge England)* (2015) 18(3):165–83. doi: 10.3109/14647273.2015.1083144
9. Practice Committee of the American Society for Reproductive Medicine and the Practice Committee of the Society for Assisted Reproductive TechnologyCriteria for Number of Embryos to Transfer: A Committee Opinion. *Fertil Steril* (2013) 99(1):44–6. doi: 10.1016/j.fertnstert.2012.09.038
10. Sun Y, Huang G, Sun H, Fan L, Shen H, Liu P(CSRM) TFSotCoCSoRM: Chinese Expert Consensus on Numbers of Embryos Transferred. *J Reprod Med* (2018) 27(10):940–5. doi: 10.3969/j.issn.1004-3845.2018.10.003
11. Evans MI, Andriole S, Britt DW. Fetal Reduction: 25 Years' Experience. *Fetal Diagnosis Ther* (2014) 35(2):69–82. doi: 10.1159/000357974
12. Antsaklis A, Souka AP, Daskalakis G, Papantoniou N, Koutra P, Kavalakis Y, et al. Pregnancy Outcome After Multifetal Pregnancy Reduction. *J maternal-fetal neonatal med: Off J Eur Assoc Perinatal Med Fed Asia Oceania Perinatal Societies Int Soc Perinatal Obstet* (2004) 16(1):27–31. doi: 10.1080/14767050410001728962
13. van de Mheen L, Everwijn SM, Knapen MF, Oepkes D, Engels M, Manten GT, et al. The Effectiveness of Multifetal Pregnancy Reduction in Trichorionic Triplet Gestation. *Am J Obstet Gynecol* (2014) 211(5):536.e531–536. doi: 10.1016/j.ajog.2014.04.023.
14. Zipori Y, Haas J, Berger H, Barzilay E. Multifetal Pregnancy Reduction of Triplets to Twins Compared With non-Reduced Triplets: A Meta-Analysis. *Reprod Biomed Online* (2017) 35(3):296–304. doi: 10.1016/j.rbmo.2017.05.012
15. Jirsova S, Mardesic T, Muller P, Huttelova R, Zvarova J, Jirkovsky M. Multi-Fetal Pregnancy Reduction Does Not Influence Perinatal Results in Twin Pregnancies. *Twin research: Off J Int Soc Twin Stud* (2001) 4(6):422–5. doi: 10.1375/twin.4.6.422
16. Hwang JL, Pan HS, Huang LW, Lee CY, Tsai YL. Comparison of the Outcomes of Primary Twin Pregnancies and Twin Pregnancies Following Fetal Reduction. *Arch Gynecol Obstet* (2002) 267(2):60–3. doi: 10.1007/s00404-001-0279-y
17. Antsaklis A, Anastasakis E. Selective Reduction in Twins and Multiple Pregnancies. *J Perinatal Med* (2011) 39(1):15–21. doi: 10.1515/jpm.2010.114
18. Zhu L, Zhang R, Zhang S, Shi W, Yan W, Wang X, et al. Chinese Neonatal Birth Weight Curve for Different Gestational Age. *Chin J Pediatr* (2015) 53 (2):97–103. doi: 10.3760/cma.j.issn.0578-1310.2015.02.007
19. Ombelet W, De Sutter P, van der Elst J, Martens G. Multiple Gestation and Infertility Treatment: Registration, Reflection and Reaction–the Belgian Project. *Hum Reprod Update* (2005) 11(1):3–14. doi: 10.1093/humupd/dmh048
20. Dodd J, Crowther C. Multifetal Pregnancy Reduction of Triplet and Higher-Order Multiple Pregnancies to Twins. *Fertil Steril* (2004) 81(5):1420–2. doi: 10.1016/j.fertnstert.2003.11.028
21. Haas J, Hourvitz A, Dor J, Yinon Y, Elizur S, Mazaki-Tovi S, et al. Pregnancy Outcome of Early Multifetal Pregnancy Reduction: Triplets to Twins Versus Triplets to Singletons. *Reprod Biomed Online* (2014) 29(6):717–21. doi: 10.1016/j.rbmo.2014.09.003
22. Evans MI, Britt DW. Multifetal Pregnancy Reduction: Evolution of the Ethical Arguments. *Semin Reprod Med* (2010) 28(4):295–302. doi: 10.1055/s-0030-1255177
23. van de Mheen L, Everwijn SM, Knapen MF, Haak MC, Engels MA, Manten GT, et al. Pregnancy Outcome After Fetal Reduction in Women With a Dichorionic Twin Pregnancy. *Hum Reprod (Oxford England)* (2015) 30 (8):1807–12. doi: 10.1093/humrep/dev132
24. Gupta S, Fox NS, Feinberg J, Klauser CK, Rebarber A. Outcomes in Twin Pregnancies Reduced to Singleton Pregnancies Compared With Ongoing Twin Pregnancies. *Am J Obstet Gynecol* (2015) 213(4):580.e581–585. doi: 10.1016/j.ajog.2015.06.018
25. Martin JA, Hamilton BE, Sutton PD, Ventura SJ, Menacker F, Kirmeyer S. Births: Final Data for 2004. *Natl vital Stat reports: Centers Dis Control Prevention Natl Center Health Stat Natl Vital Stat System* (2006) 55(1):1–101.
26. Haas J, Mohr Sasson A, Barzilay E, Mazaki Tovi S, Orvieto R, Weisz B, et al. Perinatal Outcome After Fetal Reduction From Twin to Singleton: To Reduce or Not to Reduce? *Fertil Steril* (2015) 103(2):428–32. doi: 10.1016/j.fertnstert.2014.10.027
27. Zipori Y, Haas J, Berger H, Barzilay E. Multifetal Reduction of Triplets to Twins Compared With non-Reduced Twins: A Meta-Analysis. *Reprod Biomed Online* (2017) 35(1):87–93. doi: 10.1016/j.rbmo.2017.04.001
28. Hershko-Klement A, Lipitz S, Wiser A, Berkovitz A. Reduced Versus Nonreduced Twin Pregnancies: Obstetric Performance in a Cohort of Interventional Conceptions. *Fertil Steril* (2013) 99(1):163–7. doi: 10.1016/j.fertnstert.2012.09.005
29. Lipitz S, Uval J, Achiron R, Schiff E, Lusky A, Reichman B. Outcome of Twin Pregnancies Reduced From Triplets Compared With Nonreduced Twin Gestations. *Obstet Gynecol* (1996) 87(4):511–4. doi: 10.1016/0029-7844(95)00469-6
30. Cheang CU, Huang LS, Lee TH, Liu CH, Shih YT, Lee MS. A Comparison of the Outcomes Between Twin and Reduced Twin Pregnancies Produced Through Assisted Reproduction. *Fertil Steril* (2007) 88(1):47–52. doi: 10.1016/j.fertnstert.2006.11.084
31. Pandian Z, Marjoribanks J, Ozturk O, Serour G, Bhattacharya S. Number of Embryos for Transfer Following *In Vitro* Fertilisation or Intra-Cytoplasmic


## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.851167/full#supplementary-material

**Supplementary Table 1 |** Pregnancy outcomes: triplets or twins reduced to singletons versus primary singletons.

**Supplementary Figure 1 |** The birth weight of newborns in different groups.

Sperm Injection. *Cochrane Database Systematic Rev* (2013) 7:Cd003416. doi: 10.1002/14651858.CD003416.pub4

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Endocrinology

ORIGINAL RESEARCH
published: 27 June 2022
doi: 10.3389/fendo.2022.875798

# Early Spontaneous Abortion in Fresh- and Frozen-Embryo Transfers: An Analysis of Over 35,000 Transfer Cycles

*Jun Shuai*[†], *Qiao-li Chen*[†], *Wen-hong Chen*, *Wei-wei Liu*, *Guo-ning Huang* and *Hong Ye*[*]

*Chongqing Reproduction and Genetics Institute, Chongqing Health Center for Women and Children, Chongqing, China*

OPEN ACCESS

**Edited by:**
*Richard Ivell,*
*University of Nottingham,*
*United Kingdom*

**Reviewed by:**
*Kok-Min Seow,*
*Shin Kong Wu Ho-Su Memorial Hospital, Taiwan*
*Nalini Mahajan,*
*Independent researcher,*
*New Delhi, India*

***Correspondence:**
*Hong Ye*
*yehong1210@163.com*

[†]*These authors have contributed equally to this work*

**Specialty section:**
*This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology*

**Received:** *14 February 2022*
**Accepted:** *25 May 2022*
**Published:** *27 June 2022*

**Citation:**
*Shuai J, Chen Q-l, Chen W-h, Liu W-w, Huang G-n and Ye H (2022) Early Spontaneous Abortion in Fresh- and Frozen-Embryo Transfers: An Analysis of Over 35,000 Transfer Cycles. Front. Endocrinol. 13:875798. doi: 10.3389/fendo.2022.875798*

**Background:** The aim of this study was to explore the risk factors for early spontaneous abortion (ESA) in fresh- and frozen-embryo transfers.

**Methods:** This retrospective cohort study comprised a total of 35,076 patients, including 15,557 women in the fresh-embryo transfer group and 19,519 women in the frozen-embryo transfer group from January 2016 to December 2020. The primary outcome of this study was ESA, which we defined as the termination of embryonic development before 12 weeks of pregnancy (i.e., an early abortion after artificial multi-fetal pregnancy reduction was excluded).

**Results:** In the 35,076 ART transfer cycles, the incidence of ESA was 5.77% (2023/35,076), and the incidence rates for ESA in fresh and frozen cycles were 4.93% (767 of 15,557) and 6.43% (1,256 of 19,519), respectively. Using a multivariate logistic regression analysis model, maternal age, body mass index (BMI), and number of embryos transferred were independent predictors for ESA. In addition, frozen-thawed transfer was a risk factor for ESA as compared with fresh transfer (OR = 1.207; 95% CI, 1.094–1.331; P = 0.000), blastocyst transfer was risk factor for ESA as compared with cleavage transfer (OR =1.373; 95% CI, 1.186–1.591; P = 0.000 in the total group; OR = 1.291; 95% CI, 1.111–1.499; P = 0.001 in the frozen-transfer group), and unexplained infertility was a protective factor for ESA only in the frozen group (OR = 0.746; 95% CI, 0.565–0.984; P = 0.038).

**Conclusions:** Maternal age, BMI, number of embryos transferred, and frozen-thawed transfer were independent risk factors for ESA in assisted reproductive technology treatment cycles.

**Keywords: early spontaneous abortion, assisted reproductive technology, fresh embryo, frozen embryo, transfer cycles**

## INTRODUCTION

Assisted reproductive technology (ART) is a common option for infertility patients who wish to achieve pregnancy (1), but women undergoing ART still face many challenges in the period from clinical pregnancy to live birth—including biochemical pregnancy loss, spontaneous abortion (SA), and premature delivery. Of these, SA caused by ART has evolved into one of the greatest challenges (2). Early spontaneous abortion (ESA)—defined as a miscarriage in which the an embryo halts development prior to 12 weeks of pregnancy (3)—exhibits high incidence rates that range from 9.3% to 18.3%, and ESAs have recently been reported in ART (4, 5). Considering the economic and mental burden that infertile couples participating in ART experience with ESA, it remains uncertain whether embryo freezing improves the ESA rate compared to fresh embryos after *in vitro* fertilization (IVF). Thus, it is necessary to explore and determine the prevalence of miscarriage and its related risk factors, and provide appropriate guidance and a valuable reference for predicting the probability of ESA in ART.

In recent years there has been a sharp rise in the number of frozen-thawed embryo-transfer cycles, and although the development and maturation of embryo-freezing technology allow embryos to be frozen and stored safely for further use, it remains unclear as to whether the cryopreservation technique always provides the greatest benefit to patients (6). Several studies have revealed that compared to fresh-ET recipients, frozen-ET cycles were more likely to display a history of spontaneous abortion (SA) (7, 8); however, these studies were more concerned with live births and did not describe the factors affecting SA. In contradistinction, investigators in several other reports detected no significant difference in the risk of SA when they compared frozen-embryo transfer with fresh-embryo transfer (5, 9–12). Unfortunately, these studies did not entail subgroup analysis of SA rates according to different population characteristics, and the results may have therefore been biased. There are also some studies depicting a freeze-all strategy as associated with fewer miscarriages (13, 14).

It is not surprising to observe inconsistent results from cohort studies, which indicates that not only is a large sample size required, but that multivariate and multi-subgroup analyses are also needed. Therefore, for this study we included more than 35,000 transfer cycles by ART at our center, so as to investigate whether embryo freezing constituted a possible risk factor for ESA in a multivariate and multi-subgroup analysis approached from multiple perspectives.

## MATERIALS AND METHODS

This retrospective cohort study consisted of all embryo-transfer patients at the Reproductive and Genetic Institute of Chongqing Health Center for Women and Children between January of 2016 and December of 2020. Exclusion criteria were patients with uterine malformation, chromosomal abnormality, those undergoing preimplantation genetic testing (PGT) cycles, having a history of artificial multiple-pregnancy reduction, and those who underwent oocyte-donation cycles. We ultimately included 35,076 embryo-transfer patients in our research analysis, of which 15,557 cycles were fresh-embryo transfers and 19,519 were frozen-embryo transfers. This study strictly followed the relevant requirements of the Declaration of Helsinki of the World Medical Association, and was approved by our Hospital Ethics Committee; and written informed consent was obtained from all patients at their first consultation.

The primary outcome of this study was ESA, which was defined as the stoppage of embryonic development before 12 weeks of pregnancy. Basic patient parameters included maternal age, body mass index (BMI), infertility diagnosis, ovarian-stimulation protocols (for fresh-transfer cycles), fresh/thawed-embryo transfers, endometrial preparation protocols (for frozen-transfer cycles), stage/number of embryos transferred, insemination method (for fresh-transfer cycles), and concomitant gynecological disorders.

### Statistical Analysis

Participants were first allocated to different groups according to their basic parameters, and the ESA rate was compared using the chi-squared test. We executed multivariate logistic regression to evaluate the association between the variables and ESA. All analyses were performed using IBM SPSS Statistics 21 (IBM Corp.), and all P values were two-sided, with statistical significance defined as $P < 0.05$.

## RESULTS

The overall incidence of ESA in the 35,076 ART transfer cycles was 5.77% (n=2023). ESA rate differed according to maternal age, BMI, infertility diagnosis (primary/secondary infertility), type of embryo transfer performed (fresh/frozen-thawed), embryonic stage (cleavage/blastocyst), and the number of embryos transferred (1, 2, or 3). In addition, in fresh-ET cycles, ESA differed among patients according to controlled ovarian stimulation (COS) protocol (GnRH agonist/GnRH antagonist, or others), and no difference was found between the IVF and ICSI insemination groups. We observed no difference in ESA rate in frozen-ET cycles among estrogen-progesterone (EP), pituitary down-regulation-EP, and natural protocols-frozen. Concomitant gynecological disorders (endometriosis/polycystic ovarian syndrome/unexplained infertility) were not different overall or in the fresh- or frozen-transfer groups (**Table 1**).

In **Table 1**, the incidence of ESA in fresh and frozen cycles was 4.93% (767 of 15,557) and 6.43% (1,256 of 19,519), respectively. For the fresh-ET group, the incidence of ESA was statistically different among maternal age, protocols-fresh cycle (GnRH agonist/GnRH antagonist or others), and the number of embryos transferred (1, 2, or 3). For the frozen-ET group, ESA varied among patients according to maternal age, BMI, and the number of embryos transferred. Moreover, ESA in women aged <35 years in the frozen-transfer group was significantly elevated relative to that of the fresh-transfer group ($P<0.001$), and we noted no disparity with

**TABLE 1** | Early spontaneous abortion in ART treatment cycles according to different parameters.

| | Early spontaneous abortion rate | | | | | | *P* (Fresh vs. Frozen) |
|---|---|---|---|---|---|---|---|
| | **All** | ***P*** | **Fresh (15,557)** | ***P*** | **Frozen (19,519)** | ***P*** | |
| Female age (years) | | 0.000** | | 0.000** | | 0.000** | |
| <30 | 4.6% (470/10289) | | 3.2% (157/4840) | | 5.7% (313/5449) | | 0.000** |
| 30–34 | 5.2% (845/16174) | | 4.3% (316/7337) | | 6.0% (529/8837) | | 0.000** |
| ≥35 | 8.2% (708/8613) | | 8.7% (294/3380) | | 7.9% (414/5233) | | 0.194 |
| BMI (kg/m²) | | 0.000** | | 0.128 | | 0.001** | |
| ≤18.5 | 5.1% (150/2964) | | 3.8% (44/1166) | | 5.9% (106/1798) | | 0.011* |
| 18.6–24.9 | 5.6% (1495/26618) | | 4.9% (585/11912) | | 6.2% (910/14703) | | 0.000** |
| ≥25 | 6.9% (378/5494) | | 5.6% (138/2476) | | 8.0% (240/3018) | | 0.000** |
| Infertility diagnosis | | 0.000** | | 0.054 | | 0.088 | |
| Primary infertility | 5.3% (924/17342) | | 4.7% (425/9139) | | 6.1% (499/8203) | | 0.000** |
| Secondary infertility | 6.2% (1099/17734) | | 5.3% (342/6418) | | 6.7% (757/11316) | | 0.000** |
| Stage of embryo transfer | | 0.000** | | 0.480 | | 0.060 | |
| Cleavage-stage embryo transfer | 5.6% (1765/31462) | | 4.9% (764/15516) | | 6.3% (1001/15944) | | 0.000** |
| Blastocyst transfer | 7.1% (258/3614)) | | 7.3% (3/41) | | 7.1% (255/3575) | | 0.964 |
| Number of embryos transferred | | 0.000** | | 0.000** | | 0.045* | |
| One embryo | 5.3% (209/3945) | | 4.3% (66/1521) | | 5.9% (143/2424) | | 0.033* |
| Two embryos | 5.7% (1745/30470) | | 4.9% (670/13791) | | 6.4% (1075/16679) | | 0.000** |
| Three embryos | 10.4% (69/661) | | 12.7% (31/245) | | 9.1% (38/416) | | 0.153 |
| Type of transfer | | 0.000** | – | – | – | – | – |
| Fresh embryo | 4.9% (767/15557) | | – | – | – | – | – |
| Thawed embryo | 6.4% (1256/19519) | | – | – | – | – | – |
| Fresh-cycle protocols | – | – | | 0.004** | – | – | – |
| GnRH agonist | – | – | 4.6% (527/11387) | | – | | – |
| GnRH antagonist/others | – | – | 5.8% (240/4170) | | – | | – |
| Insemination method-fresh cycle | – | – | | 0.451 | – | – | – |
| IVF | – | – | 4.9% (624/12814) | | – | – | – |
| ICSI | – | – | 5.2% (143/2743) | | – | – | – |
| Frozen-cycle protocols | – | – | – | | | 0.704 | |
| EP | – | – | – | | 6.4% (875/13588) | | – |
| Pituitary down-regulation-EP | | | | | 6.5% (368/5679) | | |
| Natural cycle | – | – | – | | 5.2% (13/252) | | – |
| Concomitant diseases, women | | | | | | | |
| Endometriosis | 6.0% (311/5175) | 0.380 | 5.0% (118/2365) | 0.886 | 6.9% (193/2792) | 0.266 | 0.004** |
| PCOS | 5.7% (134/2332) | 0.963 | 4.5% (31/689) | 0.593 | 6.3% (103/1643) | 0.775 | 0.094 |
| Unexplained infertility | 6.3% (86/1355) | 0.351 | 4.2% (28/673) | 0.345 | 8.5% (58/682) | 0.025 | 0.001** |

*BMI, body mass index; GnRH, gonadotrophin-releasing hormone; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; PCOS, polycystic ovarian syndrome; EP, estrogen-progesterone; *P < 0.05; **P < 0.01.*

women over 35 years of age. The ESA among the three subgroups of BMI was higher in the frozen-transfer group than in the fresh-transfer group, and the ESA rate in the latter was augmented over that in the fresh-transfer group regardless of the presence of primary or secondary infertility. In the cleavage-stage embryo-transfer group, the ESA rate in the frozen-transfer group was enhanced relative to the fresh-transfer group [6.3% (1001/15944) vs. 4.9% (764/15516), P=0.000], but there was no difference with respect to the blastocyst- transfer group [7.1% (255/3575) vs. 7.3% (3/41), P=0.964]. When the number of embryos transferred was one or two, the ESA rate in the frozen-transfer group was higher than that in the fresh-transfer group, but there was no difference when three embryos were transferred. Among the three subgroups of women with concomitant disease such as endometriosis or unexplained infertility, the ESA rate was higher in the former than in the latter group. Nevertheless, we observed no difference the ESA rate in young women (<35 years) between the diminished ovarian reserve (DOR) group and non-DOR group (P>0.05), and advanced age women (≥35 years), consistent results were also observed. After stratification according to fresh embryo and frozen embryo, there was still no statistical difference in ESA rate between the DOR group and the non-DOR group (P>0.05). However, after stratification according to the DOR and non-DOR, the ESA rate of the advanced age group was significantly higher than that of the younger group (P<0.05) (**Table S1**).

In **Table 2**, all of the ESA-related factors depicted above were re-analyzed simultaneously using a multivariate logistic regression analysis model with adjusted data. In the total and fresh- and frozen-transfer groups, maternal age, BMI, and the number of embryos transferred were independent predictors of ESA. In addition, frozen-thawed transfer was a risk factor for ESA as compared with fresh transfer (OR = 1.207; 95% CI, 1.094–1.331; P = 0.000). In both the total and frozen-transfer groups, blastocyst transfer was a risk factor for ESA compared with the transfer of cleavage-stage embryos (OR =1.373; 95% CI, 1.186–1.591; P = 0.000 in the total group and OR =1.291; 95% CI, 1.111–1.499; P = 0.001 in the frozen-transfer group); while unexplained infertility was a protective factor with regard to ESA only in the frozen-transfer group (OR = 0.746; 95% CI, 0.565–0.984; P = 0.038).

**TABLE 2** | Factors associated with ESA rate using logistic regression analysis.

| | All (35,076) | | Fresh (15,557) | | Frozen (19,519) | |
|---|---|---|---|---|---|---|
| | Adjusted OR (95% CI) | *P* | Adjusted OR (95% CI) | *P* | Adjusted OR (95% CI) | *P* |
| Female age | 1.044 (1.035–1.054) | 0.000** | 1.084 (1.067–1.102) | 0.000** | 1.026 (1.014–1.037) | 0.000** |
| BMI (kg/m$^2$) | 1.035 (1.019–1.052) | 0.000** | 1.028 (1.002–1.056) | 0.035* | 1.041 (1.020–1.062) | 0.000** |
| Type of transfer (frozen/fresh) | 1.207 (1.094–1.331) | 0.000** | - | - | - | - |
| Stage of embryo (blastocyst/cleavage) | 1.373 (1.186–1.591) | 0.000** | - | - | 1.291 (1.111–1.499) | 0.001** |
| Number of embryos transferred | 1.295 (1.141–1.469) | 0.000** | 1.326 (1.074–1.637) | 0.009** | 1.239 (1.058–1.450) | 0.008** |
| Unexplained infertility | - | - | - | - | 0.746 (0.565–0.984) | 0.038* |

*BMI, body mass index; *P < 0.05; **P < 0.01.*

## DISCUSSION

The use of fresh-embryo transfers in relation to frozen transfers is diminishing readily worldwide. Freeze-all embryo protocols followed by elective frozen-embryo transfer constitute an effective method for the prevention of ovarian hyperstimulation syndrome (OHSS), as a recent Cochrane review by Zaat and co-authors showed that the risk of OHSS was reduced by 75% with their freeze-thawing procedures (15). Frozen-embryo transfer also significantly lowers the risk of a baby being born small-for-gestational age (SGA) or with a low birthweight compared to babies born from fresh transfers, as shown in the systematic review by Maheshwari and co-workers in 2018 (16). Several randomized controlled trials (RCTs) have shown that frozen-embryo transfer portends better pregnancy and live-birth rates than with fresh-embryo transfer (17–20). Frozen-embryo transfer is also being increasingly used in clinical practice instead of fresh embryo transfer due to the benefits reported above, but whether this relatively new transfer strategy affects ESA is presently unknown; and there is a current lack of studies that entail both a large sample size and multi-dimensional research.

Infertile women today face tremendous biological and societal pressures, and a miscarriage during ART treatment further aggravates their psychological and financial stresses (21). It is conventional scientific knowledge that advanced age and obesity in women increase their risk of ESA, and our research reached the same conclusion; but whether frozen-embryo transfer will ultimately increase ESA rates remains controversial, and whether it can bring added benefit has aroused the concerns of both physicians and patients (22, 23). Our study first showed that the incidence of ESA in overall ART transfer cycles was 5.77%, that in fresh- and frozen-embryo transfer cycles the rates were 4.93% and 6.43%, respectively; and that the frozen-embryo transfer rate was significantly higher than in fresh-embryo transfers. Investigators uncovered an early miscarriage rate as slightly higher in women conceiving with frozen-embryo transfer compared with those conceiving with intrauterine insemination (IUI) (24). IUI, which only involves sperm preparation and/or ovulation induction, emulates natural conception more closely. The disparity in ESA rates between frozen-embryo transfer and IUI cycles highlights the impact of embryonic *in- vitro* manipulation on ESA, and the distinction in ESA between frozen-embryo and fresh-embryo transfer cycles also further accentuates the effects of the *in vitro* manipulation of frozen-thawed embryo on ESA. However, we need to further consider that the different population characteristics may have interfered with our results.

In view of the basic patient characteristics, we explored the impacts of maternal age, BMI, and infertility diagnosis on ESA with respect to the total ART transfer cycles, as well as on the fresh-embryo and frozen-embryo transfer subgroups. Our data revealed that women of advanced maternal age, who were obese, and/or with secondary infertility were at greatest risk of ESA in the 35,076 ART transfer cycles; and this was consistent with previous findings (25–27). Whereas only advanced maternal age was correlated with a greater risk for ESA in the fresh-embryo and frozen-embryo transfer subgroups, only obese women in the frozen-embryo transfer subgroup exhibited a greater risk of ESA. This suggests that age, obesity, and frozen-thawed embryo protocols adversely impact ESA rates collectively. Nevertheless, we observed no difference the ESA rate in young women (≤35 years) between the DOR group and non-DOR group, and advanced age women (>35 years), consistent results were also observed. After stratification according to fresh embryo and frozen embryo, there was still no statistical difference in ESA rate between the DOR group and the non-DOR group. However, after stratification according to the DOR and non-DOR, the ESA rate of the advanced age group was significantly higher than that of the younger group. These results suggest that women of advanced age rather than DOR increase ESA rates. In the fresh-embryo transfer subgroup, the ESA rate in GnRH antagonist (GnRH-ant) or other COS protocols was significantly higher than in GnRH agonist (GnRH-a) protocols, which was also congruent with previous RCT findings; but this may have also been due to the small sample size in the aforementioned study (28). However, a previous multivariate logistic regression analysis of 18,853 patients recruited to our Center revealed that the cumulative live-birth rate in the GnRH-ant group was lower than that in the GnRH-a group (OR=2.11; 95% CI, 1.69–2.63), particularly with respect to the suboptimal ovarian responders (where 4–9 oocytes were retrieved) (29). In another retrospective analysis, authors compared the efficiency of the GnRH-ant protocol with that of the GnRH-a protocol in patients with DOR, and demonstrated that the former possessed a lower ET-cancellation rate and higher implantation rate than the latter (30). Our data revealed that a GnRH-a protocol may be superior to a GnRH-ant protocol in terms of ESA rates, and from their different outcome indicators the previous authors suggested that GnRH-a protocols may relate to improved embryo quality or endometrial receptivity; this, however, warrants further

investigation. The ESA rate for blastocyst transfers was markedly higher than for cleavage-stage embryo transfers, but we noted no difference in the subgroup analysis of fresh and frozen cycles—which was inconsistent with previous studies (31, 32). This discrepancy may be caused by data bias between the groups, and the data divergence between the groups therefore needs to be further adjusted to the numerical disparities; and we need to balance the confounders between groups. Needless to say, the higher the number of embryos transferred, the higher the abortion rate. And ESA rates with respect to the insemination method were indistinguishable between fresh-embryo transfer and frozen-embryo transfer cycles (i.e., estrogen-progesterone/natural cycles), which agreed with previous findings (33). Recognizing that some concomitant diseases in women who undergo ART may also affect ESA, we selected endometriosis, PCOS, and unexplained infertility as representatives for our subgroup analysis; and uncovered no statistical difference in ESA rates among the total, fresh-, and frozen-transfer groups. However, ESA was higher in the frozen-transfer group than in the fresh-transfer group regardless of the presence of endometriosis or unexplained infertility. This suggests that embryo freezing itself increases the ESA rate after excluding the influences of the aforementioned accompanying diseases.

Another intriguing principal finding from the current study was that the ESA rate in women under 35 years of age in the frozen-transfer group was significantly higher than that in the fresh-transfer group, while the difference was not statistically significant for women over 35. This indicated that by excluding the influence of advanced reproductive age on abortion, embryonic freezing itself still increased ESA rates. In the BMI subgroup analysis, we also ascertained that by discounting the BMI subgroup, the ESA rate of the frozen-embryo group was still higher than that of the fresh embryo-transfer group, indicating that after excluding the influence of BMI on abortion, embryo-freezing technology still increased the risk of miscarriage (7, 8). The same results were also shown in the subgroup analysis of infertility diagnosis and the number of transferred embryos. In addition, multivariate logistic regression analysis further confirmed the above conclusion: that compared with fresh-embryo transfer, frozen-thawed-embryo transfer was an independent risk factor for ESA. The novelty of the present study, then, was our analysis from the perspective of multiple dimensions that showed that the ESA rate with frozen-embryo transfer was higher than that with fresh-embryo transfer, while ESA was observed to be either higher or not different between fresh-embryo transfers and frozen-embryo transfers in the majority of the previous studies (9–14). We posit that several reasons may explain the discrepancy among studies. First, most investigators—including ourselves—did not exclude all possible confounding factors related to ESA in their analyses. In addition, small-sample, single-center cohort studies may generate data bias, while large-sample cohort studies contain data from multiple centers or are performed over long periods of time where the methods of embryo cryopreservation, embryo thawing, and *in vitro* culture may have changed dramatically during follow-ups. These bias effects are difficult to avoid in actual work, so it is challenging to strictly control confounding factors in real-world investigations. Finally, it is unfortunate that our study lacked data on whether patients experienced a spontaneous abortion before undergoing ART; this partially limited our conclusions, as patients with a history of spontaneous abortion may manifest a higher risk of recurrent spontaneous abortion.

## CONCLUSIONS

This cohort study confirmed that the transfer of frozen embryos increased the risk of ESA during ART cycles relative to fresh embryos. In addition, maternal age, BMI, and number of embryos transferred also increased ESA. Therefore, frozen-transplantation strategies need to be more cautiously assessed so as to provide patients with the greatest benefits possible with respect to safety.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board (IRB) of Chongqing Health Center for Women and Children. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

JS designed the cohort; JS, Q-LC, W-HC, W-WL, G-NH, and HY conducted the trial; JS and Q-LC executed the statistical analyses and prepared the tables with oversight by HY; and JS, Q-LC, and WHC drafted the manuscript. All authors were involved in the data collection, interpreted the data, provided critical input to the manuscript, and approved the final submitted version.

## FUNDING

This study was supported by a grant from the Medical Research Foundation of Chongqing to Qiao-Li Chen (grant number 2020MSXM090).

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.875798/full#supplementary-material

## REFERENCES


1. Messerli F, Meister T, Soria R, Rexhaj E, Scherrer U. Late Repercussions of Assisted Reproductive Technology. *Eur Heart J* (2019) 40(44):3655. doi: 10.1093/eurheartj/ehz806
2. Li F, Niu A, Feng X, Yan Y, Chen Y. The Threshold Effect of Factors Associated With Spontaneous Abortion in Human-Assisted Reproductive Technology. *Sci Rep* (2021) 11(1):11368. doi: 10.1038/s41598-021-90970-5
3. Westhoff C. A Better Medical Regimen for the Management of Miscarriage. *New Engl J Med* (2018) 378(23):2232–3. doi: 10.1056/NEJMe1803491
4. Cai H, Mol B, Gordts S, Wang H, Wang T, Li N, et al. Early and Late Pregnancy Loss in Women With Polycystic Ovary Syndrome Undergoing IVF/ICSI Treatment: A Retrospective Cohort Analysis of 21 820 Pregnancies. *BJOG* (2021) 128(7):1160–9. doi: 10.1111/1471-0528.16590
5. Insogna I, Lanes A, Lee M, Ginsburg E, Fox J. Association of Fresh Embryo Transfers Compared With Cryopreserved-Thawed Embryo Transfers With Live Birth Rate Among Women Undergoing Assisted Reproduction Using Freshly Retrieved Donor Oocytes. *JAMA* (2021) 325(2):156–63. doi: 10.1001/jama.2020.23718
6. Kalinderis M, Kalinderi K, Srivastava G, Homburg R. When Should We Freeze Embryos? Current Data for Fresh and Frozen Embryo Replacement IVF Cycles. *Reprod Sci (Thousand Oaks Calif)* (2021) 28:3061–72. doi: 10.1007/s43032-021-00628-w
7. Gerber R, Fazzari M, Kappy M, Cohen A, Galperin S, Lieman H, et al. Differential Impact of Controlled Ovarian Hyperstimulation on Live Birth Rate in Fresh Versus Frozen Embryo Transfer Cycles: A Society for Assisted Reproductive Technology Clinic Outcome System Study. *Fertil steril* (2020) 114(6):1225–31. doi: 10.1016/j.fertnstert.2020.06.021
8. Acharya K, Acharya C, Bishop K, Harris B, Raburn D, Muasher S. Freezing of All Embryos in *In Vitro* Fertilization is Beneficial in High Responders, But Not Intermediate and Low Responders: An Analysis of 82,935 Cycles From the Society for Assisted Reproductive Technology Registry. *Fertil steril* (2018) 110(5):880–7. doi: 10.1016/j.fertnstert.2018.05.024
9. Ho V, Braam S, Pham T, Mol B, Vuong L. The Effectiveness and Safety of *In Vitro* Maturation of Oocytes Versus *In Vitro* Fertilization in Women With a High Antral Follicle Count. *Hum Reprod (Oxford England)* (2019) 34 (6):1055–64. doi: 10.1093/humrep/dez060
10. Stormlund S, Sopa N, Zedeler A, Bogstad J, Prætorius L, Nielsen H, Kitlinski M, et al. Freeze-All Versus Fresh Blastocyst Transfer Strategy During *In Vitro* Fertilisation in Women With Regular Menstrual Cycles: Multicentre Randomised Controlled Trial. *BMJ (Clinical Res ed)* (2020) 370:m2519. doi: 10.1136/bmj.m2519
11. Roeca C, Johnson R, Truong T, Carlson N, Polotsky A. Birth Outcomes are Superior After Transfer of Fresh Versus Frozen Embryos for Donor Oocyte Recipients. *Hum Reprod (Oxford England)* (2020) 35(12):2850–9. doi: 10.1093/humrep/deaa245
12. Wei D, Liu J, Sun Y, Shi Y, Zhang B, Liu J, et al. Frozen Versus Fresh Single Blastocyst Transfer in Ovulatory Women: A Multicentre, Randomised Controlled Trial. *Lancet (London England)* (2019) 393(10178):1310–8. doi: 10.1016/S0140-6736(18)32843-5
13. Wong K, van Wely M, Mol F, Repping S, Mastenbroek S. Fresh Versus Frozen Embryo Transfers in Assisted Reproduction. *Cochrane Database systematic Rev* (2017) 3:CD011184. doi: 10.1002/14651858.CD011184.pub2
14. Wong K, van Wely M, Verhoeve H, Kaaijk E, Mol F, van der Veen F, et al. Transfer of Fresh or Frozen Embryos: A Randomised Controlled Trial. *Hum Reprod (Oxford England)* (2021) 36(4):998–1006. doi: 10.1093/humrep/deaa305
15. Zaat T, Zagers M, Mol F, Goddijn M, van Wely M, Mastenbroek S. Fresh Versus Frozen Embryo Transfers in Assisted Reproduction. *Cochrane Database systematic Rev* (2021) 2:CD011184. doi: 10.1002/14651858.CD011184.pub3
16. Maheshwari A, Pandey S, Amalraj Raja E, Shetty A, Hamilton M, Bhattacharya S. Is Frozen Embryo Transfer Better for Mothers and Babies? Can Cumulative Meta-Analysis Provide a Definitive Answer? *Hum Reprod Update* (2018) 24(1):35–58. doi: 10.1093/humupd/dmx031
17. Shapiro BS, Daneshmand ST, Garner FC, Aguirre M, Hudson C, Thomas S. Evidence of Impaired Endometrial Receptivity After Ovarian Stimulation for *In Vitro* Fertilization: A Prospective Randomized Trial Comparing Fresh and Frozen-Thawed Embryo Transfer in Normal Responders. *Fertil Steril* (2011) 96(2):344–8. doi: 10.1016/j.fertnstert.2011.05.050
18. Shi Y, Sun Y, Hao C, Zhang H, Wei D, Zhang Y, et al. Transfer of Fresh Versus Frozen Embryos in Ovulatory Women. *New Engl J Med* (2018) 378(2):126–36. doi: 10.1056/NEJMoa1705334
19. Chen ZJ, Shi Y, Sun Y, Zhang B, Liang X, Cao Y, et al. Fresh Versus Frozen Embryos for Infertility in the Polycystic Ovary Syndrome. *New Engl J Med* (2016) 375(6):523–33. doi: 10.1056/NEJMoa1513873
20. Le KD, Vuong LN, Ho TM, Dang VQ, Pham TD, Pham CT, et al. A Cost-Effectiveness Analysis of Freeze-Only or Fresh Embryo Transfer in IVF of non-PCOS Women. *Hum Reprod* (2018) 33(10):1907–14. doi: 10.1093/humrep/dey253
21. Golshani F, Hasanpour S, Mirghafourvand M, Esmaeilpour K. Effect of Cognitive Behavioral Therapy-Based Counseling on Perceived Stress in Pregnant Women With History of Primary Infertility: A Controlled Randomized Clinical Trial. *BMC Psychiatry* (2021) 21(1):278. doi: 10.1186/s12888-021-03283-2
22. Roque M. Freeze-All Policy: Is it Time for That? *J Assist Reprod Genet* (2015) 32(2):171–6. doi: 10.1007/s10815-014-0391-0
23. Arvis P, Lehert P, Guivarc'h-Leveque A. Both High and Low HCG Day Progesterone Concentrations Negatively Affect Live Birth Rates in IVF/ICSI Cycles. *Reprod BioMed Online* (2019) 39(5):852–9. doi: 10.1016/j.rbmo.2019.07.001
24. Riishede I, Berndt Wulff C, Kvist Ekelund C, Pinborg A, Tabor A. Risk of Miscarriage in Women Conceiving After Medically Assisted Reproduction With an Ultrasound-Verified Viable Pregnancy at 6-8 Weeks' Gestation. *Reprod biomed Online* (2019) 39(5):819–26. doi: 10.1016/j.rbmo.2019.06.010
25. Sarkar P, Jindal S, New E, Sprague R, Tanner J, Imudia A. The Role of Preimplantation Genetic Testing for Aneuploidy in a Good Prognosis IVF Population Across Different Age Groups. *Syst Biol Reprod Med* (2021) 2021:1–8. doi: 10.1080/19396368.2021.1954725
26. Sermondade N, Huberlant S, Bourhis-Lefebvre V, Arbo E, Gallot V, Colombani M, et al. Female Obesity is Negatively Associated With Live Birth Rate Following IVF: A Systematic Review and Meta-Analysis. *Hum Reprod Update* (2019) 25(4):439–51. doi: 10.1093/humupd/dmz011
27. Bu Z, Hu L, Su Y, Guo Y, Zhai J, Sun Y. Factors Related to Early Spontaneous Miscarriage During IVF/ICSI Treatment: An Analysis of 21,485 Clinical Pregnancies. *Reprod biomed Online* (2020) 40(2):201–6. doi: 10.1016/j.rbmo.2019.11.001
28. Tomás C, Toftager M, Løssl K, Bogstad J, Prætorius L, Zedeler A, Bryndorf T, Andersen A, et al. Perinatal Outcomes in 521 Gestations After Fresh and Frozen Cycles: A Secondary Outcome of a Randomized Controlled Trial Comparing GnRH Antagonist Versus GnRH Agonist Protocols. *Reprod Biomed Online* (2019) 39(4):659–64. doi: 10.1016/j.rbmo.2019.05.010
29. Yang J, Zhang X, Ding X, Wang Y, Huang G, Ye H. Cumulative Live Birth Rates Between GnRH-Agonist Long and GnRH-Antagonist Protocol in One ART Cycle When All Embryos Transferred: Real-Word Data of 18,853 Women From China. *Reprod Biol Endocrinol* (2021) 19(1):124. doi: 10.1186/s12958-021-00814-0
30. Huang M, Tzeng S, Lee C, Chen H, Huang C, Lee T, et al. GnRH Agonist Long Protocol Versus GnRH Antagonist Protocol for Various Aged Patients With Diminished Ovarian Reserve: A Retrospective Study. *PLoS One* (2018) 13(11): e0207081. doi: 10.1371/journal.pone.0207081
31. Zhu Q, Zhu J, Wang Y, Wang B, Wang N, Yin M, et al. Live Birth Rate and Neonatal Outcome Following Cleavage-Stage Embryo Transfer Versus Blastocyst Transfer Using the Freeze-All Strategy. *Reprod Biomed Online* (2019) 38(6):892–900. doi: 10.1016/j.rbmo.2018.12.034
32. Shi W, Zhang W, Li N, Xue X, Liu C, Qu P, et al. Comparison of Perinatal Outcomes Following Blastocyst and Cleavage-Stage Embryo Transfer: Analysis of 10 Years' Data From a Single Centre. *Reprod Biomed Online* (2019) 38(6):967–78. doi: 10.1016/j.rbmo.2018.12.031
33. Wang C, Feng G, Zhang B, Shu J, Zhou H, Gan X, et al. Influence of the Insemination Method on the Outcomes of Elective Blastocyst Culture. *Clin Exp Reprod Med* (2017) 44(2):85–9. doi: 10.5653/cerm.2017.44.2.85


**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Endocrinology

CLINICAL TRIAL
published: 29 June 2022
doi: 10.3389/fendo.2022.875779

# GnRH Antagonist Protocol Versus GnRH Agonist Long Protocol: A Retrospective Cohort Study on Clinical Outcomes and Maternal-Neonatal Safety

*Jieru Zhu, Weijie Xing, Tao Li, Hui Lin and Jianping Ou**

*Center for Reproductive Medicine, The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, China*

OPEN ACCESS

**Edited by:**
*Yimin Zhu,*
*Zhejiang University, China*

**Reviewed by:**
*Jingmei Hu,*
*Reproductive Hospital Affiliated to Shandong University, China*
*Rong Li,*
*Peking University Third Hospital, China*
*Yihong Guo,*
*First Affiliated Hospital of Zhengzhou University, China*

***Correspondence:**
*Jianping Ou*
*oujp3@mail.sysu.edu.cn*

**Specialty section:**
*This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology*

**Received:** *14 February 2022*
**Accepted:** *19 May 2022*
**Published:** *29 June 2022*

**Citation:**
*Zhu J, Xing W, Li T, Lin H and Ou J (2022) GnRH Antagonist Protocol Versus GnRH Agonist Long Protocol: A Retrospective Cohort Study on Clinical Outcomes and Maternal-Neonatal Safety. Front. Endocrinol. 13:875779. doi: 10.3389/fendo.2022.875779*

**Objective:** To evaluate the clinical outcomes and maternal-neonatal safety of gonadotropin releasing hormone antagonist (GnRH-ant) and gonadotropin releasing hormone agonist (GnRH-a) protocols.

**Methods:** A total of 2505 women undergoing their first *in vitro* fertilization (IVF)/ intracytoplasmic sperm injection (ICSI) were retrospectively analyzed. Patients were divided into GnRH-ant group (n = 1514) and GnRH-a group (n = 991) according their stimulation protocol. Propensity Score Matching (PSM) was used for balancing the baseline of two groups. The pregnancy outcomes were analyzed in fresh transfer cycles, and the obstetric and perinatal outcomes were calculated in singleton live births of fresh cycles. The primary outcome was the live birth rate. The secondary outcome measures were maternal complications, preterm birth rate, low birthweight rate, multiple pregnancy rate, and moderate-severe OHSS rate.

**Results:** After 1:1 PSM, baseline characteristics of the GnRH-ant group and GnRH-a group were matched and assigned 991 cycles in each group. Before PSM, there were 700 fresh cycles including 237 singleton live births in the GnRH-ant group and 588 fresh cycles including 187 singleton live births in the GnRH-a group. After PSM, there were 471 fresh cycles including 166 singleton live births in the GnRH-ant group and 588 fresh cycles including 187 singleton live births in the GnRH-a group. No significant differences were observed in the live birth rate (44.6% vs 48.8%), maternal complications, preterm birth rate (9.0% vs 6.4%), and low birthweight rate (17.5% vs 24.1%) between two groups after PSM ($P > 0.05$). The moderate-severe OHSS rate (2.9% vs 6.0%, $P = 0.002$) and multiple pregnancy rate (24.5% vs 33.1%, $P = 0.025$) was significantly lower in the GnRH-ant group than that in the GnRH-a group after PSM.

**Conclusion:** GnRH-ant protocol was comparable with GnRH-a protocol in clinical outcomes, obstetric and perinatal outcomes, and with a lower risk of OHSS. For those who want to get an effective and safe outcome, and a shorter treatment period, GnRH-ant is a suitable choice.

**Keywords: GnRH antagonist, GnRH agonist, fresh embryo transfer, live birth, obstetric, perinatal, safety**

# 1 INTRODUCTION

Gonadotropin releasing hormone agonist (GnRH-a) has been applied in controlled ovarian stimulation (COS) in assisted reproductive technology (ART) for several decades (1). It binds the GnRH receptor of the pituitary gland and then greatly depletes the receptors, thereby reducing the secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH). Thanks to the effect of pituitary down- regulation of GnRH-a, the occurrence of premature LH surge is prevented and the homogeneity of follicle development improves (2). However, due to the 'flare up' effect and subsequent inhibition of the pituitary gland, the dosage and duration of gonadotropin also increase, and may lead to luteal insufficiency and increase the incidence of ovarian hyperstimulation syndrome (OHSS) (3). Recently, gonadotropin releasing hormone antagonist (GnRH-ant) protocol is increasingly favored in clinical practice because of its physiological advantages. GnRH-ant directly competes with the receptor for binding without pituitary stimulation, therefore inhibiting the secretion of endogenous gonadotropin in a short time. Its strengths include lower dosage and shorter duration of medication, and a reduction of the risk of OHSS (4).

Considerable stimulation outcomes such as the quality of oocytes and embryos, and pregnancy outcomes especially live birth rate are the concerns of reproductive doctors. Several studies have focused on these indicators but the results are controversial (5–7). Evidence shows ART may be related to a higher risk of maternal complications and adverse neonatal outcomes including preterm birth, low birth weight compared with natural pregnancy (3, 8, 9). GnRH-ant protocol and GnRH-a protocol are two mainstream protocols in reproductive centers of China. Few studies have simultaneously analyzed the efficiency and maternal-neonatal safety of the two stimulation protocols, which is essential for reproductive doctors to make the decision for women undergoing *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI).

The aim of this study was to evaluate the clinical outcomes and maternal-neonatal safety of GnRH-ant protocol and GnRH-a protocol.

# 2 MATERIALS AND METHODS

## 2.1 Study Design and Population

This is a retrospective cohort study. The information of patients was obtained from the electronic medical records system. Patients receiving their IVF/ICSI treatment in the Center for Reproductive Medicine, The Third Affiliated Hospital of Sun Yat-sen University who met the inclusion criteria and exclusion criteria from January 2016 to May 2021 were retrospectively analyzed. Inclusion criteria (1): Patients undergoing their first IVF/ICSI cycles; (2) Infertility caused by only pelvic oviduct disorder or male sterility; (3) Age between 21~39 years old; (4) Body mass index (BMI) between 18.5~23.9 kg/m$^2$; (5) Menstrual cycle between 21~35 days; (6) Received GnRH-ant protocol or GnRH-a protocol for their COS treatment. Patients with uterine malformations, endometriosis, polycystic ovarian syndrome (PCOS), and physical diseases such as hypertension and diabetes mellitus were excluded. Finally, a total of 2505 women were included in the present study, and 1514 women underwent treatment with GnRH-ant protocol and 991 patients underwent treatment with GnRH-a protocol.

## 2.2 Controlled Ovarian Stimulation Protocols

### 2.2.1 GnRH Antagonist Protocol

Gonadotropin (Merck Serono, Switzerland; MSD, USA; Lizhu Pharmaceutical Trading, China) was administered on the second or third day of the menstrual cycle with a dose of 75~300 IU, depending on the age, BMI, and ovarian reserve of patients. Cetrorelix (Merck Serono, Switzerland) or ganirelix (Organon, The Netherlands; Zhengdatianqing Pharmaceutical Group, China) of 0.25 mg/day was initiated once the diameter of dominant follicle >14 mm or serum estradiol ($E_2$) >400 ng/mL or serum luteinizing hormone (LH) >10 IU/L (10), continue to trigger day.

### 2.2.2 GnRH Agonist Long Protocol

Subcutaneous injection of triptorelin (Ipsen, France; Ferring GmbH, Germany) at a dose of 0.1 mg/day was commenced in the middle luteal phase of the previous menstrual cycle. If the pituitary down-regulation criteria achieved (serum $E_2$ <50 pg/mL, FSH <5 IU/L, LH <5 IU/L and the endometrial thickness <5 mm), gonadotropin (Merck Serono, Switzerland; MSD, USA; Lizhu Pharmaceutical Trading, China) was administered of 75~300 IU, based on the age, BMI, and ovarian reserve of patients, until the trigger day.

## 2.3 Trigger, Embryo Transfer, and Luteal Support

6000 IU human chorionic gonadotropin (hCG. Lizhu Pharmaceutical Trading, China) or 250 μg rhCG (Merck Serono, Italy) was injected when at least two follicles >18 mm in diameter. Part of patients with a high risk of OHSS from GnRH-ant cycles was administered 0.2 mg GnRH-a or GnRH-a plus hCG for trigger. Oocyte pick-up (OPU) was performed after

34~36 hours. Fresh embryo transfer was carried out 3 days (cleavage embryo) or 5 days (blastocyst) after OPU. Whole embryos were frozen if patients with a high risk of OHSS, high progesterone level, severe hydrosalpinx, or endometrial polyp. Oral progesterone combined vaginal progesterone or intramuscular progesterone was used for luteal support since the day of OPU for fresh transfer cycles.

## 2.4 Follow-Up

The obstetrical complications and neonatal information were obtained from the medical records if women receiving their prenatal care and delivery in our hospital, otherwise women were contacted by telephone during pregnancy and after delivery.

## 2.5 Outcome Measures

To minimize confounding factors of comparing the two stimulation protocols, all the pregnancy indexes including implantation rate, biochemical pregnancy rate, clinical pregnancy rate, multiple pregnancy rate and live birth rate were analyzed in fresh transfer cycles. The indexes of maternal complications, preterm birth rate, birth weight, low birthweight rate, macrosomia rate and neonatal malformation rate were counted in singleton live births of fresh cycles. The primary outcome was the live birth rate, which was defined as the rate of at least one live-born baby in one fresh transfer cycle. The secondary outcome measures were maternal complications, preterm birth rate, low birthweight rate, multilpe pregnancy rate, and moderate-severe OHSS rate. Preterm birth was defined as live birth greater than 26 and less than 37 weeks' gestation. Low birthweight was defined as <2500 g birth weight, and macrosomia rate was defined as ≥4000 g birth weight.

## 2.6 Statistical Analysis

Data analysis was performed by IBM SPSS Statistics software, version 26.0. The K-S test was used for the normality test. The Student's t-test was used to compare the measurement data conforming to normal distribution, and the results were expressed by mean ± standard deviation (SD). Otherwise, the Mann-Whitney U-test was adopted, and the results were expressed by median (quartile range). The Chi-square test was performed for the comparison of counted data, and the results were expressed by percentage (%). Propensity Score Matching (PSM) was used for sampling by 1:1 matching with 'maximize execution performance' and caliper (0.02) to balance the baseline of the two groups. The significant level was set at P<0.05.

# 3 RESULTS

## 3.1 Subjects and Stimulation Characteristics

A total of 2505 women were included in this study and divided into the GnRH-ant (n = 1514) and GnRH-a (n = 991) groups. The results of the normality test showed that all the measurement data in the present study were non-normally distributed. Before PSM, significant differences existed in age, basal FSH, basal LH ($P < 0.05$). After 1:1 PSM, patients in the two groups were matched in age, basal FSH, basal LH, and assigned 991 cycles in each group (**Table 1**).

The age, BMI, duration of infertility, basal LH, basal E2, type of infertility, and fertilization type were comparable in the two groups after PSM. The basal FSH level was a bit higher in the GnRH-a group ($P < 0.05$). The duration of stimulation, dosage of gonadotropins, E2 values on the trigger day, No. of oocytes retrieved, moderate-severe OHSS rate were significantly lower in the GnRH-ant group than those in the GnRH-a group ($P < 0.05$). The MII rate, fertilization rate, and D3 high qualified embryo rate were significantly higher in the GnRH-ant group than those in the GnRH-a group ($P < 0.05$) (**Table 1**).

## 3.2 Pregnancy Outcomes of Fresh Transfer Cycles

There were 700 fresh cycles in the GnRH-ant group and 588 fresh cycles in the GnRH-a group before PSM, and 471 fresh cycles in the GnRH-ant group and 588 fresh cycles in the GnRH-a group after PSM. The average number of embryos transferred and the multiple pregnancy rate after fresh embryo transfer was significantly lower in the GnRH-ant group than that in the GnRH-a group no matter before or after PSM ($P < 0.05$). No significant differences were observed in the embryo type of transfer, implantation rate, biochemical pregnancy rate, clinical pregnancy rate, miscarriage rate, and live birth rate of fresh cycles between two groups before and after PSM ($P > 0.05$) (**Table 2**).

## 3.3 Maternal Complications and Neonatal Outcomes of Singleton Live Births

There were 237 singleton live births of fresh cycles in the GnRH-ant group and 187 singleton live births of fresh cycles in the GnRH-a group before PSM, and 166 singleton live births of fresh cycles in the GnRH-ant group and 187 singleton live births of fresh cycles in the GnRH-a group after PSM. There were no significant differences in the maternal complications such as gestational diabetes mellitus (GDM), gestational hypertension, with GDM and gestational hypertension at the same time, and other complications like anemia, thrombocytopenia, intrahepatic cholestasis of pregnancy (ICP) between the two groups before and after PSM ($P > 0.05$). The preterm birth rate, birth weight, low birthweight rate, macrosomia rate and neonatal malformation rate were comparable between the two groups before and after PSM ($P > 0.05$) (**Table 3**). There was one case of ventricular septal defect (VSD), one case of patent ductus arteriosus (PDA) and one case of persistent left superior vena cava (PLSVC) in the GnRH-ant group, and one case of inherited metabolic disorder (IMD), one case of patent ductus arteriosus (PDA) and one case of patent foramen ovale (PFO) in the GnRH-a group after PSM.

# 4 DISCUSSION

In recent years, the advantages of GnRH-ant have gradually emerged with its promotion and application in ART treatments.

**TABLE 1** | Baseline information and stimulation characteristics of women before and after PSM.

| | Before matched/After matched | GnRH-ant group | GnRH-a group | P value |
|---|---|---|---|---|
| Cycles/(n) | B | 1514 | 991 | |
| | A | 991 | 991 | |
| Age/(year) | B | 31 (28~34) | 31 (29~35) | 0.002 |
| | A | 31 (28~34) | 31 (29~35) | 0.207 |
| BMI/(kg/m$^2$) | B | 20.83 (19.72~22.10) | 20.81 (19.72~22.10) | 0.517 |
| | A | 20.83 (19.72~22.04) | 20.81 (19.72~22.10) | 0.732 |
| Duration of infertility/(year) | B | 3.00 (1.75~4.00) | 3.00 (1.00~4.00) | 0.959 |
| | A | 3.00 (2.00~4.00) | 3.00 (1.00~4.00) | 0.887 |
| bFSH (U/L) | B | 6.67 (5.66~7.47) | 6.84 (5.92~7.84) | <0.001 |
| | A | 6.73 (5.72~7.60) | 6.84 (5.92~7.84) | 0.032 |
| bLH (U/L) | B | 5.42 (3.97~6.56) | 4.99 (3.76~6.31) | 0.001 |
| | A | 5.22 (3.91~6.27) | 4.99 (3.76~6.31) | 0.240 |
| b$E_2$ (pg/mL) | B | 40.26 (29.78~48.53) | 39.44 (29.86~50.71) | 0.763 |
| | A | 40.14 (29.53~48.02) | 39.44 (29.86~50.71) | 0.555 |
| Type of infertility | | | | |
| Primary | B | 720/1514 (47.6) | 465/991 (46.9) | 0.756 |
| | A | 467/991 (47.1) | 465/991 (46.9) | 0.928 |
| Secondary | B | 794/1514 (52.4) | 526/991 (53.1) | |
| | A | 524/991 (52.9) | 526/991 (53.1) | |
| Fertilization type | | | | |
| IVF | B | 1256/1514 (83.0) | 795/991 (80.2) | 0.082 |
| | A | 821/991 (82.8) | 795/991 (80.2) | 0.132 |
| ICSI | B | 258/1514 (17.0) | 196/991 (19.8) | |
| | A | 170/991 (17.2) | 196/991 (19.8) | |
| Duration of stimulation/(days) | B | 8 (7~9) | 10 (8~11) | <0.001 |
| | A | 8 (7~9) | 10 (8~11) | <0.001 |
| Dosage of gonadotropins/(IU) | B | 1500 (1125~1800) | 1950 (1250~2400) | <0.001 |
| | A | 1550 (1200~1900) | 1950 (1250~2400) | <0.001 |
| $E_2$ values on the trigger day/(pg/mL) | B | 2682 (1744~3765) | 2821 (1920~3880) | 0.027 |
| | A | 2635 (1701~3699) | 2821 (1920~3880) | 0.005 |
| No. of oocytes retrieved/(n) | B | 12 (7~16) | 12 (8~16) | 0.290 |
| | A | 11 (7~17) | 12 (8~16) | 0.049 |
| MII rate | B | 15596/19019 (82.0) | 9921/12611 (78.7) | <0.001 |
| | A | 9992/12171 (82.1) | 9921/12611 (78.7) | <0.001 |
| Fertilization rate | B | 14822/19019 (77.9) | 9679/12611 (76.8) | 0.014 |
| | A | 9492/12171 (78.0) | 9679/12611 (76.8) | 0.020 |
| D3 high qualified embryo rate | B | 6326/12360 (51.2) | 3839/8304 (46.2) | <0.001 |
| | A | 4059/7985 (50.8) | 3839/8304 (46.2) | <0.001 |
| Moderate-severe OHSS rate | B | 44/1514 (2.9) | 59/991 (6.0) | 0.002 |
| | A | 33/991 (3.3) | 59/991 (6.0) | 0.030 |

*Data are presented as the M(P25~P75) for continuous variables and n (%) for categorical variables. BMI, body mass index; bFSH, basal FSH; bLH, basal LH; b$E_2$, basal $E_2$; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection.*

Compared with GnRH-a, GnRH-ant competitively binds to the GnRH receptor of the pituitary gland, without waiting for receptor exhaustion and desensitization, and there is no 'flare up' effect. It inhibits the secretion of gonadotropin within a few hours and avoids excessive pituitary inhibition. Therefore, GnRH-ant can effectively reduce the consumption of gonadotropin and greatly shorten the treatment time (11, 12). The results of the present study suggested that the duration of stimulation and the dosage of gonadotropins of GnRH-ant group were significantly lower than those of GnRH-a group, which was consistent with the previous studies (6, 13).

Live birth is the common goal of patients and reproductive doctors. Optimistic ovarian stimulation outcomes are closely related to favourable live birth rate. Although the number of oocytes retrieved of the GnRH-ant group was slightly inferior to the GnRH-a group in our study, the MII rate, fertilization rate, and D3 high qualified embryo rate were significantly higher. These results indicated that the GnRH-ant protocol had a positive effect on follicular maturity, fertilization ability, and embryonic developmental potential (5). In order to obtain more objective results, evaluate the impact of stimulation protocols on subsequent pregnancy outcomes, and avoid some confounding factors of frozen cycles, we only selected fresh cycles to analyze the pregnancy outcomes of the two protocols. The implantation rate, clinical pregnancy rate, and live birth rate were comparable in the GnRH-ant and GnRH-a protocols, suggesting GnRH-ant protocol was as effective as GnRH-a protocol. Recently, a real-world study of 18853 women from China concluded that the cumulative live birth rate (CLBR) was similar in GnRH-ant and GnRH-a groups (5). Its large sample and real-world data provided a powerful reference for clinical practice. Although we focused on live birth rate but not CLBR in the present study, the conclusion was consistent, and was significative for explaining to patients the success chance of one fresh cycle.

**TABLE 2 |** Pregnancy outcomes of fresh cycles before and after PSM matching.

| | Before matched/After matched | GnRH-ant group | GnRH-a group | P value |
|---|---|---|---|---|
| Fresh transferred cycles/(n) | B | 700 | 588 | |
| | A | 471 | 588 | |
| Cleavage embryo or blastocyst transfer | | | | |
| Cleavage embryo | B | 511/700 (73.0) | 451/588 (76.7) | 0.229 |
| | A | 351/471 (74.5) | 451/588 (76.7) | 0.411 |
| Blastocyst | B | 189/700 (27.0) | 137/588 (23.3) | |
| | A | 120/471 (25.5) | 137/588 (23.3) | |
| Implantation rate | B | 460/1126 (40.9) | 436/1030 (42.3) | 0.487 |
| | A | 301/751 (40.1) | 436/1030 (42.3) | 0.341 |
| Average No. of embryos transferred | B | 2 (1~2, 1.61 ± 0.50) | 2 (1~2, 1.75 ± 0.47) | <0.001 |
| | A | 2 (1~2, 1.59 ± 0.50) | 2 (1~2, 1.75 ± 0.47) | <0.001 |
| Biochemical pregnancy rate | B | 29/700 (4.1) | 22/588 (3.7) | 0.713 |
| | A | 23/471 (4.9) | 22/588 (3.7) | 0.360 |
| Clinical pregnancy rate | B | 366/700 (52.3) | 329/588 (56.0) | 0.188 |
| | A | 241/471 (51.2) | 329/588 (56.0) | 0.121 |
| Multiple pregnancy rate | B | 94/366 (25.7) | 109/329 (33.1) | 0.031 |
| | A | 59/241 (24.5) | 109/329 (33.1) | 0.025 |
| Miscarriage rate | B | 23/366 (6.3) | 32/329 (9.7) | 0.093 |
| | A | 14/241 (5.8) | 32/329 (9.7) | 0.090 |
| Live birth rate | B | 329/700 (47.0) | 287/588 (48.8) | 0.517 |
| | A | 210/471 (44.6) | 287/588 (48.8) | 0.171 |

*Data are presented as the M(P25~P75, mean ± SD) for the average No. of embryos transferred, and the n (%) for categorical variables.*

Several studies have reported live birth rates with GnRH-ant protocol and yielded the same conclusions as to the present study (7, 14, 15). A meta-analysis including 29 randomized controlled trials (RCTs) showed that there were no significant differences in the clinical pregnancy rate, ongoing pregnancy rate, and live birth rate between the GnRH-ant and GnRH-a groups (14). A clinical research reported that there were no significant differences in the clinical pregnancy rate and live birth rate among the modified agonist, mild-stimulation and antagonist protocols (7). Some studies have come to different conclusions. Another meta-analysis suggested that the live birth rate with GnRH-ant protocol averaged 1.5% lower than GnRH-a protocol (16), but it only included 9 RCTs involving 1515 women, the sample of which was too small.

The safety of treatments was another considerable thing, including maternal and neonatal safety. In the present study, the

**TABLE 3 |** Obstetric and perinatal outcomes of singleton live births before and after PSM matching.

| | Before matched/After matched | GnRH-ant group | GnRH-a group | P value |
|---|---|---|---|---|
| Singletons live births/(n) | B | 237 | 187 | |
| | A | 166 | 187 | |
| Maternal complications | | | | |
| No complications | B | 190/237 (80.2) | 153/187 (81.8) | 0.915 |
| | A | 132/166 (79.5) | 153/187 (81.8) | 0.759 |
| GDM | B | 28/237 (11.8) | 17/187 (9.1) | |
| | A | 22/166 (13.3) | 17/187 (9.1) | |
| Gestational hypertension | B | 9/237 (3.8) | 8/187 (4.3) | |
| | A | 6/166 (3.6) | 8/187 (4.3) | |
| Both GDM and gestational hypertension | B | 5/237 (2.1) | 4/187 (2.1) | |
| | A | 3/166 (1.8) | 4/187 (2.1) | |
| Others | B | 5/237 (2.1) | 5/187 (2.7) | |
| | A | 3/166 (1.8) | 5/187 (2.7) | |
| Preterm birth rate | B | 21/237 (8.9) | 12/187 (6.4) | 0.351 |
| | A | 15/166 (9.0) | 12/187 (6.4) | 0.355 |
| Birth weight | B | 3020 (2090~3050) | 3020 (2095~3050) | 0.613 |
| | A | 3025 (2095~3055) | 3020 (2095~3050) | 0.564 |
| Low birthweight rate | B | 41/237 (17.3) | 45/187 (24.1) | 0.085 |
| | A | 29/166 (17.5) | 45/187 (24.1) | 0.129 |
| Macrosomia rate | B | 2/237 (0.8) | 6/187 (3.2) | 0.076 |
| | A | 2/166 (1.2) | 6/187 (3.2) | 0.207 |
| Neonatal malformation rate | B | 3/237 (1.3) | 3/187 (1.6) | 0.770 |
| | A | 3/166 (1.8) | 3/187 (1.6) | 0.883 |

*Data are presented as the M(P25~P75) for continuous variables and n (%) for categorical variables. GDM, gestational diabetes mellitus.*

moderate-severe OHSS incident rate and multiple pregnancy rate of GnRH-ant group were significantly lower than those of GnRH-a group, which was consistent with previous reports (17–19). The follicle development of GnRH-ant protocol is not as synchronous as that of the GnRH-a protocol, and the gonadotropin dosage and estrogen levels of trigger day were lower, which may be the reasons for reducing the occurrence of OHSS (17). Because of a bit less number of embryos transferred and slightly lower implantation rate in the GnRH-ant group, its multiple pregnancy rate was lower. Previous studies indicated higher rates of pregnancy complications and adverse neonatal outcomes in women receiving ART (8, 20, 21). All women of fresh transfer cycles will receive exogenous progesterone as luteal support, which increases the risk of insulin resistance and leads to gestational diabetes mellitus (GDM) (22). Studies also suggested that hyperphysiological doses of estrogen were associated with preeclampsia and low birth weight (3, 9, 23). However, few researches focused on the relationship between stimulation protocols and perinatal outcomes. Our results suggested that there were no significant differences in the maternal complications, preterm birth rates, birth weight, low birthweight rates, macrosomia rates, and neonatal malformation rates between GnRH-ant and GnRH-a groups. That was to say, the differences in steroid hormone levels, placental gene imprinting and epigenetic changes caused by the two protocols did not seem to affect perinatal outcomes (21). The results of an RCT including 521 gestations showed that in singletons after fresh embryo transfer, the preterm birth rates, mean birthweight, and low birthweight rates were similar of GnRH-ant and GnRH-a protocols (3). A large prospective, pregnancy and infant follow-up trial indicated that there were more multiple pregnancies in the GnRH agonist, but no significant differences in major congenital malformations in fetuses of GnRH antagonist and GnRH agonist (19). These results were consistent with the results of the present study.

The greatest advantage of this study was using PSM for balancing the baseline characteristics of two groups. Not only the stimulation and pregnancy outcomes, but also the obstetric and perinatal conditions of the two protocols were evaluated with strict inclusion and exclusion criteria. The limitation was that this was a retrospective analysis with a limited sample. The control for confounding factors and the preciseness of the conclusions were not as good as prospective RCTs. The information may be inaccurate because part of women were followed up only by telephone. However, this was a study that was from the real world, which was closer to the clinical practice.

In summary, GnRH-ant protocol was comparable with GnRH-a protocol in clinical outcomes, obstetric and perinatal outcomes, and with a lower risk of OHSS. For patients who want to get an effective and safe outcome, and a shorter treatment period, GnRH-ant is a suitable choice. The results of the present study require a well-designed RCT and larger samples to be identified.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the medical ethics committee of Third Affiliated Hospital of Sun Yat-sen University. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

JZ and JO designed the study. WX selected the study population that met the criteria and exclusion criteria. JZ and WX performed the statistical analysis. JO, TL and HL reviewed the data. JZ drafted the manuscript. All authors contributed to the article and approved the submitted version.

## REFERENCES


1. Porter RN, Smith W, Craft IL, Abdulwahid NA, Jacobs HS. Induction of Ovulation for *In-Vitro* Fertilisation Using Buserelin and Gonadotropins. *Lancet* (1984) 2:1284–85. doi: 10.1016/S0140-6736(84)92840-X
2. Zhang J, Sun Y, Xu Y, Shi B, Han Y, Luo Z, et al. Effect of Endometrium Thickness On Clinical Outcomes in Luteal Phase Short-Acting GnRH-A Long Protocol and GnRH-Ant Protocol. *Front Endocrinol* (2021) 12:578783. doi: 10.3389/fendo.2021.578783
3. Tomás C, Toftager M, Løssl K, Bogstad J, Prætorius L, Zedeler A, et al. Perinatal Outcomes in 521 Gestations After Fresh and Frozen Cycles: A Secondary Outcome of a Randomized Controlled Trial Comparing GnRH Antagonist Versus GnRH Agonist Protocols. *Reprod BioMed Online* (2019) 39:659–64. doi: 10.1016/j.rbmo.2019.05.010
4. Li J, Sun Y, Mo S, Wang S, Luo W. Effects of Oral Contraceptive for Different Responder Women Before GnRH Antagonists: A Systematic Review and Meta-Analysis. *Gynecol Endocrinol* (2021) 37:977–86. doi: 10.1080/09513590.2021.1918664
5. Yang J, Zhang X, Ding X, Wang Y, Huang G, Ye H. Cumulative Live Birth Rates Between GnRH-Agonist Long and GnRH-Antagonist Protocol in One ART Cycle When All Embryos Transferred: Real-Word Data of 18,853 Women From China. *Reprod Biol Endocrin* (2021) 19:124–32. doi: 10.1186/s12958-021-00814-0
6. Trenkić M, Popović J, Kopitović V, Bjelica A, Živadinović R, Pop-Trajković S. Flexible GnRH Antagonist Protocol vs. Long GnRH Agonist Protocol in Patients With Polycystic Ovary Syndrome Treated for IVF: Comparison of Clinical Outcome and Embryo Quality. *Polish Gynaecol* (2016) 87:265–70. doi: 10.17772/gp/62205
7. Yu R, Jin H, Huang X, Lin J, Wang P. Comparison of Modified Agonist, Mild-Stimulation and Antagonist Protocols Forin Vitro Fertilization in Patients With Diminished Ovarian Reserve. *J Int Med Res* (2018) 46:2327–37. doi: 10.1177/0300060518770346
8. Sunkara SK, LaMarca A, Polyzos NP, Seed PT, Khalaf Y. Live Birth and Perinatal Outcomes Following Stimulated and Unstimulated IVF: Analysis of Over Two Decades of a Nationwide Data. *Hum Reprod* (2016) 31:2261–67. doi: 10.1093/humrep/dew184

9. Sunkara SK, La Marca A, Seed PT, Khalaf Y. Increased Risk of Preterm Birth and Low Birthweight With Very High Number of Oocytes Following IVF: An Analysis of 65 868 Singleton Live Birth Outcomes. *Hum Reprod* (2015) 30:1473–80. doi: 10.1093/humrep/dev076
10. Lainas T, Zorzovilis J, Petsas G, Stavropoulou G, Cazlaris H, Daskalaki V, et al. In a Flexible Antagonist Protocol, Earlier, Criteria-Based Initiation of GnRH Antagonist is Associated With Increased Pregnancy Rates in IVF. *Hum Reprod* (2005) 20:2426–33. doi: 10.1093/humrep/dei106
11. Luo X, Pei L, Li F, Li C, Huang G, Ye H. Fixed Versus Flexible Antagonist Protocol in Women With Predicted High Ovarian Response Except PCOS: A Randomized Controlled Trial. *BMC Pregnancy Childbirth* (2021) 21:348. doi: 10.1186/s12884-021-03833-2
12. Papamentzelopoulou M, Stavros S, Mavrogianni D, Kalantzis C, Loutradis D, Drakakis P. Meta-Analysis of GnRH-Antagonists Versus GnRH-Agonists in Poor Responder Protocols. *Arch Gynecol Obstet* (2021) 304:547–57. doi: 10.1007/s00404-020-05954-z
13. Hershko KA, Berkovitz A, Wiser A, Gonen O, Amichay K, Cohen I, et al. GnRH-Antagonist Programming Versus GnRH Agonist Protocol: A Randomized Trial. *Eur J Obstet Gynecol Reprod Biol* (2015) 185:170–73. doi: 10.1016/j.ejogrb.2014.12.021
14. Wang R, Lin S, Wang Y, Qian W, Zhou L. Comparisons of GnRH Antagonist Protocol Versus GnRH Agonist Long Protocol in Patients With Normal Ovarian Reserve: A Systematic Review and Meta-Analysis. *PloS One* (2017) 12:e175985. doi: 10.1371/journal.pone.0175985
15. Al-Inany HG, Youssef M, Ayeleke R. Gonadotrophin-Releasing Hormone Antagonists for Assisted Reproductive Technology. *Cochrane Database Systematic Rev* (2016) 4:D1750. doi: 10.1002/14651858.CD001750.pub4
16. Al-Inany HG, Youssef MAFM, Aboulghar M, Broekmans F, Sterrenburg M, Smit J, et al. GnRH Antagonists are Safer Than Agonists: An Update of a Cochrane Review. *Hum Reprod Update* (2011) 17:435. doi: 10.1093/humupd/dmr004
17. Toftager M, Bogstad J, Bryndorf T, Løssl K, Roskær J, Holland T, et al. Risk of Severe Ovarian Hyperstimulation Syndrome in GnRH Antagonist Versus GnRH Agonist Protocol: RCT Including 1050 First IVF/ICSI Cycles. *Hum Reprod* (2016) 31:1253–64. doi: 10.1093/humrep/dew051
18. Qiao J, Zhang Y, Liang X, Ho T, Huang HY, Kim SH, et al. A Randomised Controlled Trial to Clinically Validate Follitropin Delta in its Individualised Dosing Regimen for Ovarian Stimulation in Asian IVF/ICSI Patients. *Hum Reprod* (2021) 36:2452–62. doi: 10.1093/humrep/deab155
19. Bonduelle M, Oberyé J, Mannaerts B, Devroey P. Large Prospective, Pregnancy and Infant Follow-Up Trial Assures the Health of 1000 Fetuses Conceived After Treatment With the GnRH Antagonist Ganirelix During Controlled Ovarian Stimulation. *Hum Reprod* (2010) 25:1433–40. doi: 10.1093/humrep/deq072
20. Sha T, Wang X, Cheng W, Yan Y. A Meta-Analysis of Pregnancy-Related Outcomes and Complications in Women With Polycystic Ovary Syndrome Undergoing IVF. *Reprod BioMed Online* (2019) 39:281–93. doi: 10.1016/j.rbmo.2019.03.203
21. Bosdou JK, Anagnostis P, Goulis DG, Lainas GT, Tarlatzis BC, Grimbizis GF, et al. Risk of Gestational Diabetes Mellitus in Women Achieving Singleton Pregnancy Spontaneously Or After ART: A Systematic Review and Meta-Analysis. *Hum Reprod Update* (2020) 26:514–44. doi: 10.1093/humupd/dmaa011
22. Brănişteanu DD, Mathieu C. Progesterone in Gestational Diabetes Mellitus: Guilty Or Not Guilty? *Trends Endocrinol Metab* (2003) 14:54–6. doi: 10.1016/s1043-2760(03)00003-1
23. McDonald SD, Han Z, Mulla S, Murphy KE, Beyene J, Ohlsson A. Preterm Birth and Low Birth Weight Among *in Vitro* Fertilization Singletons: A Systematic Review and Meta-Analyses. *Eur J Obstet Gyn R B* (2009) 146:138–48. doi: 10.1016/j.ejogrb.2009.05.035


**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers | Frontiers in Endocrinology

ORIGINAL RESEARCH
published: 05 July 2022
doi: 10.3389/fendo.2022.817555

# Risk of Higher Blood Pressure in 3 to 6 Years Old Singleton Born From OHSS Patients Undergone With Fresh IVF/ICSI

*Yimin Zhu*[*†], *Yanling Fu*[†], *Minyue Tang*, *Huanmiao Yan*, *Fanghong Zhang*, *Xiaoling Hu*, *Guofang Feng*, *Yu Sun* and *Lanfeng Xing*

*Department of Reproductive Endocrinology, Women's Hospital, School of Medicine, Zhejiang University, Hangzhou, China*

**Background:** A large registry-based study found the increasing disorders of cardiovascular and metabolism in IVF children but underlying mechanism is still unknown. Few studies have investigated any association between OHSS and cardiovascular or metabolic function in subsequent children.

**Objective:** To evaluate the effect of ovarian hyperstimulation syndrome (OHSS) on blood pressure of singletons after *in vitro* fertilization (IVF) with or without intracytoplasmic sperm injection (ICSI).

**Study Design:** The singlet-center corhort study included 1780 singletons born with IVF/ICSI and 83 spontaneously conceived children from 2003 to 2014. Follow-up has lasted more than 10 years, and is still ongoing. This study analyzed data from follow-up surveys at 3 to 6 years of age.

**Participants, Setting and Methods:** We recruited 83 children (Group E) spontaneously conceived (SC) as control group and 1780 children born with IVF/ICSI including 126 children born to OHSS-fresh embryo transfer (ET) women (Group A), 1069 children born to non OHSS-ET women (Group B), 98 children conceived by women who developed into moderate or severe OHSS after oocyte retrieval and selected the frozen-thawed embryo transfer (FET) (Group C), 487 children conceived with non OHSS-FET (Group D). We evaluated cardiometabolic function, assessed BP in mmHg, heart rate, anthropometrics, and metabolic index including glucose, serum lipid (triglyceride, total cholesterol, low density lipoprotein, high density lipoprotein), thyroid function, of those children. The BP and heart rate were measured twice on the same day. We applied several multiple regression analyses to investigate the effect of OHSS in the early pregnancy.

**Main Findings:** By the single factor analysis, the SBP and DBP in the SC group (SBP: 99.84 ± 8.9; DBP: 55.27 ± 8.8) were significantly lower than OHSS-ET group's, while the blood pressure was similar between the SC group and other three ART groups. Children had higher BP in the OHSS-ET group (SBP: 101.93 ± 8.17; DBP: 58.75 ± 8.48) than in the non OHSS-ET (SBP: 99.49 ± 8.91; DBP: 56.55 ± 8.02) or OHSS-FET group (SBP: 99.38 ± 8.17; DBP: 55.72 ± 7.94). After using multiple regression analysis to adjust current, early life, parental and

OPEN ACCESS

***Edited by:***
*Marta Tesone, Universidad de Buenos Aires, Argentina*

***Reviewed by:***
*Mingru Yin, Shanghai Jiao Tong University, China*
*Haiyan Wang, Peking University Third Hospital, China*

***Correspondence:**
*Yimin Zhu*
*zhuyim@zju.edu.cn*

[†]*These authors share first authorship*

***Specialty section:***
*This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology*

***Received:*** *18 November 2021*
***Accepted:*** *30 May 2022*
***Published:*** *05 July 2022*

***Citation:***
*Zhu Y, Fu Y, Tang M, Yan H, Zhang F, Hu X, Feng G, Sun Y and Xing L (2022) Risk of Higher Blood Pressure in 3 to 6 Years Old Singleton Born From OHSS Patients Undergone With Fresh IVF/ICSI. Front. Endocrinol. 13:817555. doi: 10.3389/fendo.2022.817555*

ART characteristics, the differences in the SBP and DBP (B (95% confidence interval)) between OHSS-ET and non OHSS-ET remained significant (SBP: 3.193 (0.549 to 2.301); DBP: 3.440 (0.611 to 2.333)). And the BP showed no significant difference complementarily when compared non OHSS-FET group with non OHSS-ET group. In addition, the anthropometrics, fast glucose, serum lipid, and thyroid index did not differ among the ART groups.

**Principal Conclusions:** OHSS might play an independent key role on offspring's BP even cardiovascular function. Electing frozen-thawed embryo transfer for high risk of OHSS population may reduce the risk of the high BP trend.

**Wider Implications of the Findings:** It is a large sample study to investigate the effect of OHSS on offspring's health. These findings provide a clinic evidence of the impact of early environment (embryo even oocyte stage) on the offspring's cardiovascular health. Our study emphasis the importance of the accuracy of IVF clinic strategy and preventing the OHSS after fresh embryo transfer.

**Keywords: OHSS, art, child development, blood pressure, IVF (ICSI)**

## INTRODUCTION

Worldwide, assisted reproductive techniques (ARTs) are used increasingly for those infertile couples. In western industrialized countries, nowadays 1–6% of all newborn children are conceived with the help of IVF with or without ICSI (1). The long-term effect on the development and health of the IVF offspring has aroused people's attention.

Nowadays a large number of studies in the reproductive area focus on adult chronic diseases which are considered of the fetal origin in the ART offspring, such as cardiovascular diseases, overfat, diabetes, chronic kidney diseases and so on. The cardiovascular disease has still been the most frequently occurring disease, also the leading cause of death in the world. Poorer cardiometabolic outcome after IVF may be due to the higher rates of preterm birth and low birth weight (2), which are risk factors for hypertension and adiposity (3). However, previous studies indicated that poorer cardiometabolic outcome in IVF offspring could not be explained by these factors alone (4–9). As the proposal of the Baker theory (10), there is a shred of increasing study evidence suggesting that the early environment shapes an individual's health in later life. IVF might compromise the environment of the early embryo (11) as the exposure of high estradiol and progesterone level in the uterus.

As a serious and potentially life-threatening complication of IVF, ovarian hyperstimulation syndrome (OHSS) is characterized by elevated serum estradiol level, massive cystic ovarian enlargement and fluid shift from the intravascular compartment into the third space of the body (12), which has a globally incidence of 23.3% and 1.5% in different published reports (13). In cycles with fresh embryo transplantation, there is still a risk for the development of severe OHSS, which occurs in around 1–14% of IVF cycles (12, 14). OHSS, which shows high estrogen and progesterone during early pregnancy (15, 16) is considered as a good model to study the effects of ovarian stimulation and high sex hormone on the health and development of offspring. Till now far fewer studies have investigated the health and development of the offspring such as cardiometabolic characteristics. Of these studies, some indications of increased cardiovascular and cognitive dysfunction among OHSS-children need further investigation (17, 18).

The objective of this prospective study was to assess the impact of the OHSS in the early pregnancy on BP (Blood Pressure), anthropometrics and some metabolic functions of 3 to 6-year-old singletons. Our primary outcomes were systolic BP (SBP) and diastolic BP (DBP) in mmHg. Secondary outcomes were the measurements of heart rate, weight, standing height, BMI, fasting glucose, parameters of serum lipid metabolism (including total cholesterol (TG), triglyceride (TC), low density lipoprotein (LDL), high density lipoprotein (HDL)) and thyroid function.

## MATERIALS AND METHODS

### Study Design and Recruitments

For the ART population, this study is a corhort, longitudinal follow-up study of children born to subfertile couples. All the reproductive information of patients was extracted from the patient database of the reproductive center, Women's Hospital, School of Medicine, Zhejiang University. Between 2003 and 2014, subfertile women who had a successful pregnancy after undergoing IVF/ICSI at the reproductive center of the Women's Hospital, School of Medicine, Zhejiang University were included in the study. And we randomly recruited SC children born in the above hospital at the same time. **Exclusion criteria**: 1) ART characteristics: sperm or oocyte donation, preimplantation genetic diagnosis/screening (PGD/PGS); 2)

couple's characteristics: woman with hypertension or diabetes before pregnancy, male with a history of hypertension or diabetes, one member of the couple smoked or abused alcohol before pregnancy, one member of the couple had family history of hypertension or diabetes; 3) pregnancy and neonatal outcomes: multiple pregnancy, abortion, fetal loss, stillbirth, neonatal death or with serious diseases; 4) data missing in database. According to Golan and Wasserman's 2009 criteria (19), besides the abdominal distension, nausea and ovary enlarged, the criteria of moderate or severe OHSS should match at least one of the two following entries in our study: 1) ultrasonic or clinical evidence of ascites or hydrothorax or breathing difficulties; 2) blood volume changed, haemoconcentration, coagulation abnormalities, and renal dysfunction. Then women who satisfied with the moderate or severe OHSS criteria were accepted into the trial, while those who were with clinically diagnosed mild OHSS (only with the abdominal distension, nausea and ovary enlarged, without the above two performance) were excluded in case of interference. After exclusion and screening, the 1780 women were divided into two groups, one group was ET group (n=1195) and the other group was FET group (n=585) on the basis of fresh transplantation or frozen-thawed embryo transfer. According to the criteria we divided those women in the trial into four subgroups: 1) Group A (OHSS-ET, n=126): women who suffered moderate or severe OHSS in their early pregnancy after fresh ET; 2) Group B (non OHSS-ET, n=1069): women without obvious OHSS in their cycle of fresh ET; 3) Group C (OHSS-FET, n=98): women who followed the adoption of the freeze-all strategy in case of the high risk of OHSS while ultimately developed into moderate or severe OHSS after oocyte retrieval; 4) Group D (non OHSS-FET, n=487): women without OHSS after oocyte retrieval and accepting elective FET due to various reasons. Until the study ends, 83 mother and their spontaneously conceived singletons who fulfill the criteria were recruited in the Group E (SC, n=83).

The study investigates the independent effects of ovarian hyperstimulation syndrome on the offspring's health and development especially the BP level and metabolic function.

Parents gave written informed consent and the study design was approved by the Ethics Committee of the Women's Hospital, School of Medicine, Zhejiang University.

## Follow-Up Examination

Between 2006 and 2017, we contacted the women whose children were at 3 to 6 years by telephone and invited them to the Reproductive Follow-up clinic of the Women's Hospital, School of Medicine, Zhejiang University to participate the follow-up assessments. Follow-up work were carryied out on every working day. The trained assessors were blinded to the mode of conception. Each child was requested to come with an empty stomach for the peripheral blood examination in the morning and avoid acute exacerbations of childhood illness including respiratory or intestinal diseases. After a brief introduction on the test to be performed (blood examination, cognitive, cardiovascular and anthropometric assessment), BP and heart rate were measured once. The BP measurement on the non-dominant arm, while the child was seated, was taken with an appropriate cuff size. Then the child would do the peripheral blood examination including the blood routine, blood biochemistry (covering fasting blood glucose, blood fat, hepatorenal function), thyroid function, hepatitis B-virus and trace element examination. It is necessary for the child to have breakfast after finishing the blood examination. Next, BP was measured for the second time. Finally, anthropometric data were collected. In total, BP and heart rate were measured twice. The two readings were averaged to obtain the BP data in mmHg and heart rate in beats/min. In the present paper, cardiovascular, anthropometric outcomes and a part of metabolic function index are reported.

## Statistical Analysis

Group differences in background variables and outcome measures were investigated with Student t-test, Mann-Whitney U tests, Pearson's Chi-square test when appropriate. In case significant differences were found between two groups, Student's t-tests and Mann-Whitney U-tests were used to specify the pair-wise differences. At the same time, it was a coincidence between the children's mother and father in their age in our analysis. Thus, in this study, we did not put father's age into the multivariable regression analysis.

We performed multivariable linear regression analyses to explore potential differences in BP between the OHSS and non OHSS groups while correcting for possible confounders. In line with other studies in the field (7, 20, 21), the mean differences were adjusted to control for certain confounders according to different models with the linear regression analysis. Models separately were performed for current risk indicators (Weight, gender, age, BMI, pulse and TSH), for early life factors (preterm birth, birth weight, cesarean section), for parental characteristics (maternal age, maternal pre-pregnancy BMI, pregnancy-induced hypertension, gestational diabetes, PCOS), for IVF characteristics (gonadotropin dosage, type of IVF) and finally for all current, early life, parental and IVF variables. Results were expressed as unstandardized regression coefficients (B) with their 95% confidence intervals (95% CI). The analyses were performed using the IBM Statistical Package for the Social Sciences version 23. Probability values of 0.05 were considered statistically significant.

# RESULTS

## Demographics/Maternal Background Data and Subfertility, ART Characteristics

It was shown in **Table 1** of the data of Parental, subfertility and ART characteristics. Most basal characteristics were similar among five or four groups, but several difference were found, like the maternal pregnancy age, the basal FSH or LH levels, estradiol or progesterone level on hCG day, the incidence of PCOS, dosage of GN. Maternal pregnancy age seemed older in the non OHSS-ET group than other four groups. When referring

**TABLE 1** | Demographic data on mothers, subfertility and ART characteristics.

| Characteristic | Group A (OHSS-ET) (N = 126) | Group B (non OHSS-ET) (N = 1069) | Group C (OHSS-FET) (N = 98) | Group D (non OHSS-FET) (N = 487) | Group E (SC group) (N = 83) | P values |
|---|---|---|---|---|---|---|
| Maternal characteristics | | | | | | |
| Pregnancy age (yr) | 29.5 (3.8) | 31.0 (3.7) | 28.9 (4.0) | 30.1 (4.0) | 28.07 (3.3) | **<0.001** |
| BMI before pregnancy[a] ($kg/m^2$) | 21.7 (1.0) | 21.6 (1.1) | 21.5 (0.8) | 21.4 (1.3) | 21.6 (1.2) | 0.178 |
| Basal sexual hormone levels[b] | | | | | | |
| FSH (IU/L) | 6.2 (1.4) | 6.9 (2.2) | 5.8 (1.3) | 6.4 (2.4) | NA | **<0.001** |
| LH (IU/L) | 5.4 (3.1) | 4.9 (2.6) | 6.6 (3.3) | 4.7 (2.6) | NA | **0.010** |
| Estradiol (pmol/L) | 123.1 (62.1) | 177.9 (67.8) | 199.2 (69.0) | 127.4 (98.0) | NA | 0.862 |
| Hormone level on hCG administration day | | | | | | |
| Estradiol (pmol/L) | 17810 (6825) | 11196 (6104) | 29129 (8900) | 13787 (6556) | NA | **<0.001** |
| Progesterone (nmol/L) | 2.9 (1.2) | 2.6 (2.2) | 4.3 (2.2) | 2.5 (1.3) | NA | **<0.001** |
| Fertility parameters | | | | | | |
| Infertility duration (yr) | 3.0 (1.0-9.0) | 4.0 (0.5-14.0) | 3.8 (1.0-10.0) | 4.1 (1.0-15.0) | NA | 0.556 |
| PCOS[c], n (%) | 10 (7.9) | 47 (4.4) | 13 (13.3)shi | 31 (6.4) | NA | **0.001** |
| ART characteristics | | | | | | |
| Gn dosage[d] (IU) | 1830 (640) | 2273 (897) | 1916 (678) | 2160 (919) | NA | **<0.001** |
| Spermatozoa origin (TESA/PESA)[e], n (%) | 9 (7.1) | 66 (6.2) | 6 (6.9) | 22 (4.6) | NA | 0.383 |
| ICSI, n (%) | 32 (25.4) | 281 (26.3) | 24 (24.5) | 118 (24.2) | NA | 0.847 |

*P values were calculated by one-way ANOVA test, Chi-square test.*

*Statistically significant results (P value<0.05) are displayed in bold numbers. Data represent mean (standard deviation), percentages, or median (range).*

[a]*Body mass index was defined as weight divided by height squared.*

[b]*The hormone level between the Day1 to Day 3 of the menstrual cycle.*

[c]*Polycystic ovary syndrome, diagnosed by the Rotterdam criteria.*

[d]*The dose of gonadotrophin during the controlled ovarian hyperstimulation.*

[e]*Testicular Sperm Aspiration/percutaneous epididymal sperm aspiration. NA, Not Available.*

to maternal hormone level, non OHSS-ET group had higher basal FSH and lower basal LH level, lower estradiol level on hCG day. The dosage of gonadotropin was lower in OHSS-ET group as compared with other three groups.

## Perinatal Characteristics and Blood Pressure, Anthropometrics, Metabolic Function of Offspring

**Tables 2**, **3** showed an overview of all outcome measures for the five groups. As to the obstetric complications, there was no significant difference among groups in the incidence of complications such as gestational hypertension, gestational diabetes. The cesarean section was more often performed in the ART groups than the SC group. Children born to non OHSS-FET had a higher weight, BMI than other groups. SBP, DBP and heart rate were differ among five groups. Metabolic index including fasting blood glucose, serum lipid, and thyroid function seemed no significantly difference.

A single factor analysis was performed for the SC with ART groups. The BP in the SC group was lower than the OHSS-ET group while similar with other 3 groups, which was showed in the **Table 4**.

## Multiple Regression Analysis on the Blood Pressure of 3 to 6-Year-Old Singletons

Subsequently, we performed multiple linear regression analysis of SBP and DBP among the ART groups in mmHg showed in **Table 5**. Children born following OHSS-ET had higher SBP and DBP in mmHg than children born following non OHSS-ET and OHSS-FET, also after correction for various sets of variables. While in the comparison of non OHSS-FET with non OHSS-ET, the blood pressure showed no obvious and unstable differences after adjusting those concomitant variables.

# COMMENT

It is a large sample and prospective study to investigate the effect of OHSS on offspring's health. Evidence is accumulating in animals and humans that ART alters the cardiovascular phenotype. For example, there is evidence in normal mice that ART causes premature vascular aging and arterial hypertension that is related to an epigenetic mechanism and associated with a shortened life span (22). In line with these findings, studies demonstrate that ART has shown to cause morphological alteration of the vascular in the systemic circulation (9) and increase arterial BP (7, 20) in apparently healthy ART children. Although variety studies demonstrating ART-induced alterations of the offspring phenotype, it is lack of the researches on a large sample or adjusting multiple relative factors like ours. This study showed that children born from OHSS mothers might have higher BP than SC children or non-OHSS children. After adjusting other risk factors, OHSS-ET children also showed significantly higher blood pressure when compared with non OHSS-ET children. Meanwhile, to our knowledge, it's the first follow-up study that set the OHSS-FET and non OHSS-FET as the control group to confirm the benefits of freeze-all strategy for those at high risk OHSS after oocyte

**TABLE 2 |** Demographic data on children and perinatal characteristics.

| Characteristic | Group A (OHSS-ET) (N = 126) | Group B (non OHSS-ET) (N = 1069) | Group C (OHSS-FET) (N = 98) | Group D (non OHSS-FET) (N = 487) | Group E (SC group) (N = 83) | P values |
|---|---|---|---|---|---|---|
| Gestational characteristics | | | | | | |
| Gestational diabetes, n (%) | 9 (7.1) | 95 (8.9) | 9 (9.2) | 48 (9.9) | 7 (8.4) | 0.907 |
| Pregnancy-induced hypertension, n (%) | 4 (3.2) | 73 (6.8) | 10 (10.2) | 33 (6.8) | 3 (3.6) | 0.123 |
| Preeclampsia, n (%) | 3 (2.4) | 70 (6.5) | 9 (9.2) | 32 (6.6) | 1 (1.2) | 0.072 |
| Perinatal characteristics | | | | | | |
| Preterm birth (<37week), n (%) | 11 (8.7) | 120 (11.2) | 9 (9.2) | 52 (10.7) | 6 (7.3) | 0.728 |
| Birth weight (g) | 3306 (525) | 3252 (543) | 3388 (424) | 3530 (373) | 3305 (653) | 0.915 |
| Cesarean section, n (%) | 93 (73.8) | 923 (86.3) | 81 (82.7) | 444 (91.2) | 44 (53.0) | **<0.001** |
| Children characteristics | | | | | | |
| Age (yr) | 4.1 (0.3) | 4.1 (0.4) | 4.1 (0.4) | 4.1 (0.2) | 4.3 (0.6) | 0.357 |
| Male gender, n (%) | 70 (55.6) | 549 (51.4) | 44 (44.9) | 251 (51.5) | 50 (60.2) | 0.283 |
| Standing Height (cm) | 109.0 (99.0-116.0) | 109.0 (94.3-130.0) | 108.4 (102.0-116.2) | 110.8 (102.0-120.0) | 109.0 (97.5-124.5) | 0.241 |
| Weight (kg) | 17.6 (14.4-28.0) | 18.0 (12.7-31.8) | 18.4 (15.3-22.0) | 19.6 (15.2-28.5) | 17.8 (14.3-29.0) | **0.040** |
| BMI[a] ($kg/m^2$) | 15.0 (13.3-20.8) | 15.1 (12.4-23.2) | 15.6 (13.9-19.4) | 16.0 (13.1-22.1) | 14.9 (12.6-19.9) | **0.023** |
| BMI>18$kg/m^2$, n(%) | 11 (8.7) | 65 (6.1) | 5 (5.1) | 45 (9.2) | 4 (4.8) | 0.142 |

*P values were calculated by one-way ANOVA test, Chi-square test. Statistically significant results (P value<0.05) are displayed in bold numbers. Data represent mean (standard deviation), percentages, or median (range).*

[a]*Body mass index was defined as weight divided by height squared.*

**TABLE 3 |** Blood pressure (in mmHg) and metabolic function for 3 to 6-year-old singletons born after IVF/ICSI with OHSS-ET (Group A), non OHSS-ET (Group B), OHSS-FET (Group C) or non OHSS-FET (Group D) and SC (Group E).

| Characteristic | Group A (OHSS-ET) (N = 126) | Group B (non OHSS-ET) (N = 1069) | Group C (OHSS-FET) (N = 98) | Group D (non OHSS-FET) (N = 487) | Group E (SC group) (N = 83) | P values |
|---|---|---|---|---|---|---|
| Blood pressure, pulse pressure, and heart rate | | | | | | |
| SBP (mm Hg) | 101.93 (8.17) | 99.49 (8.91) | 99.38 (8.17) | 99.56 (8.50) | 99.84 (8.9) | **0.048** |
| DBP (mm Hg) | 58.75 (8.48) | 56.55 (8.02) | 55.72 (7.94) | 56.14 (7.77) | 55.27 (8.8) | **0.008** |
| SBP>110mmHg, n(%) | 20 (15.9) | 117 (10.9) | 9 (9.2) | 45 (9.2) | 6 (7.2) | 0.199 |
| DBP>70mmHg, n(%) | 7 (5.6) | 16 (1.5) | 6 (6.1) | 4 (0.8) | 3 (3.6) | **<0.001** |
| Pulse pressure (mm Hg) | 42.10 (8.60) | 43.12 (9.33) | 42.06 (8.15) | 42.74 (9.50) | 44.58 (8.88) | 0.481 |
| Heart rate (beats/min) | 95.23 (69-124) | 97.17 (70-126) | 92.29 (69-110) | 99.66 (75-122) | 93.98 (70-122) | **0.004** |
| Fasting blood-glucose (mmol/L) | 4.95 (0.52) | 4.87 (0.39) | 4.85 (0.41) | 4.85 (0.43) | 4.84 (0.4) | 0.428 |
| Serum lipid | | | | | | |
| Triglyceride (mmol/L) | 0.65 (0.50-1.62) | 0.69 (0.35-3.34) | 0.61 (0.36-0.84) | 0.70 (0.36-1.70) | 0.66 (0.36-1.53) | 0.065 |
| Total cholesterol (mmol/L) | 4.49 (1.12) | 4.29 (0.76) | 4.29 (0.62) | 4.25 (0.72) | 4.34 (0.73) | 0.847 |
| LDL (mmol/L) | 2.40 (0.94) | 2.23 (0.60) | 2.05 (0.46) | 2.16 (0.58) | 2.30 (0.61) | 0.826 |
| HDL (mmol/L) | 1.42 (0.32) | 1.44 (0.30) | 1.51 (0.26) | 1.41 (0.32) | 1.40 (0.28) | **0.049** |
| Uric acid (μmol/L) | 265.55 (58.09) | 251.58 (51.58) | 261.00 (54.17) | 266.72 (49.47) | 262.90 (56.72) | 0.532 |
| TSH (mIU/L) | 1.98 (1.23-4.84) | 2.23 (0.32-8.41) | 2.32 (1.00-5.73) | 2.17 (0.72-4.91) | 2.21 (0.86-7.71) | 0.063 |

*P values were calculated by one-way ANOVA test, Chi-square test. Statistically significant results are displayed in bold numbers. Data represent mean (standard deviation), percentages, or median (range).*

*SBP, systolic blood pressure; DBP, diastolic blood pressure; Pulse pressure: SBP minus DBP; LDL, low density lipoprotein; HDL, high density lipoprotein; TSH, thyrotropin hormone.*

**TABLE 4** | Comparison the BP of SC (group E) and ART group.

| Covariate | Reference | SBP mean difference (95% CI) unadjusted | P value | DBP mean difference (95% CI) unadjusted | P value |
|---|---|---|---|---|---|
| SC (E, n = 83) | OHSS-ET (A, n = 126) | -2.097 (-4.930 to -0.090) | **0.042** | -3.481 (-5.700 to -1.260) | **0.002** |
| | non OHSS-ET (B, n = 1069) | -0.064 (-2.010 to 1.890) | 0.949 | -1.284 (-3.080 to 0.510) | 0.160 |
| | OHSS-FET (C, n = 98) | 0.044 (-2.510 to 2.600) | 0.973 | 0.701 (-2.810 to 1.890) | 0.701 |
| | non OHSS-FET (D, n = 487) | -0.137 (-2.170 to 1.890) | 0.895 | -0.873 (-2.740 to 0.990) | 0.360 |

*P value were calculated by one-way ANOVA test.*
*Statistically significant results are displayed in bold numbers.*

retrieval and their offspring. The BP displayed no obvious differences when compared SC with non-OHSS ET/FET, non OHSS-FET with non OHSS-ET. Through setting multiple control groups and preforming multiple regression analysis, our study indicated that OHSS in early pregnancy might play an impendent key role on offspring's BP even cardiovascular function. As we known, an OHSS pregnancy compared with a spontaneously conceived pregnancy showed high estrogen and progesterone in early pregnancy (16). To some extent, it indicates that higher estrogen and progesterone probably influence the offspring's cardiovascular. In the present study, we found that 4-year-old IVF children born after ovarian stimulation have higher blood pressure (23). OHSS children showed an alteration of cardiovascular functions, which may be related to the mother's super-physiological dose of estrogen and progesterone (17). To date, the association between prenatal exposure of high estradiol/progesterone and cardiovascular changes of offspring is still unclear. Also, the angiotensin II was significantly increased in the ascites of OHSS pregnancy (24). Angiotensin II is been considered as a consensus cardiovascular factor. The mechanism under those researches needs further data support and experimental research.

From a clinical point of view, it may seem like a tiny gap that OHSS children have 2 to 4-mm Hg higher systolic and diastolic blood pressure than non OHSS children. And researches shows the prevalence of hypertension in children is low: for example, a study conducted in Switzerland suggested a prevalence of only 2 % (25). However, a slight increase in blood pressure may significantly raise the risk of future cardiovascular disease. Hypertension is a major risk factor for coronary heart disease, peripheral artery occlusive disease, stroke, and even chronic kidney disease (26–29). For instance, lowering mean systolic blood pressure in adults by 2 mm Hg corresponds to an 8% reduction in the risk of stroke. Furthermore, it cannot be excluded that increased blood pressure after IVF may be amplified throughout life because blood pressure is known to track from childhood into adult life (30, 31).

Existing research shows that use of hCG to trigger ovulation or luteal support was significantly associated with early-onset OHSS (32–34). Elevate endogenous hCG in early pregnancy is associated with late-onset OHSS. Selective embryo freezing is a routine method

**TABLE 5** | Multiple linear regression analysis of the effect of OHSS on blood pressure.

| Model | Covariate | Reference | SBP mean difference (95% CI) unadjusted | P value | DBP mean difference (95% CI) unadjusted | P value |
|---|---|---|---|---|---|---|
| Unadjusted | OHSS-ET (A) | non OHSS-ET (B) | 2.443 (0.830 to 4.050) | **0.003** | 2.197 (0.720 to 3.680) | **0.004** |
| | | OHSS-FET (C) | 2.551 (0.250 to 4.850) | **0.021** | 3.022 (0.900 to 5.140) | **0005** |
| | non OHSS-FET (D) | non OHSS-ET (B) | 0.073 (-0.860 to 1.010) | 0.878 | -0.412 (-1.263 to 0.440) | 0.348 |
| Current risk indicators | OHSS-ET (A) | non OHSS-ET (B) | 3.306 (0.574 to 2.249) | **0.001** | 3.260 (0.507 to 2.038) | **0.001** |
| | | OHSS-FET (C) | 2.142 (0.185 to 4.450) | **0.033** | 2.889 (1.006 to 5.330) | **0.004** |
| | non OHSS-FET (D) | non OHSS-ET (B) | -0.369 (-1.268 to 0.531) | 0.421 | -0.838 (-1.651 to -0.025) | 0.043 |
| Early life factors | OHSS-ET (A) | non OHSS-ET (B) | 3.046 (0.463 to 2.139) | **0.002** | 3.077 (0.435 to 1.966) | **0.002** |
| | | OHSS-FET (C) | 2.339 (0.407 to 4.776) | **0.020** | 2.872 (1.018 to 5.470) | **0.004** |
| | non OHSS-FET (D) | non OHSS-ET (B) | 0.070 (-0.875 to 1.015) | 0.885 | -0.420 (-1.274 to 0.434) | 0.335 |
| Parental characteristic | OHSS-ET (A) | non OHSS-ET (B) | 3.178 (0.521 to 2.201) | **0.002** | 3.251 (0.506 to 2.045) | **0.001** |
| | | OHSS-FET (C) | 2.210 (0.370 to 6.604) | **0.029** | 1.787 (-0.307 to 6.103) | 0.076 |
| | non OHSS-FET (D) | non OHSS-ET (B) | -0.202 (-1.686 to 1.282) | 0.789 | -0.429 (-1.761 to 0.904) | 0.528 |
| IVF characteristic | OHSS-ET (A) | non OHSS-ET (B) | 2.910 (0.415 to 2.132) | **0.004** | 3.028 (0.429 to 2.010) | **0.003** |
| | | OHSS-FET (C) | 2.420 (0.726 to 7.198) | **0.017** | 1.293 (-1.137 to 5.442) | 0.198 |
| | non OHSS-FET (D) | non OHSS-ET (B) | -0.317 (-1.835 to 1,201) | 0.682 | -0.649 (-2.020 to 0.722) | 0.353 |
| All above | OHSS-ET (A) | non OHSS-ET (B) | 3.193 (0.549 to 2.301) | **0.001** | 3.440 (0.611 to 2.233) | **0.001** |
| | | OHSS-FET (C) | 2.147 (0.290 to 7.271) | **0.034** | 0.747 (-2.269 to 5.012) | 0.457 |
| | non OHSS-FET (D) | non OHSS-ET (B) | -1.187 (-2.674 to 0.299) | 0.117 | -1.213 (-2.572 to 0.145) | 0.080 |

*Data is represented by the mean difference (unadjusted 95% confidence interval). P values were calculated by multiple regression analysis. Statistically significant results (P value<0.05) are displayed in bold numbers.*
*SBP, systolic blood pressure; DBP, diastolic blood pressure.*
*Current risk indicators: adjust standing height, weight, gender, age, BMI, pulse, and TSH of the child for the BP analysis.*
*Early life factors: adjust preterm birth, birthweight, and cesarean section.*
*Parental characteristics: adjust maternal age, maternal BMI before pregnancy, pregnancy-induced hypertension, gestational diabetes, PCOS, infertility duration and type of infertility for the BP analysis.*
*IVF characteristic: adjust for the dosage of gonadotrophin, ICSI, Spermatozoa origin.*

for preventing late-onset OHSS by avoiding continued exposure to hCG during the luteal phase, avoiding cycle cancellation, and guaranteeing cumulative pregnancy rates. Current clinical pregnancy rates for FET cycles are comparable to fresh embryo transfer cycles (35). Although selective embryo freezing cannot completely avoid the occurrence of early-onset OHSS, it can more effectively reduce the incidence of early-onset OHSS and avoid the occurrence of late-onset OHSS compared with other methods. Therefore, the establishment of the FET group in our study has certain practical significance.

The purpose of this study was primarily to focus on the health of ART offspring. Therefore, the follow-up nodes set up cover almost the entire period of children, including birth, infancy, preschool, school, and adolescence. Follow-up studies have been ongoing for more than 10 years and are continuing. This sub-project study focuses on the cardiovascular and metabolic changes of the offspring. The starting point of the first study is in the preschool age (3 to 6 years old). And a series of follow-up studies will be carried out in the future, aiming to discover the dynamic changes of the relevant indicators throughout childhood and even adulthood through very early follow-up examinations.

When interpreting our study, the limitations of our study also need to be considered. In our study, BP was measured in office twice on one day and the mean of the two measurements was used for analysis. This may deviate the final blood pressure value from the actual value and miss differences between groups. Similarly, we found other published studies in the area also have the difficulty to measure the BP multiple times. Studies evaluating the effects of reproductive technologies most opted to measure BP on a single day (7, 8, 20). Using gold standard assessment (24h-ambulatory blood pressure measurements, ABPM) of arterial blood pressure, evidence for increased blood pressure in young adult ART participants have been presented at the ESC meeting (36). Such measurements, in addition to settling the issue of ART-induced hypertension in humans, also would provide important information on additional independent predictors of cardiovascular risk (i.e. night-time dipping, blood pressure variability). In future research, we will consider using ABPM in the hope of getting more accurate cardiovascular data.

There is abundant evidence that essential hypertension is a highly heritable condition. Although at the beginning of the research, we had excluding the couple who had the history of hypertension, diabetes, and family history of those diseases, we did not collect the basic blood pressure indicators of both husband and wife. It, therefore, appears possible that BP in the parents of Group A is higher than in the parents of the other groups and that they transmit this trait to the offspring. Additionally, although the pregnancy-induced hypertension is as a well-established cardiovascular risk factor in naturally conceived humans, the effect of the ART combined with pregnancy-induced hypertension on the blood pressure of the offspring was not studied separately in this research. We put the pregnancy-induced hypertension as a factor into the multiple regression analysis that showed pregnancy-induced hypertension also affect offspring blood pressure (data was not labeled).

In addition, there were some differences in the baseline data among these groups, which may seem to affect the outcomes. First women in the SC group seemed much younger than the ART group. Since the SC group is a randomly selected voluntary participant who meets the criteria, the age of infertile patients is generally larger than that of non-infertile patients in the clinical status. In the OHSS group, the basal LH value of the mother was higher than that in the control group, and the basal FSH value was also increased in the non OHSS group. It was consistent with clinical that the ovarian function of the OHSS patients was superior to that of the non-OHSS women. It can also explain the result that mothers in the OHSS group were younger than those in the control group, as the ovarian function is known to decrease linearly with age. At the same time, the higher total dose of Gn during ovulation induction in the OHSS group can be fully explained from a clinical point of view. Out of clinician's experience, younger infertile women of better ovarian function often take the ovulation induction programs in low doses to avoid OHSS although eventually developed into OHSS. In our study, we took multivariable regression analyses to assess the outcome to control these confounders. In another way, it also reflects the reliability and authenticity of the data in this study. In addition, we also did a propensity matching analysis for Group A (OHSS-ET) and Group B (non OHSS-ET). Although the sample size after matching (maternal age, basal FSH and trigger day estradiol levels) was only 76 cases in each group, the final univariate analysis showed that Children had higher BP in the OHSS-ET group than in the non OHSS-ET (SBP: 101.79 ± 8.02 vs. 98.63 ± 8.34, P=0.019; DBP: 59.87 ± 8.18 vs. 56.18 ± 7.47, P=0.004). There were no significant differences in other metabolic indicators between the two groups.

The study also included IVF and ICSI children. As it is unknown whether IVF and ICSI share the same cardiometabolic risk for offspring (20), we performed a linear regression analysis. Indeed, several studies suggested cardiometabolic alterations in IVF/ICSI offspring (5, 6, 8, 9).

It can be found that the cesarean section rate in our study was extremely high. The main reason was that the offspring conceived through IVF/ICSI was viewed as "precious child". Most parents view cesarean section as a safer way to deliver. There is also a focus on having a "perfect baby" under the "one child policy" in the past (37).

In conclusion, the results of the present study suggest that OHSS in the early pregnancy is associated with higher blood pressure in mmHg including systolic blood pressure and diastolic blood pressure in 3 to 6-year-old offspring. While electing frozen-thawed embryo transfer for high risk of OHSS population may reduce the risk of that high BP trend. In addition, we found no significant evidence for an adverse effect of OHSS on anthropometrics and metabolic function. Future research is needed to confirm the role of OHSS or early environment for oocyte and embryo in a poorer cardiometabolic outcome and investigate the underlying mechanisms. Our findings emphasize the importance of the accuracy of the IVF clinic strategy and preventing the OHSS after fresh embryo transfer, calling for the cardiovascular monitoring of the growing number of children conceived with IVF worldwide.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Women's Hospital, School of Medicine, Zhejiang University. The participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

All authors fulfil the criteria for authorship; YZ initiated the study, YF, MT and HY collected the data. YF interpreted and analysed the data, who finally drafted the report. All authors commented on the drafts, and have seen and approved the final version.

## FUNDING

The study was supported by the Ministry of Health of PRC Science Foundation (NO. WKJ-ZJ-1522), the Natural Science Foundation of Zhejiang Province (No. LZ15H040001), the National science & Technology Pillar Program during the 13th Five-year Plan Period (NO. 2016YFC1000302), and the Zhejiang Provincial & Ministry of Health Research Fund For Medical Sciences WKJ-ZJ-1722, and the Program for Key Subjects of Zhejiang Province in Medicine & Hygiene. The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

## ACKNOWLEDGMENTS

We thank the parents and children who participated in the study. We are grateful to Professor Yu Yunxian for his help in statistics.

## REFERENCES

1. European IVF-Monitoring Consortium (EIM) for the European Society of Human Reproduction and Embryology (ESHRE), Calhaz-Jorge C, de Geyter C, Kupka MS, de Mouzon J, Erb K, et al. Assisted Reproductive Technology in Europe, 2012: Results Generated From European Registers by ESHRE. *Hum Reprod* (2016) 31:1638–52. doi: 10.1093/humrep/dew151
2. Helmerhorst FM, Perquin DA, Donker D, Keirse MJ. Perinatal Outcome of Singletons and Twins After Assisted Conception: A Systematic Review of Controlled Studies. *Bmj* (2004) 328:261. doi: 10.1136/bmj.37957.560278.EE
3. Wells JC, Chomtho S, Fewtrell MS. Programming of Body Composition by Early Growth and Nutrition. *Proc Nutr Soc* (2007) 66:423–34. doi: 10.1017/S0029665107005691
4. Belva F, Henriet S, Liebaers I, Van Steirteghem A, Celestin-Westreich S, Bonduelle M. Medical Outcome of 8-Year-Old Singleton ICSI Children (Born >or=32 Weeks' Gestation) and a Spontaneously Conceived Comparison Group. *Hum Reprod* (2007) 22:506–15. doi: 10.1093/humrep/del372
5. Belva F, Painter R, Bonduelle M, Roelants M, Devroey P, De Schepper J. Are ICSI Adolescents at Risk for Increased Adiposity? *Hum Reprod* (2012) 27:257–64. doi: 10.1093/humrep/der375
6. Ceelen M, van Weissenbruch MM, Roos JC, Vermeiden JP, van Leeuwen FE, Delemarre-van deWaal HA. Body Composition in Children and Adolescents Born After *In Vitro* Fertilization or Spontaneous Conception. *J Clin Endocrinol Metab* (2007) 92:3417–23. doi: 10.1210/jc.2006-2896
7. Ceelen M, van Weissenbruch MM, Vermeiden JP, van Leeuwen FE, Delemarre-van deWaal HA. Cardiometabolic Differences in Children Born After *In Vitro* Fertilization: Follow-Up Study. *J Clin Endocrinol Metab* (2008) 93:1682–8. doi: 10.1210/jc.2007-2432
8. Sakka SD, Loutradis D, Kanaka-Gantenbein C, Margeli A, Papastamataki M, Papassotiriou I, et al. Absence of Insulin Resistance and Low-Grade Inflammation Despite Early Metabolic Syndrome Manifestations in Children Born After *In Vitro* Fertilization. *Fertil Steril* (2010) 94:1693–9. doi: 10.1016/j.fertnstert.2009.09.049
9. Scherrer U, Rimoldi SF, Rexhaj E, Stuber T, Duplain H, Garcin S, et al. Systemic and Pulmonary Vascular Dysfunction in Children Conceived by Assisted Reproductive Technologies. *Circulation* (2012) 125:1890–6. doi: 10.1161/CIRCULATIONAHA.111.071183
10. Barker DJ. The Fetal and Infant Origins of Disease. *Eur J Clin Invest* (1995) 25:457–63. doi: 10.1111/j.1365-2362.1995.tb01730.x
11. Watkins AJ, Fleming TP. Blastocyst Environment and its Influence on Offspring Cardiovascular Health: The Heart of the Matter. *J Anat* (2009) 215:52–9. doi: 10.1111/j.1469-7580.2008.01033.x
12. Nastri CO, Ferriani RA, Rocha IA, Martins WP. Ovarian Hyperstimulation Syndrome: Pathophysiology and Prevention. *J Assist Reprod Genet* (2010) 27:121–8. doi: 10.1007/s10815-010-9387-6
13. Golan A, Ron-el R, Herman A, Soffer Y, Weinraub Z, Caspi E. Ovarian Hyperstimulation Syndrome: An Update Review. *Obstet Gynecol Surv* (1989) 44:430–40. doi: 10.1097/00006254-198906000-00004
14. Youssef MAFM, van Wely M, Hassan MA, et al. Can Dopamine Agonists Reduce the Incidence and Severity of OHSS in IVF/ICSI Treatment Cycles? *A Syst Rev Meta-analysis. Human Reprod Update* (2010) 16:459–66. doi: 10.1093/humupd/dmq006
15. Delvigne A, Rozenberg S. Epidemiology and Prevention of Ovarian Hyperstimulation Syndrome (OHSS): A Review. *Hum Reprod Update* (2002) 8:559–77. doi: 10.1093/humupd/8.6.559
16. Kosaka K, Fujiwara H, Yoshioka S, Fujii S. Vascular Endothelial Growth Factor Production by Circulating Immune Cells is Elevated in Ovarian Hyperstimulation Syndrome. *Hum Reprod* (2007) 22:1647–51. doi: 10.1093/humrep/dem042
17. Xu GF, Zhang JY, Pan HT, Tian S, Liu ME, Yu TT, et al. Cardiovascular Dysfunction in Offspring of Ovarian-Hyperstimulated Women and Effects of Estradiol and Progesterone: A Retrospective Cohort Study and Proteomics Analysis. *J Clin Endocr Metab* (2014) 99:E2494–E503. doi: 10.1210/jc.2014-2349
18. Xu GF, Zhou CL, Xiong YM, Li JY, Yu TT, Tian S, et al. Reduced Intellectual Ability in Offspring of Ovarian Hyperstimulation Syndrome: A Cohort Study. *EBioMedicine* (2017) 20:263–7. doi: 10.1016/j.ebiom.2017.05.020
19. Golan A, Weissman A. Symposium: Update on Prediction and Management of OHSS. *A Mod Classif OHSS Reproductive Biomed Online* (2009) 19:28–32. doi: 10.1016/S1472-6483(10)60042-9
20. Belva F, Roelants M, De Schepper J, Roseboom TJ, Bonduelle M, Devroey P, et al. Blood Pressure in ICSI-Conceived Adolescents. *Hum Reprod* (2012) 27:3100–8. doi: 10.1093/humrep/des259
21. Seggers J, Haadsma ML, La Bastide-VanGemert S, Heineman MJ, Middelburg KJ, Roseboom TJ, et al. Is Ovarian Hyperstimulation Associated With Higher Blood Pressure in 4-Year-Old IVF Offspring? *Part I: Multivariable Regression Anal Human Reprod* (2014) 29:502–9. doi: 10.1093/humrep/det396

22. Rexhaj E, Paoloni-Giacobino A, Rimoldi SF, Fuster DG, Anderegg M, Somm E, et al. Mice Generated by *In Vitro* Fertilization Exhibit Vascular Dysfunction and Shortened Life Span. *J Clin Invest* (2013) 123:5052–60. doi: 10.1172/JCI68943
23. La Bastide-Van Gemert S, Seggers J, Haadsma ML, Heineman MJ, Middelburg KJ, Roseboom TJ, et al. Is Ovarian Hyperstimulation Associated With Higher Blood Pressure in 4-Year-Old IVF Offspring? Part II: An Explorative Causal Inference Approach. *Hum Reprod* (2014) 29:510–7. doi: 10.1093/humrep/det448
24. Delbaere A, Bergmann PJ, Gervy-Decoster C, Deschodt-Lanckman M, de Maertelaer V, Staroukine M, et al. Increased Angiotensin II in Ascites During Severe Ovarian Hyperstimulation Syndrome: Role of Early Pregnancy and Ovarian Gonadotropin Stimulation. *Fertil Steril* (1997) 67:1038–45. doi: 10.1016/S0015-0282(97)81436-7
25. Chiolero A, Cachat F, Burnier M, Paccaud F, Bovet P. Prevalence of Hypertension in Schoolchildren Based on Repeated Measurements and Association With Overweight. *J Hypertens* (2007) 25:2209–17. doi: 10.1097/HJH.0b013e3282ef48b2
26. MacMahon S, Peto R, Cutler J, Collins R, Sorlie P, Neaton J, Abbott R, et al. Blood Pressure, Stroke, and Coronary Heart Disease. Part 1, Prolonged Differences in Blood Pressure: Prospective Observational Studies Corrected for the Regression Dilution Bias. *Lancet* (1990) 335:765–74. doi: 10.1016/0140-6736(90)90878-9
27. Klag MJ, Whelton PK, Randall BL, Neaton JD, Brancati FL, Ford CE, et al. Blood Pressure and End-Stage Renal Disease in Men. *New Engl J Med* (1996) 334:13–8. doi: 10.1056/NEJM199601043340103
28. Tozawa M, Iseki K, Iseki C, Kinjo K, Ikemiya Y, Takishita S. Blood Pressure Predicts Risk of Developing End-Stage Renal Disease in Men and Women. *Hypertension* (2003) 41:1341–5. doi: 10.1161/01.HYP.0000069699.92349.8C
29. Hadaegh F, Mohebi R, Khalili D, Hasheminia M, Sheikholeslami F, Azizi F. High Normal Blood Pressure Is an Independent Risk Factor for Cardiovascular Disease Among Middle-Aged but Not in Elderly Populations: 9-Year Results of a Population-Based Study. *J Hum Hypertens* (2013) 27:18–23. doi: 10.1038/jhh.2011.112
30. Law CM, de Swiet M, Osmond C, Fayers PM, Barker DJ, Cruddas AM, et al. Initiation of Hypertension *In Utero* and its Amplification Throughout Life. *Bmj* (1993) 306:24–7. doi: 10.1136/bmj.306.6869.24
31. Chen X, Wang Y. Tracking of Blood Pressure From Childhood to Adulthood: A Systematic Review and Meta-Regression Analysis. *Circulation* (2008) 117:3171–80. doi: 10.1161/CIRCULATIONAHA.107.730366
32. Neulen J, Yan Z, Raczek S, Weindel K, Keck C, Weich HA, et al. Human Chorionic Gonadotropin-Dependent Expression of Vascular Endothelial Growth Factor/vascular Permeability Factor in Human Granulosa Cells: Importance in Ovarian Hyperstimulation Syndrome. *J Clin Endocrinol Metab* (1995) 80:1967–71. doi: 10.1210/jcem.80.6.7775647
33. McClure N, Healy DL, Rogers PA, Sullivan J, Beaton L, Haning RVJr, et al. Vascular Endothelial Growth Factor as Capillary Permeability Agent in Ovarian Hyperstimulation Syndrome. *Lancet* (1994) 344:235–6. doi: 10.1016/S0140-6736(94)93001-5
34. Pellicer A, Albert C, Mercader A, Bonilla-Musoles F, Remohí J, Simón C. The Pathogenesis of Ovarian Hyperstimulation Syndrome: *In Vivo* Studies Investigating the Role of Interleukin-1beta, Interleukin-6, and Vascular Endothelial Growth Factor. *Fertil Steril* (1999) 71:482–9. doi: 10.1016/S0015-0282(98)00484-1
35. Herrero L, Martínez M, Garcia-Velasco JA. Current Status of Human Oocyte and Embryo Cryopreservation. *Curr Opin Obstet Gynecol* (2011) 23:245–50. doi: 10.1097/GCO.0b013e32834874e2
36. Scherrer U, Rexhaj E, Allemann Y, Sartori C, Rimoldi SF. Cardiovascular Dysfunction in Children Conceived by Assisted Reproductive Technologies. *Eur Heart J* (2015) 36:1583–9. doi: 10.1093/eurheartj/ehv145
37. Hellerstein S, Feldman S, Duan T. China's 50% Caesarean Delivery Rate: Is it Too High? *BJOG* (2015) 122:160–4. doi: 10.1111/1471-0528.12971

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Endocrinology

ORIGINAL RESEARCH
published: 14 July 2022
doi: 10.3389/fendo.2022.900499

# Prognosis of Congenital Anomalies in Conceptions Following *In Vitro* Fertilization: A Multicenter Retrospective Cohort Study in China

*Jie Bao*[1,2,3,4†], *Lixue Chen*[1,2,3,4†], *Yongxiu Hao*[1,2,3,4], *Hongping Wu*[1,2,3,4], *Xiaojin He*[5], *Chuncheng Lu*[6], *Xinhua Ji*[7], *Jie Qiao*[1,2,3,4], *Yuanyuan Wang*[1,2,3,4*] and *Hongbin Chi*[1,2,3,4*]

[1] Center for Reproductive Medicine, Department of Obstetrics and Gynecology, Peking University Third Hospital, Beijing, China, [2] National Clinical Research Center for Obstetrics and Gynecology, Peking University Third Hospital, Beijing, China, [3] Key Laboratory of Assisted Reproduction, Peking University, Ministry of Education, Beijing, China, [4] Beijing Key Laboratory of Reproductive Endocrinology and Assisted Reproductive Technology, Beijing, China, [5] Center for Reproductive Medicine, The First Affiliated Hospital of Anhui Medical University, Hefei, China, [6] School of Public Health, Nanjing Medical University, Nanjing, China, [7] International Peace Maternity and Child Health Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China

OPEN ACCESS

**Edited by:**
*Yimin Zhu,*
*Zhejiang University, China*

**Reviewed by:**
*Xuefeng Lu,*
*Shanghai Jiao Tong University, China*
*Yuhua Shi,*
*Guangdong Provincial People's Hospital, China*

***Correspondence:**
*Hongbin Chi*
*chihb@163.com*
*Yuanyuan Wang*
*yyuanwang@163.com*

[†]*These authors have contributed equally to this work and share first authorship*

**Specialty section:**
*This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology*

**Received:** *20 March 2022*
**Accepted:** *17 June 2022*
**Published:** *14 July 2022*

**Citation:**
*Bao J, Chen L, Hao Y, Wu H, He X, Lu C, Ji X, Qiao J, Wang Y and Chi H (2022) Prognosis of Congenital Anomalies in Conceptions Following In Vitro Fertilization: A Multicenter Retrospective Cohort Study in China. Front. Endocrinol. 13:900499. doi: 10.3389/fendo.2022.900499*

**Background:** Conceptions following *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) have an increased risk of congenital anomalies. Few studies have explored the prognosis of fetuses with congenital anomalies. This study aimed to investigate the prevalence and prognosis of congenital anomalies in IVF/ICSI pregnancies, and to analyze the influencing factors contributing to poor prognosis.

**Methods:** In this multicenter retrospective cohort study, we followed 405,473 embryo transfer cycles at 15 reproductive centers between January 2010 and December 2019 and enrolled 2,006 intrauterine pregnancies with congenital anomalies. The relatively positive prognosis group with one or more live births and neonatal survival for more than 7 days was compared with the poor prognosis group with poorer outcomes.

**Results:** Among the 168,270 ongoing intrauterine pregnancy cycles, the prevalence of congenital anomalies was 1.19%, wherein the malformation rates of cycles with late abortion and delivery were 2.37% (716/30,202) and 0.93% (1,290/138,068), respectively. Among all IVF/ICSI cycles with congenital anomalies, the relatively positive prognosis rate was 61.39%. Moreover, the fertilization failure rate (2 pro-nuclei rate < 25%) in the poor prognosis group was significantly higher than that in the relatively positive prognosis group (10.89% vs. 5.09%, $p < 0.001$). Multivariate logistic regression analysis revealed no significant differences in the relatively positive prognosis rate among the various IVF/ICSI protocols. The relatively positive prognosis rate of fertilization failure cycles was 0.180 times that of normal fertilization cycles.

**Conclusion:** Poor fertilization rates during IVF/ICSI treatments are more likely to have poor prognosis in fetuses or neonates with congenital anomalies, and obstetric management should be strengthened in pregnant women, with which pregnant women should be recommended to strengthen obstetric management.

**Keywords: congenital anomalies, *in vitro* fertilization, fertilization failure, pregnancy outcome, prognosis**

## INTRODUCTION

Over the decades, with the continuous development and social acceptance of assisted reproductive technologies (ARTs), an increasing number of infertile couples have conceived by undergoing *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI). In European countries, approximately 2% of infants are born annually through ART (1). In Beijing, the rate of births attributed to ART has reached 1.4%, which is close to the estimated rate of ART conceptions in China (1%–2%) (2). Although this technique primarily aims to improve pregnancy outcomes in infertile couples, there remains widespread concern and controversy about whether IVF/ICSI increases the incidence of adverse obstetric and perinatal outcomes due to its unnatural *in vitro* procedures.

Congenital anomalies are a leading cause of fetal or neonatal death in the perinatal period. Their treatment is complicated, resulting in great psychological and financial burden on the child and his family. Recently, several studies have demonstrated that pregnancies conceived with IVF/ICSI have an increased risk of congenital anomalies compared to spontaneous conceptions (3–7), and some meta-analyses have concluded that the pooled risk estimation ranges from 1.32 to 1.37 (8–10).

The underlying mechanisms explaining the association between the risk of congenital anomalies and ART remain unclear, including infertility itself (6) and the increased proportion of multiple births (4, 11). In addition, specific ART procedures, such as ICSI and embryos frozen and thawed, may increase the risk of birth defects (8).

However, few studies have assessed the clinical pregnancy outcomes of fetuses or infants with congenital anomalies born after IVF/ICSI. This study aimed to evaluate the prevalence and prognosis of congenital anomalies among pregnancies conceived through IVF/ICSI treatments from 2010 to 2019, and to explore the factors contributing to poor prognosis.

## MATERIALS AND METHODS

### Study Design

This retrospective, multicenter, cohort study collected infertile patients undergoing IVF/ICSI cycles at 15 reproductive centers in China. This study was approved by the Medical Science Research Ethics Committee of Peking University Third Hospital (IRB00006761-M2019487). All data collection and analysis procedures conducted in this trial were performed in accordance with the Declaration of Helsinki. Individual informed consent was waived, as it is a retrospective study.

### Patients

From January 2010 to December 2019, we followed up 199,591 IVF-fresh cycles and 114,816 IVF-frozen cycles from 15 reproductive centers. Then, a total of 405,473 embryo transfers were performed. Finally, 174,639 women obtained clinical pregnancy, including 6,369 heterotopic pregnancy or early miscarriage and 168,270 intrauterine pregnancy of more than 12 weeks (**Figure 1**).

We collected and recorded detailed information from all centers in this study, including parental basic characteristics (age, body mass index, infertility duration, and parity), IVF/ICSI indications, IVF/ICSI specific techniques (oocyte retrieval protocols, type of fertilization, and fresh or frozen embryos), and IVF/ICSI outcomes (mature oocytes, 2 pro-nuclei [2PN], and fertilized embryos).

Clinical pregnancy outcomes were primarily follow-up data recorded at the end of the pregnancy. Pregnancies ≥28 weeks and <28 weeks but with a live birth were considered deliveries. Pregnancy loss referred to late abortions between 12 and 28 weeks, including spontaneous abortions, embryo damage, and induced abortions for various reasons, divided into miscarriages <20 and ≥20 weeks.

After confirming intrauterine pregnancy, women were scheduled for a systematic prenatal examination in the

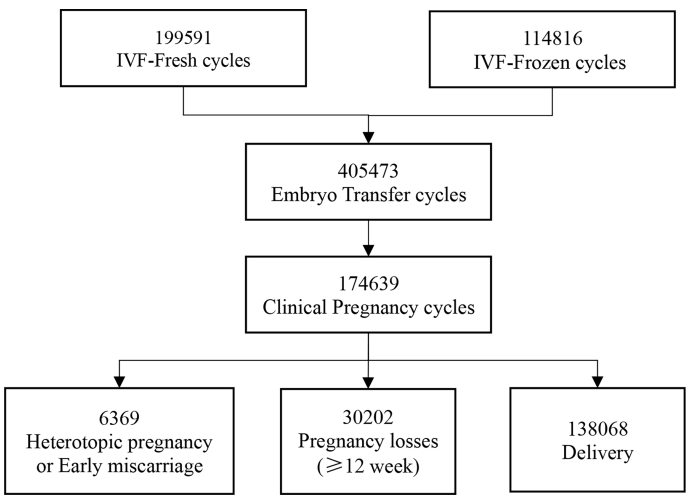



**FIGURE 1** | Flowchart showing patient selection.

obstetrics department to identify the presence of deformities or chromosomal abnormalities. In addition to the malformations detected during pregnancy, any structural, functional, and genetic anomalies diagnosed after late abortion or delivery were defined as congenital anomalies (12), which were classified according to the diagnostic codes of the International Classification of Diseases version 10 (ICD-10).

IVF/ICSI cycles with congenital anomalies were grouped into relatively positive prognosis and poor prognosis based on the outcomes of fetuses or neonates. Based on the Manual of Maternal and Child Health Surveillance of China (13), hospital monitoring on birth defects is from 28 weeks of gestation to 7 days after birth. Thus, we defined the relatively positive prognosis as having one or more live births and neonatal survival for more than 7 days. Poor prognosis covered intrauterine deaths, stillbirths, neonatal deaths within 7 days of birth, therapeutic abortions for fetuses with severe fatal and disabling malformations detected by prenatal examination or non-fatal malformations but requested by their parents, and spontaneous abortions after 12 weeks of gestation with chromosomal abnormalities.

## Influencing Factors

To explore the potential factors influencing the prognosis of offspring with congenital anomalies, we made a comparison on the details collected between the relatively positive prognosis group and the poor prognosis group.

Between the two groups, we also compared the rate of fertilization failure, when the number of 2PN embryos accounted for was less than 25% of the number of oocytes collected in one IVF/ICSI cycle, and their pregnancy outcomes, including pregnancy loss rate, delivery rate, live birth rate, birth weight, and birth height.

## Statistical Analysis

All statistical analyses were conducted using SPSS software, version 26.0 for Windows (SPSS, USA). Continuous variables were reported as mean ± standard deviation (SD). The characteristics of two groups were compared using an independent sample *t*-test or the chi-squared test, as appropriate. Given that many factors may affect the prognosis of pregnancies with congenital anomalies, multivariate logistic regression analysis was performed to analyze the potential influencing factors contributing to poor prognosis, with using the odds ratios (ORs) and corresponding 95% confidence intervals (CIs). Statistical significance was defined as a two-sided $p < 0.05$.

# RESULTS

Over the study period, we followed up a total of 168,270 IVF/ICSI cycles with ongoing intrauterine pregnancies and finally enrolled 2,006 pregnancies with congenital anomalies. The overall prevalence of congenital anomalies in the study population was 1.19%, wherein the prevalence of cycles with late pregnancy loss and delivery was 2.37% (716/30,202) and 0.93% (1,290/138,068), respectively. Based on ICD-10 codes, the incidence of congenital anomalies in specific organ systems is shown in **Table 1**. Malformations of the circulatory system were the most common in all pregnancies following IVF/ICSI, with a frequency of 0.34%, while genital malformations were the least common (0.02%). In IVF/ICSI cycles with late abortion, chromosomal abnormality was the main congenital anomaly, accounting for 37.99% of cases (272/716).

Among all IVF/ICSI cycles with congenital anomalies, the rate of relatively positive prognosis was 61.39% (1,258/2,006). Mothers in the relatively positive prognosis group tended to be younger (31.77 ± 4.30 vs. 32.95 ± 4.80, $p < 0.001$); the trend continued for the fathers (33.44 ± 5.27 vs. 34.75 ± 5.93, $p < 0.001$). Furthermore, they were more likely to have primary infertility (57.87% vs. 51.34%, $p = 0.004$) and be nulliparous

**TABLE 1** | Congenital anomalies from *in vitro* fertilization cycles.

| ICD-10 code | Item | Pregnancy losses | Delivery | All* | OR 95% CI |
|---|---|---|---|---|---|
| / | No. of cycles (%) | 30,202 | 138,068 | 168,270 | / |
| / | No. of congenital anomalies (%) | 716 (2.37%) | 1,290 (0.93%) | 2,006 (1.19%) | 2.575 (2.348, 2.823) |
| Q00–Q07 | Congenital malformations of the nervous system | 69 (0.23%) | 96 (0.07%) | 165 (0.10%) | 3.291 (2.415, 4.485) |
| Q10–Q18 | Congenital malformations of eye, ear, face, and neck | 8 (0.03%) | 93 (0.07%) | 101 (0.06%) | 0.393 (0.191, 0.809) |
| Q20–Q28 | Congenital malformations of the circulatory system | 134 (0.44%) | 440 (0.32%) | 574 (0.34%) | 1.394 (1.148, 1.692) |
| Q30–Q34 | Congenital malformations of the respiratory system | 3 (0.01%) | 54 (0.04%) | 57 (0.03%) | 0.254 (0.079, 0.812) |
| Q35–Q37 | Cleft lip and cleft palate | 28 (0.09%) | 67 (0.05%) | 95 (0.06%) | 1.911 (1.229, 2.971) |
| Q38–Q45 | Other congenital malformations of the digestive system | 7 (0.02%) | 68 (0.05%) | 75 (0.04%) | 0.470 (0.216, 1.024) |
| Q50–Q56 | Congenital malformations of genital organs | 0 (0.00%) | 27 (0.02%) | 27 (0.02%) | / ($p = 0.015$) |
| Q60–Q64 | Congenital malformations of the urinary system | 18 (0.06%) | 86 (0.06%) | 104 (0.06%) | 0.957 (0.576, 1.590) |
| Q65–Q79 | Congenital malformations and deformations of the musculoskeletal system | 41 (0.14%) | 156 (0.11%) | 197 (0.12%) | 1.202 (0.852, 1.696) |
| Q80–Q89 | Other congenital malformations | 43 (0.14%) | 86 (0.06%) | 129 (0.08%) | 2.288 (1.586, 3.299) |
| Q90–Q99 | Chromosomal abnormalities, not elsewhere classified | 272 (0.90%) | 38 (0.03%) | 310 (0.18%) | 33.010 (23.504, 46.362) |
| / | Multi-malformations | 93 (0.31%) | 79 (0.06%) | 172 (0.10%) | 5.395 (3.996, 7.284) |

**Persistent intrauterine pregnancy, intrauterine pregnancy of 12 weeks or more.*

(89.43% vs. 85.70%, $p$ = 0.013) than were those in the poor prognosis group. No statistically significant differences were found in mean body mass index (BMI) of the couples, infertility duration, or IVF/ICSI indications (**Table 2**).

Of the 2,006 IVF/ICSI cycles, 522 frozen cycles had missing IVF/ICSI laboratory information. There was a statistically significant difference in the method of fertilization between the two study groups. The rate of ICSI was higher in the relatively positive prognosis group (35.93% vs. 29.46%, $p$ = 0.011). In cycles treated with ICSI, the rates of mature oocytes (84.41% vs. 87.30%, $p$ = 0.002) and 2PN embryos (68.84% vs. 72.90%, $p$ = 0.002) were lower in pregnancies with poor prognosis. Furthermore, the rate of fertilization failure (2PN rate <25%) in the poor prognosis group was significantly higher than in the relatively positive prognosis group (10.89% vs. 5.09%, $p$ < 0.001) (**Table 3**). In addition, no differences were observed when comparing the oocyte retrieval protocols, fresh or frozen embryo transfer, and the embryo stage at transfer during IVF/ICSI conception between the two groups.

The clinical outcomes of the study populations were demonstrated in **Table 4**. Newborns with relatively positive prognosis yielded a significantly superior birth weight (2,616.06 ± 754.53 g vs. 1,936.07 ± 1,186.61 g, $p$ = 0.001) and birth height (47.25 ± 4.29 cm vs. 40.33 ± 7.58 cm, $p$ < 0.001).

Multivariate logistic regression analysis showed that pluriparity, early preterm birth (delivery at 28–34 weeks), and fertilization failure were factors for poor prognosis in IVF/ICSI pregnancies with congenital anomalies after adjusting for all confounding variables (**Table 5**). The resulting adjusted OR for fertilization failure was 0.180 (95% CI: 0.061–0.528, $p$ = 0.002) for patients with a relatively positive prognosis.

## DISCUSSION

In this 2010–2019 multicenter comprehensive follow-up study in China, the overall prevalence of congenital anomalies among intrauterine pregnancies ≥12 weeks conceived through IVF/ICSI

**TABLE 2** | Basal characteristics of patients with congenital anomalies after *in vitro* fertilization.

| | Relatively positive prognosis | Poor prognosis | All | *P*-value |
|---|---|---|---|---|
| Treatment cycles | 1,258 | 748 | 2,006 | / |
| Maternal age (years) | 31.77 ± 4.30 | 32.95 ± 4.80 | 32.21 ± 4.53 | <0.001 |
| <30 (%) | 401 (31.88%) | 194 (25.94%) | 595 (29.66%) | <0.001 |
| 30–<35 (%) | 532 (42.29%) | 284 (37.97%) | 816 (40.68%) | |
| 35–<40 (%) | 274 (21.78%) | 195 (26.07%) | 469 (23.38%) | |
| ≥40 (%) | 51 (4.05%) | 75 (10.03%) | 126 (6.28%) | |
| Maternal body mass index (kg/m$^2$) | 22.39 ± 3.16 | 22.66 ± 3.52 | 22.49 ± 3.30 | 0.115 |
| <18.5 (%) | 81 (7.72%) | 42 (6.94%) | 123 (7.44%) | 0.533 |
| 18.5–<24.0 (%) | 681 (64.92%) | 392 (64.79%) | 1,073 (64.87%) | |
| 24.0–<28.0 (%) | 230 (21.93%) | 128 (21.16%) | 358 (21.64%) | |
| ≥28.0 (%) | 57 (5.43%) | 43 (7.11%) | 100 (6.05%) | |
| Unknown (%) | 209 (16.61%) | 143 (19.12%) | 352 (17.55%) | / |
| Paternal age (years) | 33.44 ± 5.27 | 34.75 ± 5.93 | 33.93 ± 5.56 | <0.001 |
| <30 (%) | 287 (22.85%) | 151 (20.19%) | 438 (21.86%) | <0.001 |
| 30–<35 (%) | 490 (39.01%) | 235 (31.42%) | 725 (36.18%) | |
| 35–<40 (%) | 335 (26.67%) | 208 (27.81%) | 543 (27.10%) | |
| ≥40 (%) | 144 (11.46%) | 154 (20.59%) | 298 (14.87%) | |
| Unknown (%) | 2 (0.16%) | 0 (0.00%) | 2 (0.10%) | / |
| Paternal body mass index (kg/m$^2$) | 24.85 ± 3.55 | 24.75 ± 3.33 | 24.81 ± 3.47 | 0.559 |
| <18.5 (%) | 27 (2.75%) | 14 (2.39%) | 41 (2.61%) | 0.784 |
| 18.5–<24.0 (%) | 382 (38.86%) | 242 (41.30%) | 624 (39.77%) | |
| 24.0–<28.0 (%) | 404 (41.10%) | 235 (40.10%) | 639 (40.73%) | |
| ≥28.0 (%) | 170 (17.29%) | 95 (16.21%) | 265 (16.89%) | |
| Unknown (%) | 275 (21.86%) | 162 (21.66%) | 437 (21.78%) | / |
| Infertility duration (years) | 4.19 ± 3.21 | 4.06 ± 3.10 | 4.14 ± 3.17 | 0.389 |
| Primary infertility (%) | 728 (57.87%) | 384 (51.34%) | 1,112 (55.43%) | 0.004 |
| Nulliparous (%) | 1,125 (89.43%) | 641 (85.70%) | 1,766 (88.04%) | 0.013 |
| IVF indications (%) | | | | |
| Pelvic and tubal disorder | 457 (36.33%) | 265 (35.43%) | 722 (35.99%) | 0.455 |
| Ovulatory disorder | 98 (7.79%) | 58 (7.75%) | 156 (7.78%) | |
| Endometriosis | 34 (2.70%) | 21 (2.81%) | 55 (2.74%) | |
| Mixed female infertility factors | 74 (5.88%) | 28 (3.74%) | 102 (5.08%) | |
| Other female infertility factors* | 6 (0.48%) | 4 (0.53%) | 10 (0.50%) | |
| Oligo-, asthen-, and/or terato-spermia | 215 (17.09%) | 132 (17.65%) | 347 (17.30%) | |
| Ejaculation disorder | 9 (0.72%) | 2 (0.27%) | 11 (0.55%) | |
| Azoospermia | 43 (3.42%) | 32 (4.28%) | 75 (3.74%) | |
| Mixed female and male infertility factors | 230 (18.28%) | 136 (18.18%) | 366 (18.25%) | |
| Chromosomal abnormality | 16 (1.27%) | 10 (1.34%) | 26 (1.30%) | |
| Unexplained | 76 (6.04%) | 60 (8.02%) | 136 (6.78%) | |

**Other female infertility factors, including uterine malformations and immune infertility.*

**TABLE 3 |** Characteristics of IVF.

| | Relatively positive prognosis | Poor prognosis | All | *p*-value |
|---|---|---|---|---|
| Treatment cycles | 1,258 | 748 | 2,006 | / |
| Protocol (%) | | | | |
| Stimulate protocol | 990 (78.70%) | 595 (79.55%) | 1,585 (79.01%) | 0.400 |
| Micro stimulate protocol | 117 (9.30%) | 57 (7.62%) | 174 (8.67%) | |
| Natural cycle | 151 (12.00%) | 96 (12.83%) | 247 (12.31%) | |
| ART (%) | | | | |
| IVF-Fresh cycles | 515 (40.94%) | 307 (41.04%) | 822 (40.98%) | 0.181 |
| IVF-Frozen cycles | 500 (39.75%) | 358 (47.86%) | 858 (42.77%) | |
| PGT | 20 (1.59%) | 12 (1.60%) | 32 (1.60%) | |
| Fertilization cycles | | | | |
| IVF-ET (%) | 592 (64.07%) | 395 (70.54%) | 987 (66.51%) | 0.011 |
| ICSI (%) | 332 (35.93%) | 165 (29.46%) | 497 (33.49%) | |
| Unknown (%) | 334 (26.55%) | 188 (25.13%) | 522 (26.02%) | / |
| IVF (include rescue ICSI) (%)* | 592 | 395 | 987 | / |
| No. of 2PN embryos/No. of oocytes collected (%) | 4,708/7,069 (66.60%) | 2,801/4,259 (65.77%) | 7,509/11,328 (66.29%) | 0.363 |
| No. of fertilization embryos/No. of oocytes collected (%) | 5,462/7,069 (77.27%) | 3,319/4,259 (77.93%) | 8,781/11,328 (77.52%) | 0.414 |
| ICSI (include half-ICSI) (%)* | 332 | 165 | 497 | / |
| No. of mature oocytes/No. of oocytes collected (%) | 3,671/4,205 (87.30%) | 1,781/2,110 (84.41%) | 5,452/6,315 (86.33%) | 0.002 |
| No. of 2PN embryos/No. of mature oocytes (%) | 2,676/3,671 (72.90%) | 1,226/1,781 (68.84%) | 3,902/5,452 (71.57%) | 0.002 |
| No. of fertilization embryos/No. of mature oocytes (%) | 2,921/3,671 (79.57%) | 1,361/1,781 (76.42%) | 4,282/5,452 (78.54%) | 0.008 |
| Fertilization failure cycles (2PN rate < 25%) (%)* | 47 (5.09%) | 61 (10.89%) | 108 (7.28%) | <0.001 |
| Embryos transferred (%) | | | | |
| 1 | 233 (18.52%) | 192 (25.63%) | 425 (21.19%) | <0.001 |
| 2 | 951 (75.60%) | 507 (67.69%) | 1,458 (72.68%) | |
| 3 | 74 (5.88%) | 49 (6.54%) | 123 (6.13%) | |
| Embryo stage at transfer (%) | | | | |
| Cleavage stage | 825 (65.58%) | 484 (64.62%) | 1,309 (65.25%) | 0.691 |
| Blastocyst stage | 433 (34.42%) | 264 (35.25%) | 697 (34.75%) | |

**Out of the 2,006 IVF cycles, IVF laboratory information was collected for 1,484 cycles (73.98%).*

*2PN, 2 pro-nuclei; ICSI, intracytoplasmic sperm injection; IVF, in vitro fertilization; PGT, preimplantation genetic testing.*

**TABLE 4 |** Clinical outcomes.

| | Relatively positive prognosis | Poor prognosis | All | *P*-value |
|---|---|---|---|---|
| Treatment cycles | 1258 | 748 | 2006 | / |
| Pregnancy outcomes | | | | |
| Pregnancy losses (weeks) | 0 (0.00%) | 665 (88.90%) | 665 (33.15%) | <0.001 |
| 12–<20 (%) | 0 (0.00%) | 204 (27.27%) | 204 (10.17%) | / |
| 20–<28 (%) | 0 (0.00%) | 461 (61.63%) | 461 (22.98%) | |
| Delivery (weeks)* | 1,258 (100.00%) | 83 (11.10%) | 1,341 (66.85%) | <0.001 |
| <28 (%) | 37 (2.94%) | 1 (0.13%) | 38 (1.89%) | / |
| 28–<34 (%) | 197 (15.66%) | 60 (8.02%) | 257 (12.81%) | |
| 34–<37 (%) | 308 (24.48%) | 8 (1.07%) | 316 (15.75%) | |
| ≥37 (%) | 716 (56.92%) | 14 (1.87%) | 730 (36.39%) | |
| Live births** (%) | 1,258 (100.00%) | 18 (2.41%) | 1,276 (63.61%) | <0.001 |
| Live birth infants | 1,749 | 22 | 1,771 | / |
| Congenital anomalies | 1,305 (74.61%) | 21 (95.45%) | 1,326 (74.87%) | 0.025 |
| Healthy | 444 (25.39%) | 1 (4.55%)*** | 445 (25.13%) | |
| Live birth weight (g) | 2,616.06 ± 754.53 | 1,936.07 ± 1186.61 | 2,610.48 ± 761.01 | 0.001 |
| <1,500 (%) | 132 (7.55%) | 7 (31.82%) | 139 (7.85%) | <0.001 |
| 1,500–<2,500 (%) | 544 (31.10%) | 2 (9.09%) | 546 (30.83%) | |
| 2,500–<4,500 (%) | 1,004 (57.40%) | 5 (22.73%) | 1,009 (56.97%) | |
| ≥4,500 (%) | 10 (0.57%) | 0 (0.00%) | 10 (0.56%) | |
| Unknown | 59 (3.37%) | 8 (36.36%) | 67 (3.78%) | / |
| Live birth height (cm) | 47.25 ± 4.29 | 40.33 ± 7.58 | 47.19 ± 4.37 | <0.001 |
| Unknown | 677 (38.71%) | 13 (59.09%) | 690 (38.96%) | / |

**Number of deliveries at or above 28 weeks gestation and deliveries under 28 weeks but with live births.*

***Number of cycles having one or more live births.*

****Newborn died with no congenital anomalies.*

**TABLE 5 |** Logistic regression of the relatively positive prognosis rate in IVF cycles.

| | *B* | *P*-value | OR | 95% CI for OR | |
|---|---|---|---|---|---|
| | | | | Lower | Upper |
| Maternal age (years) | | | | | |
| <30 | | 0.120 | | | |
| 30–<35 | 0.307 | 0.511 | 1.359 | 0.545 | 3.391 |
| 35–<40 | 1.145 | 0.098 | 3.141 | 0.808 | 12.208 |
| ≥40 | 3.693 | 0.032 | 40.158 | 1.382 | 1167.321 |
| Paternal age (years) | | | | | |
| <30 | | 0.241 | | | |
| 30–<35 | 0.502 | 0.295 | 1.653 | 0.645 | 4.232 |
| 35–<40 | –0.390 | 0.499 | 0.677 | 0.219 | 2.098 |
| ≥40 | –0.475 | 0.555 | 0.622 | 0.128 | 3.009 |
| Primary infertility | –0.100 | 0.794 | 0.904 | 0.426 | 1.920 |
| Nulliparous | 1.420 | 0.007 | 4.138 | 1.478 | 11.581 |
| ART | | | | | |
| IVF-Fresh cycles | | 0.808 | | | |
| IVF-Frozen cycles | 0.225 | 0.514 | 1.253 | 0.637 | 2.465 |
| PGT | 15.323 | 0.996 | 4,515,108 | 0.000 | . |
| Pregnancy outcomes (weeks) | | | | | |
| Delivery, ≥37 | | 0.000 | | | |
| Pregnancy losses, 12–<20 | –36.392 | 0.990 | 0.000 | 0.000 | . |
| Pregnancy losses, 20–<28 | –36.846 | 0.986 | 0.000 | 0.000 | . |
| Delivery, <28 | 17.039 | 0.998 | 25,118,306 | 0.000 | . |
| Delivery, 28–<34 | –2.657 | 0.000 | 0.070 | 0.030 | 0.163 |
| Delivery, 34–<37 | –0.625 | 0.226 | 0.535 | 0.194 | 1.473 |
| Congenital anomalies | | | | | |
| Chromosomal abnormalities, not elsewhere classified | | 0.004 | | | |
| Congenital malformations of the nervous system | 1.521 | 0.021 | 4.575 | 1.253 | 16.709 |
| Congenital malformations of eye, ear, face, and neck | 3.380 | 0.004 | 29.363 | 3.037 | 283.891 |
| Congenital malformations of the circulatory system | 2.418 | 0.000 | 11.228 | 3.631 | 34.715 |
| Congenital malformations of the respiratory system | 3.180 | 0.006 | 24.037 | 2.451 | 235.762 |
| Cleft lip and cleft palate | 1.613 | 0.079 | 5.016 | 0.829 | 30.334 |
| Other congenital malformations of the digestive system | 2.113 | 0.019 | 8.271 | 1.420 | 48.187 |
| Congenital malformations of genital organs | 20.081 | 0.998 | 525,983,590 | 0.000 | . |
| Congenital malformations of the urinary system | 17.320 | 0.990 | 33,278,477 | 0.000 | . |
| Congenital malformations and deformations of the musculoskeletal system | 2.605 | 0.001 | 13.536 | 3.085 | 59.390 |
| Other congenital malformations | 1.212 | 0.054 | 3.360 | 0.980 | 11.512 |
| Multi-malformations | 2.504 | 0.001 | 12.228 | 2.779 | 53.808 |
| ICSI | 0.540 | 0.161 | 1.716 | 0.807 | 3.649 |
| Fertilization failure cycles (2PN rate < 25%) | –1.716 | 0.002 | 0.180 | 0.061 | 0.528 |
| Embryos transferred | | | | | |
| 1 | | 0.931 | | | |
| 2 | –0.180 | 0.750 | 0.836 | 0.278 | 2.516 |
| 3 | –0.002 | 0.999 | 0.998 | 0.160 | 6.239 |
| Constant | 1.768 | 0.999 | 5.859 | | |

*OR, odds ratio; CI, confidence interval; 2PN, 2 pro-nuclei; ICSI, intracytoplasmic sperm injection; IVF, in vitro fertilization; PGT, preimplantation genetic testing.*

was 1.19%. Nearly two-thirds could obtain a relatively positive prognosis with one or more live births surviving more than 7 days. Moreover, patients with fertilization failure were more likely to have a poor prognosis for fetuses with congenital anomalies.

The prevalence of congenital anomalies in the present study was similar to the rate of 1.23% observed in a multicenter study conducted from 2004 to 2008 (14). Another single-center study indicated that congenital anomalies among infants conceived using ART ranged from 1.10% to 1.20% (15). However, both studies were limited to live births, and other pregnancy outcomes, such as stillbirths or therapeutic labor induction, were not considered. In contrast, our study showed a lower prevalence of birth defects (0.93%) in cycles that resulted in delivery.

Consistent with previous studies (5, 15), the most common anomalies were malformations of the circulatory system in pregnancies after IVF/ICSI. Chromosomal and musculoskeletal anomalies followed behind. The incidence of chromosomal abnormalities in patients who experienced abortion was 33 times higher than in women who gave birth (odds ratio [OR] = 33.010, 95% CI = 23.504, 46.362). Infertile patients may have underlying genetic anomalies, and some chromosomal abnormalities and genetic pathogenic variants could lead to infertility (16). The genetic defects might transmit to offspring, associated with congenital anomalies (17). In the process of IVF/ICSI treatment, preimplantation genetic testing (PGT) or single-nucleotide polymorphism (SNP) technology can effectively reduce the prevalence of recurrent pregnancy loss or chromosomal

karyotype abnormalities (18, 19). Therefore, a PGT biopsy should be performed in patients with indications. Among the infants born, circulatory malformations remain the most common birth defects, followed by musculoskeletal deformities, while chromosomal abnormalities were the rarest after genital malformations. This finding was different from the results of Han et al., in which gastrointestinal anomalies were the second and cheilopalatognathus was the third.

To date, several studies have indicated that ART use is associated with an increased risk of congenital anomalies (2–7, 11, 20, 21). Levi et al. enrolled pregnancies of more than 12 weeks and early spontaneous abortions and ectopic pregnancies (3). They reported a prevalence of 3.8% for congenital anomalies after ART, which was significantly higher than the general population (2.05%). Shechter et al. only included live births and found that newborns conceived with ART were more likely to have birth defects compared with those conceived without ART in the US (OR = 2.14, 95% CI = 1.94, 2.35) (7). Another recent research on offspring obtained through ART or non-ART in Beijing showed a higher rate of birth defects in ART offspring (crude RR = 1.49; 95% CI = 1.26, 1.76) (2). A meta-analysis by Hansen et al. (8) reviewed 45 cohort studies and identified a pooled relative risk estimation of 1.32 (95% CI = 1.24, 1.42). The observed increased incidence of congenital anomalies may be explained by advanced parental age (22, 23), multiple pregnancies (4, 11), and underlying causes of infertility (3, 6, 24) among infertile patients undergoing ART treatments.

Few studies have explored the prognosis of fetuses with congenital anomalies. Our study showed that the relatively positive prognosis rate was 61.39% (1,258/2,006). Previously, Zhang et al. recorded the outcomes of fetuses with congenital heart disease (25). In this study, less than one in five newborns was born alive (346/1,851), of whom 34 died within 7 days after birth. They indicated that gestational age at delivery was the only risk factor contributing to neonatal death in the first week of life ($p < 0.001$).

The present study found statistically significant differences between female sex, male mean age, and delivery history (nulliparous or not) between the two prognosis groups. Older women have a lower quality of oocytes and an increased risk of chromosomal abnormalities in their offspring (26), resulting in a poor prognosis for fetuses with congenital anomalies. Primary infertility and nulliparity were more common in the relatively positive prognosis group. After controlling for other underlying influencing factors, nulliparity still showed a relatively positive outcome. The hypothesis was that a history of abnormal gestation and birth might impact pregnancy outcomes in infertile patients undergoing IVF/ICSI cycles.

It has been proposed that specific ART procedures, such as fresh or frozen embryo transfer after IVF/ICSI or ICSI, have little impact on the prevalence of congenital anomalies (27, 28). A 2012 meta-analysis did not find any difference in risk between the two insemination methods (10). Furthermore, in our study, although more ICSI cycles were observed in the relatively positive prognosis group (35.93% vs. 29.46%, $p = 0.011$), there was no substantial difference in the effect on prognosis whether patients were treated with ICSI ($p = 0.161$) after excluding other confounding factors. When comparing fresh and frozen-thawed embryo transfers, our study showed a similar rate of relatively positive prognosis ($p = 0.808$). The consensus is that the transfer of a single embryo has better perinatal outcomes for both mothers and offspring (29, 30). In contrast to previous reports, our study found that single-embryo transfer was associated with a lower chance of favorable prognosis; however, the multivariate logistic regression analysis identified that the number of transferred embryos had no significant effect on prognosis ($p = 0.931$).

More importantly, pregnancies with a poor prognosis had a lower rate of mature oocytes and a higher fertilization failure rate (2PN rate < 25%). Multivariate logistic regression analysis showed that the relatively positive prognosis rate of fertilization failure cycles was 0.180 times that of normal fertilization cycles. Epigenetic remodeling, including DNA methylation, chromatin accessibility, and histone modifications, occurs primarily during human gametogenesis and early embryonic development (31). ART procedures may affect epigenetic reprogramming processes, causing severe defects in offspring (32, 33). Additionally, several genes reportedly cause oocyte maturation arrest, fertilization failure, embryonic arrest, and preimplantation embryonic lethality (34). Combined with the results of the current study, infertility patients with fewer mature oocytes and more failed fertilization have a plausible increased risk of abnormal gametes, consequently leading to poor quality embryos. Poor quality day 5 embryos transferred were more likely to have major anomalies and chromosomal abnormalities (35) and had a further poor prognosis.

Several limitations exist in our study. First, there are no clear guidelines for defining favorable or unfavorable prognosis. In this study, we defined a relatively positive prognosis as having one or more live births surviving for more than 7 days. However, quite a few babies suffering from congenital anomalies die within the first month of life. At present, in majority of reproductive centers in China, the routine follow-up endpoint for ongoing pregnancies conceived by IVF/ICSI is 1–2 weeks after the expected date of confinement. The terminal point of the Chinese hospital-based birth defect surveillance system is 7 days after birth (35). As a multicenter study, it was difficult to obtain the outcomes of newborns aged 1 month age or older. Second, we did not compare the clinical outcomes of fetuses or infants with congenital anomalies after IVF/ICSI with those conceived naturally, as the participants were recruited from centers for reproductive medicine. Further research is needed to compare the prognosis of IVF/ICSI and spontaneous conceptions with congenital anomalies. Third, some laboratory data for fresh oocyte retrieval cycles corresponding to frozen-thawed embryo transfer cycles were unavailable, because some participating centers had limited electronic medical record systems. However, these deletions were completely random and did not significantly influence the results of our study.

In conclusion, this is the first multicenter study on the prognosis of pregnancies with congenital anomalies after IVF/ICSI. Moreover, our study suggests that poor fertilization rates

during IVF treatment are associated with a poor prognosis in fetuses or neonates with congenital anomalies. Hence, couples experiencing fertilization failure should be recommended to strengthen obstetric management and active prenatal diagnosis.

## DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because:

According to the requirements of this research funding, the original data are uniformly managed by the database of the Information Center for the National Health Commission of the People's Republic of China and cannot be freely shared.

Requests to access the datasets should be directed to:

Wang Yuanyuan, yyuanwang@163.com.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Peking University Third Hospital Medical Science Research Ethics Committee. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

## AUTHOR CONTRIBUTIONS

HC, YW, and JQ contributed to conception and design of the study. YH, HW, XH, CL, and XJ were in charge of data collection. JB and LC performed the statistical analysis and wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version. All authors contributed to the article and approved the submitted version.

## FUNDING

This work was supported by the National Key Research and Development Program of China (No. 2019YFC1005106).

## ACKNOWLEDGMENTS

The authors thank the following 15 reproductive centers for providing data and strongly supporting this project: Peking University Third Hospital, Peking University First Hospital, Beijing Chao-Yang Hospital, Beijing Obstetrics and Gynecology Hospital, Peking Union Medical College Hospital, Beijing Haidian District Maternal and Child Health Care Hospital, Beijing Perfect Family Hospital, Anhui Province Maternity & Child Health Hospital, The First Affiliated Hospital of Anhui Medical University, Nanjing Medical University, No. 901 Hospital of the Joint Logistics Support Force, Wanbei Coal-Electricity Group General Hospital, The First Affiliated Hospital of Wannan Medical College, The Armed Police Frontier Force General Hospital, and the International Peace Maternity & Child Health Hospital of China Welfare Institute.

## REFERENCES

1. Iacusso C, Iacobelli BD, Morini F, Totonelli G, Viggiano M, Caforio L, et al. Assisted Reproductive Technology and Anorectal Malformation: A Single-Center Experience. *Front Pediatr* (2021) 9:705385. doi: 10.3389/fped.2021.705385
2. Zhang L, Zhang W, Xu H, Liu K. Birth Defects Surveillance After Assisted Reproductive Technology in Beijing: A Whole of Population-Based Cohort Study. *BMJ Open* (2021) 11(6):e044385. doi: 10.1136/bmjopen-2020-044385
3. Levi Setti PE, Moioli M, Smeraldi A, Cesaratto E, Menduni F, Livio S, et al. Obstetric Outcome and Incidence of Congenital Anomalies in 2351 IVF/ICSI Babies. *J Assisted Reprod Genet* (2016) 33(6):711–7. doi: 10.1007/s10815-016-0714-4
4. Wen SW, Miao Q, Taljaard M, Lougheed J, Gaudet L, Davies M, et al. Associations of Assisted Reproductive Technology and Twin Pregnancy With Risk of Congenital Heart Defects. *JAMA Pediatr* (2020) 174(5):446–54. doi: 10.1001/jamapediatrics.2019.6096
5. Galdini A, Fesslova VME, Gaeta G, Candiani M, Pozzoni M, Chiarello C, et al. Prevalence of Congenital Heart Defects in Pregnancies Conceived by Assisted Reproductive Technology: A Cohort Study. *J Clin Med* (2021) 10(22):5363. doi: 10.3390/jcm10225363
6. Seggers J, de Walle HE, Bergman JE, Groen H, Hadders-Algra M, Bos ME, et al. Congenital Anomalies in Offspring of Subfertile Couples: A Registry-Based Study in the Northern Netherlands. *Fertil Steril* (2015) 103(4):1001–10.e3. doi: 10.1016/j.fertnstert.2014.12.113
7. Shechter-Maor G, Czuzoj-Shulman N, Spence AR, Abenhaim HA. The Effect of Assisted Reproductive Technology on the Incidence of Birth Defects Among Livebirths. *Arch Gynecol Obstetrics* (2018) 297(6):1397–403. doi: 10.1007/s00404-018-4694-8
8. Hansen M, Kurinczuk JJ, Milne E, de Klerk N, Bower C. Assisted Reproductive Technology and Birth Defects: A Systematic Review and Meta-Analysis. *Hum Reprod Update* (2013) 19(4):330–53. doi: 10.1093/humupd/dmt006
9. Qin J, Sheng X, Wang H, Liang D, Tan H, Xia J. Assisted Reproductive Technology and Risk of Congenital Malformations: A Meta-Analysis Based on Cohort Studies. *Arch Gynecol Obstetrics* (2015) 292(4):777–98. doi: 10.1007/s00404-015-3707-0
10. Wen J, Jiang J, Ding C, Dai J, Liu Y, Xia Y, et al. Birth Defects in Children Conceived by *In Vitro* Fertilization and Intracytoplasmic Sperm Injection: A Meta-Analysis. *Fertil Steril* (2012) 97(6):1331–7.e1-4. doi: 10.1016/j.fertnstert.2012.02.053
11. Liberman RF, Getz KD, Heinke D, Luke B, Stern JE, Declercq ER, et al. Assisted Reproductive Technology and Birth Defects: Effects of Subfertility and Multiple Births. *Birth Defects Res* (2017) 109(14):1144–53. doi: 10.1002/bdr2.1055
12. Zegers-Hochschild F, Adamson GD, de Mouzon J, Ishihara O, Mansour R, Nygren K, et al. International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) Revised Glossary of ART Terminology, 2009. *Fertil Steril* (2009) 92(5):1520–4. doi: 10.1016/j.fertnstert.2009.09.009
13. National Health and Family Planning Commission. *The Manual of Maternal and Child Health Surveillance of China* (2018). Available at: http://www.mchscn.cn/National-22/450.html (Accessed August 30, 2018).
14. Yan J, Huang G, Sun Y, Zhao X, Chen S, Zou S, et al. Birth Defects After Assisted Reproductive Technologies in China: Analysis of 15,405 Offspring in Seven Centers (2004 to 2008). *Fertil Sterility* (2011) 95(1):458–60. doi: 10.1016/j.fertnstert.2010.08.024

15. Han Y, Luo H, Zhang Y. Congenital Anomalies in Infants Conceived by Infertile Women Through Assisted Reproductive Technology: A Cohort Study 2004-2014. *Exp Ther Med* (2018) 16(4):3179–85. doi: 10.3892/etm.2018.6572
16. Wu J, Li D, Liu X, Li Q, He X, Wei J, et al. IDDB: A Comprehensive Resource Featuring Genes, Variants and Characteristics Associated With Infertility. *Nucleic Acids Res* (2021) 49(D1):D1218–24. doi: 10.1093/nar/gkaa753
17. Buckett WM, Tan SL. Congenital Abnormalities in Children Born After Assisted Reproductive Techniques: How Much Is Associated With the Presence of Infertility and How Much With its Treatment? *Fertil Sterility* (2005) 84(5):1318–9; discussion 27. doi: 10.1016/j.fertnstert.2005.04.065
18. Kimelman D, Pavone ME. Non-Invasive Prenatal Testing in the Context of IVF and PGT-A. *Best Pract Res Clin Obstetrics Gynaecol* (2021) 70:51–62. doi: 10.1016/j.bpobgyn.2020.07.004
19. Rajcan-Separovic E. Next Generation Sequencing in Recurrent Pregnancy Loss-Approaches and Outcomes. *Eur J Med Genet* (2020) 63(2):103644. doi: 10.1016/j.ejmg.2019.04.001
20. Boulet SL, Kirby RS, Reefhuis J, Zhang Y, Sunderam S, Cohen B, et al. Assisted Reproductive Technology and Birth Defects Among Liveborn Infants in Florida, Massachusetts, and Michigan, 2000-2010. *JAMA Pediatr* (2016) 170(6):e154934. doi: 10.1001/jamapediatrics.2015.4934
21. Giorgione V, Parazzini F, Fesslova V, Cipriani S, Candiani M, Inversetti A, et al. Congenital Heart Defects in IVF/ICSI Pregnancy: Systematic Review and Meta-Analysis. *Ultrasound Obstetrics Gynecol Off J Int Soc Ultrasound Obstetrics Gynecol* (2018) 51(1):33–42. doi: 10.1002/uog.18932
22. Frederiksen LE, Ernst A, Brix N, Braskhøj Lauridsen LL, Roos L, Ramlau-Hansen CH, et al. Risk of Adverse Pregnancy Outcomes at Advanced Maternal Age. *Obstetrics Gynecol* (2018) 131(3):457–63. doi: 10.1097/aog.0000000000002504
23. Lean SC, Derricott H, Jones RL, Heazell AEP. Advanced Maternal Age and Adverse Pregnancy Outcomes: A Systematic Review and Meta-Analysis. *PloS One* (2017) 12(10):e0186287. doi: 10.1371/journal.pone.0186287
24. Davies MJ, Moore VM, Willson KJ, Van Essen P, Priest K, Scott H, et al. Reproductive Technologies and the Risk of Birth Defects. *New Engl J Med* (2012) 366(19):1803–13. doi: 10.1056/NEJMoa1008095
25. Zhang W, Xu HY, Zhang YC, Liu KB. Delayed Diagnosis of Critical Congenital Heart Defects Predicting Risk Factors and Survival Rate in Newborns in Beijing: A Retrospective Study. *J Int Med Res* (2021) 49(7):3000605211028028. doi: 10.1177/03000605211028028
26. Mikwar M, MacFarlane AJ, Marchetti F. Mechanisms of Oocyte Aneuploidy Associated With Advanced Maternal Age. *Mutat Res Rev Mutat Res* (2020) 785:108320. doi: 10.1016/j.mrrev.2020.108320
27. Beltran Anzola A, Pauly V, Montjean D, Meddeb L, Geoffroy-Siraudin C, Sambuc R, et al. No Difference in Congenital Anomalies Prevalence Irrespective of Insemination Methods and Freezing Procedure: Cohort Study Over Fourteen Years of an ART Population in the South of France. *J Assisted Reprod Genet* (2017) 34(7):867–76. doi: 10.1007/s10815-017-0903-9
28. Maheshwari A, Pandey S, Shetty A, Hamilton M, Bhattacharya S. Obstetric and Perinatal Outcomes in Singleton Pregnancies Resulting From the Transfer of Frozen Thawed Versus Fresh Embryos Generated Through *In Vitro* Fertilization Treatment: A Systematic Review and Meta-Analysis. *Fertil Sterility* (2012) 98(2):368–77.e1-9. doi: 10.1016/j.fertnstert.2012.05.019
29. Grady R, Alavi N, Vale R, Khandwala M, McDonald SD. Elective Single Embryo Transfer and Perinatal Outcomes: A Systematic Review and Meta-Analysis. *Fertil Steril* (2012) 97(2):324–31. doi: 10.1016/j.fertnstert.2011.11.033
30. Kissin DM, Kulkarni AD, Kushnir VA, Jamieson DJ. Number of Embryos Transferred After *In Vitro* Fertilization and Good Perinatal Outcome. *Obstetrics Gynecol* (2014) 123(2 Pt 1):239–47. doi: 10.1097/aog.0000000000000106
31. Wang Y, Liu Q, Tang F, Yan L, Qiao J. Epigenetic Regulation and Risk Factors During the Development of Human Gametes and Early Embryos. *Annu Rev Genomics Hum Genet* (2019) 20:21–40. doi: 10.1146/annurev-genom-083118-015143
32. Uyar A, Seli E. The Impact of Assisted Reproductive Technologies on Genomic Imprinting and Imprinting Disorders. *Curr Opin Obstetrics Gynecol* (2014) 26(3):210–21. doi: 10.1097/gco.0000000000000071
33. Hattori H, Hiura H, Kitamura A, Miyauchi N, Kobayashi N, Takahashi S, et al. Association of Four Imprinting Disorders and ART. *Clin Epigenet* (2019) 11(1):21. doi: 10.1186/s13148-019-0623-3
34. Sang Q, Zhou Z, Mu J, Wang L. Genetic Factors as Potential Molecular Markers of Human Oocyte and Embryo Quality. *J Assisted Reprod Genet* (2021) 38(5):993–1002. doi: 10.1007/s10815-021-02196-z
35. Abel K, Healey M, Finch S, Osianlis T, Vollenhoven B. Associations Between Embryo Grading and Congenital Malformations in IVF/ICSI Pregnancies. *Reprod Biomed Online* (2019) 39(6):981–9. doi: 10.1016/j.rbmo.2019.07.035

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Endocrinology

ORIGINAL RESEARCH
published: 22 July 2022
doi: 10.3389/fendo.2022.865807

# Recurrent Implantation Failure May Be Identified by a Combination of Diagnostic Biomarkers: An Analysis of Peripheral Blood Lymphocyte Subsets

*Jun-Ying Cai, Yuan-Yuan Tang, Xi-He Deng, Yan-Juan Li, Gui Liang, Ya-Qing Meng and Hong Zhou* *

*Department of Reproductive Center, Maternal and Child Health Hospital and Obstetrics and Gynecology Hospital of Guangxi Zhuang Autonomous Region, Nanning, China*

OPEN ACCESS

**Edited by:**
*Yimin Zhu,*
*Zhejiang University, China*

**Reviewed by:**
*Etienne Marbaix,*
*Catholic University of Louvain, Belgium*
*Caroline E. Dunk,*
*Toronto General Research Institute (TGRI), Canada*

***Correspondence:**
*Hong Zhou*
*yuhongting@hotmail.com*

**Specialty section:**
*This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology*

**Received:** *30 January 2022*
**Accepted:** *17 June 2022*
**Published:** *22 July 2022*

**Citation:**
*Cai J-Y, Tang Y-Y, Deng X-H, Li Y-J, Liang G, Meng Y-Q and Zhou H (2022) Recurrent Implantation Failure May Be Identified by a Combination of Diagnostic Biomarkers: An Analysis of Peripheral Blood Lymphocyte Subsets. Front. Endocrinol. 13:865807. doi: 10.3389/fendo.2022.865807*

**Background:** Recurrent implantation failure (RIF) is a challenge during assisted reproductive technology (ART). In the present study, potential diagnostic biomarkers for the immune status of peripheral blood lymphocyte subsets in patients with RIF were analyzed, with the aim of identifying novel biomarkers that may predict RIF.

**Methods:** A total of 41 participants, including 21 women with RIF and 20 fertile controls, were included in the present study. Functional analysis was performed and the cytokine status of natural killer (NK), T, CD8+ T, T helper (Th), and γδ T cells which are lymphocyte subsets in peripheral blood was measured using flow cytometry. Binary logistic regression analysis adjusted for T follicular helper 1 (Tfh1), Tfh2, Tfh17, and early NK cells was performed to determine the relationship between the peripheral blood lymphocyte subsets and RIF. Potential diagnostic biomarkers were assessed by logistic regression analysis and receiver operating characteristic curves.

**Results:** There were significantly more Tfh1, Tfh17, and NK cells in the RIF group compared with the control group (all $P < 0.05$). However, the percentage of T, regulatory T (Tregs), and Tfh2 cells, as well as early inhibitory NK cells, was significantly lower in the RIF group compared with the control group (all $P < 0.05$). Following logistics regression analysis, Treg, Tfh17, and early inhibitory NK cells exhibited significant differences between the two groups. Combination diagnosis using these 3 biomarkers had a higher area under the curve of 0.900 (95% confidence interval: 0.808–0.992, $P < 0.001$) in the RIF group compared with that in the control group.

**Conclusion:** T, Tregs, Tfh1, Tfh2, Tfh17, NK cells, and early inhibitory NK cells may play important regulatory roles in embryo implantation. The combination of 3 molecular

markers (Treg, Tfh17, and early inhibitory NK cells) could provide a high diagnostic value for women with RIF, thus providing novel potential biomarkers for RIF in ART. The present findings could provide a reference either for the clinical treatment of patients with RIF or for future large, well-designed studies.

**Keywords: peripheral blood lymphocyte subsets, T cells, natural killer cells, diagnostic biomarker, flow cytometry, recurrent implantation failure**

## INTRODUCTION

Recent advances in the optimization of assisted reproductive technology (ART) have led to marked improvements in embryo implantation. However, low implantation rates remain a challenge. *In vitro* fertilization (IVF) is associated with low pregnancy rates due to recurrent implantation failure (RIF), which has become a research hotspot in ART. RIF, which can lead to considerable financial losses, as well as inflict physical or mental pressures on patients and their families, has been reported to have an incidence rate of 5–10% in women undergoing IVF cycles and therefore requires urgent attention (1). However, no unified and standardized diagnostic methods have been reported for RIF worldwide.

Embryo implantation mainly includes three stages: apposition, adhesion, and invasion (2). RIF may be caused by multiple factors, including parental chromosomal abnormalities, embryo quality, endometrial receptivity, and immunological disturbance (3). Among them, immunologic factors are thought to play an important role in RIF. At the maternal–fetal interface, a multitude of immune cells, including T cells, natural killer (NK) cells, macrophages, and dendritic cells, form a vast network of cellular connections (4). A cellular immunological abnormality in any of these cell types may lead to pregnancy failure. Currently, the etiology of RIF is unclear. Considerable evidence suggests that RIF is caused as a result of maternal immune activation in semi-allograft embryos that will be rejected by the mothers' endometrium (5, 6).

NK cells are a type of lymphocyte found in human peripheral blood and the endometrium; they are primarily responsible for nonspecific immunity. NK cells can recognize target cells through natural cytotoxicity receptors (7). Approximately 90% of peripheral blood NK cells turn into cytotoxic NK cells (4). Compared with NK cells, T cells, which take up 10–20% of the lymphocytes in the decidua, are responsible for cellular immunity. A total of 30–45% of the T cells are $CD4^+$ and 45–75% are $CD8^+$ (8). A relationship between T cell activation and Th1 immunity was reported in women with RIF and recurrent pregnancy losses (RPLs) (9). The findings by Yin et al. indicated that peripheral $CD8^+$ T cells may contribute to immune disorders in women with RIF (10).

In recent years, the detection of peripheral blood lymphocyte subsets has been used for the diagnoses of other diseases, including diffuse large B-cell lymphomas and coronavirus disease 2019 (COVID-19) (11, 12). The diagnostic value for women with RIF remains unknown. In the present study, a comprehensive analysis was conducted on the subsets of peripheral blood lymphocytes, including T cells, NK cells, and γδ T cells, in patients with RIF, as compared with the same subsets in patients with successful pregnancies by embryo transfer. The aim of the present study was to explore the regulatory mechanisms of peripheral blood lymphocytes in patients with RIF during the implantation window and attempt to find novel and valuable diagnostic biomarkers for RIF in ART.

## MATERIALS AND METHODS

### Study Population

The present study was carried out on patients from the Reproductive Medicine Center, Maternal and Child Health Hospital of Guangxi Zhuang Autonomous Region and approved by the Ethics Committee of the Maternal and Child Health Hospital of Guangxi Zhuang Autonomous Region for Reproductive Medicine. Informed consent was obtained from all individual participants.

Participants were recruited between January and December 2018 according to the following inclusion criteria: (i) age or <35 years; (ii) normal menstrual cycle of 21–35 days; (iii) single frozen-thawed blastocyst transfer cycle; and (iv) embryo grade of ≥3BB, according to the Garden and Lane criteria (13). Patients with one or more than one of the following conditions were excluded: uterine adenomyosis, endometriosis, intrauterine occupational disease, intrauterine adhesions, an endocrine disorder, abnormal chromosome, genital tract malformation, and inflammation. In the end, a total of 41 participants were selected and divided into two groups: the RIF and control groups. The participants who experienced pregnancy failure after at least 3 consecutive IVF attempts (involving either fresh or frozen-thawed cycles) and transplantation of 1–2 embryos of high-grade quality in each cycle were included in the RIF group (14). Participants with fallopian tube malfunctions who were able to achieve successful IVF-assisted pregnancies and their babies (aged >1 years) were included in the control group. The information collected from each participant included age, body mass index (BMI), infertility time, endometrial thickness, and basal and mid-luteal period (luteinizing hormone day 5–7) sex hormone levels.

### Sample Collection and Flow Cytometry

Peripheral blood samples were collected during the mid-luteal period. Blood samples (100 μl) and a mixture of antibodies for $CD4^+$ T, $CD8^+$ T, NK, γδT, Th, and B cells (10 μl) were added to six tubes, respectively, according to the manufacturers' instructions. The color scheme of antibodies and the combination of surface antibodies for each cell are detailed in **Supplementary Tables S1**,

**S2**. An additional step-wise gating procedure for the flow cytometry can be seen in **Supplementary Figure S1**, including the gate for $CD4^+$ T, $CD8^+$ T, NK, and γδ cells and their subsets. Following shaking for 30 s, cells were incubated at room temperature to avoid light for 15 min. Cells were then lysed and fixed with 800 μl AKC lysing solution (Becton, Dickinson and Company) in an incubator in the dark at 4°C for 15 min, followed by centrifugation at 1,000×*g* for 5 min. After removing the supernatant, 200 μl AKC lysing solution was added to each tube while avoiding the light, followed by further incubation for 3 min after which the pellets were washed with 2 ml PBS and centrifuged at 1,000×*g* for 5 min. Finally, 350 μl PBS was added to each tube and centrifuged, and the supernatants were removed. The pellets were measured in a FACSCanto flow cytometer (Mountain View; Becton, Dickinson and Company). The results were analyzed using FlowJo software (TreeStar).

## Statistical Analysis

All statistical analysis was performed with SPSS (version 23.0; IBM Corp.). The Kolmogorov–Smirnov test was used to confirm whether the data were normally distributed. All data are presented as the mean ± standard deviation or a median (25–75% quartiles). Differences between two groups were compared using a Student's *t* test or Mann–Whitney *U* tests. Considering that T and NK cells are large group of lymphocytes, their fluctuation and significance are markedly affected by cell changes in each of the other lymphocyte subsets. Therefore, binary logistic regression analysis adjusted for the 5 indicators was performed to determine differences among them, and an ROC curve was created. Logistic regression analysis and areas under the curve (AUC) of ROC curves with 95% CI were used to predict diagnostic value. The statistical tests were two-tailed and $P < 0.05$ was considered to indicate a statistically significant difference.

# RESULTS

## Baseline Characteristics

There were 21 patients in the RIF group and 20 in the control group. The median age of participants in the RIF group and fertile controls were 32.00 (28.50–32.50) and 30.00 (28.00–31.50) years, respectively. The characteristics of the two groups are presented in **Table 1**. There was no significant difference in age, BMI, infertility time, baseline hormone levels of estradiol (E2) and progesterone (P), mid-luteal period of endometrial thickness, E2, P, and luteinizing hormone levels between the two groups (all $P > 0.05$).

## Comparisons of Lymphocyte Subsets Between Groups

To investigate the potential relationship between the immune status of peripheral blood lymphocytes and RIF, the lymphocyte subsets were first assessed between the two groups. Results showed that patients with RIF had a significantly lower percentage of T cells ($P = 0.010$) and a significantly higher percentage of NK cells ($P = 0.019$) in their peripheral blood samples (**Table 2**). No significant differences were observed in the other lymphocyte subsets (T helper, killer T cells, and double-positive T lymphocytes) or in the T helper (Th) to T cytotoxic (Tc) ratio, between the two groups (**Table 2**).

## Comparisons of Functions and Differentiation of T Cell Subsets Between Groups

To assess the functions and differentiation of T cells between groups, an analysis of T cells in peripheral blood samples was performed (**Table 3**). The percentage of $CD3^+$ regulatory T cells (Tregs) in the RIF group was found to be significantly lower than that in the control group ($P = 0.005$). No significant differences were observed in the other subsets. As compared with the control group, the RIF group exhibited a higher percentage of Tfh1 ($P = 0.024$) and Tfh17 ($P = 0.004$) cells and a lower percentage of Tfh2 cells ($P = 0.008$) among the total number of Tfh cells. In addition, the RIF group exhibited a significantly higher percentage of Th17 to Th2 ($P = 0.003$) and $Th1^+Th17$ to Th2 ($P = 0.002$). No differences were observed in the functions of $CD8^+$ T and γδ T cell subsets between the two groups.

## Comparisons of NK Cell Subsets Between Groups

NK cell subsets in the peripheral blood were also measured. It was found that the percentage of early inhibitory NK cells was lower in the RIF group than that in the control group ($P = 0.004$; **Table 4**). No significant differences in the percentages of T, immature, mature,

**TABLE 1** | Baseline characteristics of the study population.

| Variables | RIF group ($n = 21$) | Control group ($n = 20$) | *P* value |
|---|---|---|---|
| Age (years) | 32 (28.5–32.5) | 30 (28–31.5) | 0.247 |
| Body mass index (kg/m$^2$) | 20.81 (19.8–22.04) | 21.08 (20.26–22.83) | 0.486 |
| Infertility time (years) | 3 (1.5–5.5) | 2 (1–3) | 0.083 |
| Baseline sex hormone level | | | |
| E2 (pmol/l) | 165.8 (129–213.25) | 137.3 (117.2–177.55) | 0.170 |
| P (nmol/l) | 1.59 ± 0.54 | 1.7 ± 0.68 | 0.597 |
| Mid-luteal period | | | |
| Endometrial thickness (mm) | 9.19 ± 2.22 | 9.90 ± 1.77 | 0.267 |
| LH (mIU/ml) | 35.25 ± 18.46 | 34.25 ± 21.15 | 0.874 |
| E2 (pmol/l) | 1,309.15 ± 475.23 | 1,250.23 ± 664.19 | 0.747 |
| P (nmol/l) | 2.33 ± 1.07 | 2.06 ± 1.15 | 0.449 |

*RIF, recurrent implantation failure; E2, estradiol; P, progesterone; LH, luteinizing hormone. Data are presented as the mean ± standard deviation or a median (25–75% quartiles). The P value was calculated using a Student's t test or Mann–Whitney U test.*

**TABLE 2 |** Comparisons of lymphocyte subsets between the recurrent implantation failure and control groups.

| Cell type | RIF group (*n* = 21) | Control group (*n* = 20) | *P* value |
|---|---|---|---|
| T cells (% of lymphocyte) | 64.57 ± 10.22 | 72.81 ± 9.17 | 0.010 |
| Natural killer cells (% of lymphocyte) | 20.27 ± 9.95 | 13.63 ± 7.13 | 0.019 |
| Th cells (% of T cells) | 54.65 ± 9.04 | 58.73 ± 8.41 | 0.143 |
| Killer T cells (% of T cells) | 38.20 (31.05, 45.00) | 35.75 (27.58, 41.88) | 0.235 |
| Double positive T lymphocytes (% of T cells) | 1.50 (0.84, 2.28) | 1.52 (1.01, 1.98) | 0.725 |
| Th/T cytotoxic | 1.55 (1.05, 1.77) | 1.63 (1.21, 2.48) | 0.24 |

*RIF, Recurrent implantation failure; Th, T helper. The data are presented as the mean ± standard deviation or a median (25–75% quartiles). A Student's t test or Mann–Whitney U test was conducted. P < 0.05 was considered to indicate a statistically significant difference.*

late inhibitory, activated, conventional killer, and virus-specific NK cells were observed between groups (all $P > 0.05$; **Table 4**).

Binary logistic regression analysis was performed for the 5 different indicators, and the results showed that Treg, Tfh17, and early inhibitory NK cells exhibited significant differences between the two groups ($P < 0.05$; **Table 5**).

## Comparisons of Lymphocytes Between Groupsin Peripheral Blood Mononuclear Cells Between Groups

To investigate the differences in peripheral blood lymphocytes between groups, their percentages in peripheral blood mononuclear cells were analyzed using flow cytometry. There

**TABLE 3 |** Comparisons of functions and differentiation of T cell subsets between the recurrent implantation failure and control groups in peripheral blood samples.

| Cell type | RIF group (*n* = 21) | Control group (*n* = 20) | *P* value |
|---|---|---|---|
| T cell functional subsets | | | |
| Naïve $CD4^+$ T cells (% of CD4 T cells) | 19.65 ± 16.22 | 22.03 ± 10.26 | 0.581 |
| Terminal differentiated $CD4^+$ T cells (% of CD4 T cells) | 27.22 ± 12.82 | 25.09 ± 11.87 | 0.584 |
| Central memory $CD4^+$ T cells (% of T cells) | 5.48 (2.66, 8.30) | 5.54 (3.84, 8.73) | 0.322 |
| Effective memory $CD4^+$ T cells (% of CD4 T cells) | 47.60 ± 13.10 | 48.23 ± 12.21 | 0.774 |
| Exhaustion $CD4^+$ T cells (% of CD4 T cells) | 2.00 (0.48, 4.58) | 2.23 (0.30, 5.59) | 0.794 |
| Functional $CD4^+$ T cells (% of CD4 T cells) | 98.00 (95.40, 99.55) | 97.80 (94.40, 99.68) | 0.834 |
| Tregs (% of CD3 T cells) | 2.24 ± 0.91 | 3.17 ± 1.09 | 0.005 |
| Naïve $CD8^+$ T cells (% of CD8 T cells) | 21.84 ± 9.05 | 25.58 ± 14.70 | 0.336 |
| Terminal differentiation $CD8^+$ T cells | 51.50 ± 13.44 | 43.93 ± 12.15 | 0.066 |
| Central memory $CD8^+$ T cells (% of CD8 T cells) | 0.32 (0.22, 0.5) | 0.36 (0.24, 0.75) | 0.498 |
| Effective memory $CD8^+$ T cells (% of CD8 T cells) | 20.00 (15.50, 41.15) | 27.60 (21.23, 36.75) | 0.285 |
| Exhaustion of $CD8^+$ T cells (% of CD8 T cells) | 10.4 (1.94, 31.00) | 13.10 (4.28, 32.28) | 0.368 |
| Inactive specificity $CD8^+$ T cells (% of effective memory $CD8^+$ T cells) | 64.74 ± 15.85 | 57.78 ± 22.08 | 0.252 |
| Inactive specificity terminal differentiation $CD8^+$ T cells (% of terminal differentiation $CD8^+$ T cells) | 57.00 ± 20.33 | 53.22 ± 22.99 | 0.579 |
| Persistent viral specificity $CD8^+$ T cells (% of effective memory $CD8^+$ T cells) | 35.25 ± 15.86 | 42,.21 ± 22.08 | 0.251 |
| Persistent viral specificity terminal differentiation $CD8^+$ T cells (% of terminal differentiation $CD8^+$ T cells) | 43.00 ± 20.33 | 46.77 ± 22.97 | 0.581 |
| T cell differentiation subsets | | | |
| Tfh (% of $CD4^+$ T cells) | 20.17 ± 5.08 | 19.73 ± 5.91 | 0.798 |
| Th1 (% of Th cells) | 10.43 ± 4.64 | 8.64 ± 3.73 | 0.182 |
| Th2 (% of Th cells) | 18.25 ± 5.60 | 20.45 ± 5.88 | 0.227 |
| Th17 (% of Th cells) | 3.25 (2.27, 7.11) | 2.63 (1.70, 3.55) | 0.050 |
| Tfh1 (% of Tfh cells) | 11.70 ± 3.34 | 9.58 ± 2.33 | 0.024 |
| Tfh2 (% of Tfh cells) | 37.72 ± 6.57 | 42.72 ± 4.56 | 0.008 |
| Tfh17 (% of Tfh cells) | 6.52 ± 2.45 | 4.42 ± 1.82 | 0.004 |
| Tc1 (% of Tc cells) | 31.69 ± 13.02 | 33.81 ± 10.26 | 0.568 |
| Tc2 (% of Tc cells) | 17.20 (14.25, 21.65) | 20.0 0 (15.18, 23.23) | 0.865 |
| Tc17 (% of Tc cells) | 8.54 (5.17, 12.20) | 9.31 (4.96, 11.18) | 0.969 |
| Th1/Th2 | 0.63 ± 0.33 | 0.45 ± 0.22 | 0.050 |
| Th17/Th2 | 0.21 (0.17,0.33) | 0.13 (0.09, 0.19) | 0.003 |
| Th1+Th17/Th2 | 0.90 ± 0.34 | 0.59 ± 0.25 | 0.002 |
| Peripheral helper T cells (% of $CD4^+$ T cells) | 56.74 ± 5.23 | 58.00 ± 6.91 | 0.514 |
| Activated Tfh (% of $CD4^+$ T cells) | 14.93 ± 4.12 | 14.65 ± 5.33 | 0.850 |
| CD8+ T cells subsets | | | |
| Inhibitory $CD8^+$ T cells (% of CD8 T cell) | 21.09 ± 8.05 | 20.33 ± 7.34 | 0.754 |
| Potential functional $CD8^+$ T cells (% of CD8 T cell) | 71.76 ± 10.22 | 73.50 ± 11.65 | 0.614 |
| Total memory $CD8^+$ T cells (% of CD8 T cell) | 2.76 ± 1.35 | 3.50 ± 2.34 | 0.227 |
| Homing memory $CD8^+$ T cells (% of CD8 T cell) | 72.70 ± 12.15 | 78.03 ± 12.28 | 0.170 |
| Terminally senescent $CD8^+$ T cells (% of CD8 T cell) | 16.73 ± 8.83 | 17.22 ± 10.52 | 0.872 |
| γδ T cells subsets | | | |

*(Continued)*

**TABLE 3** | Continued

| Cell type | RIF group (*n* = 21) | Control group (*n* = 20) | *P* value |
|---|---|---|---|
| γδ T cells (% of T cells) | 5.25 ± 2.66 | 4.24 ± 1.77 | 0.162 |
| Vδ 1$^+$ (% of γδ T cells) | 47.16 ± 22.88 | 54.96 ± 22.12 | 0.274 |
| Vδ 2$^+$ γδ T cells | 50.36 ± 22.95 | 44.45 ± 22.26 | 0.408 |
| Vδ 1$^+$/Vδ 2$^+$ | 0.70 (0.39, 1.77) | 1.30 (0.42, 3.03) | 0.251 |
| NKG2D$^+$Vδ 2$^+$ (% of γδ T cells) | 96.20 (86.85,98.50) | 94.35 (87.50, 99.15) | 0.948 |
| PD1$^+$ Vδ 2$^+$ (% of γδ T cells) | 5.45 (3.65,10.60) | 5.87 (3.17, 11.78) | 0.917 |
| NKP30$^+$Vδ 2$^+$ (% of γδ T cells) | 0.43 (0.13,0.92) | 0.88 (0.43, 1.67) | 0.074 |
| NKP46$^+$Vδ 2$^+$ (% of γδ T cells) | 1.76 (0.77,3.90) | 1.41 (0.47, 2.92) | 0.465 |
| NKG2D$^+$Vδ 1$^+$ (% of γδ T cells) | 58.87 ± 16.14 | 58.40 ± 11.86 | 0.917 |
| PD1$^+$Vδ 1$^+$ (% of γδ T cells) | 27.60 ± 13.06 | 25.15 ± 12.42 | 0.543 |
| NKP30$^+$Vδ 1$^+$ (% of γδ T cells) | 9.21 (3.18, 13.85) | 10.25 (4.01, 15.05) | 0.725 |
| NKP46$^+$Vδ 1$^+$ (% of γδ T cells) | 25.66 ± 17.05 | 18.34 ± 12.48 | 0.126 |

*Tregs, regulatory T cells; Tfh, T follicle helper cell; Th, T helper; Tc, T cytotoxic; NKG2D, activated receptor of NK cells; NKP30, natural cytotoxicity triggering receptor 3; NKP46, natural cytotoxicity triggering receptor 1; PD1, programmed cell death protein 1. Data are presented as the mean ± standard deviation or a median (25–75% quartiles). A Student's t test or Mann–Whitney U test was conducted. P < 0.05 was considered to indicate a statistically significant difference.*

**TABLE 4** | Comparisons of NK cell subsets between the RIF and control groups.

| Cell type | RIF group (*n* = 21) | Control group (*n* = 20) | *P* value |
|---|---|---|---|
| NKT cells (% of lymphocyte) | 7.89 ± 3.56 | 7.40 ± 2.92 | 0.629 |
| Immature NK cells (% of NK cells) | 60.20 (20.45, 86.45) | 64.60 (33.93, 78.03) | 0.774 |
| Mature NK cells (% of NK cells) | 37.90 (12.25, 79.60) | 33.95 (20.33, 64.13) | 0.648 |
| Early inhibitory NK cells (% of NK cells) | 41.79 ± 13.17 | 55.10 ± 14.62 | 0.004 |
| Late inhibitory NK cells (% of NK cells) | 6.44 (4.47, 10.65) | 5.23 (2.21, 11.00) | 0.335 |
| Activated NK cells (% of NK cells) | 45.12 ± 14.29 | 50.60 ± 16.92 | 0.269 |
| Conventional NK cells (% of NK cells) | 57.03 ± 23.35 | 52.38 ± 17.22 | 0.474 |
| Viral specific NK cells (% of NK cells) | 71.05 ± 16.24 | 61.68 ± 20.78 | 0.115 |

*NK, natural killer; NKT, Natural killer T cell; RIF, recurrent implantation failure. The data are presented as the mean ± standard deviation or a median (25–75% quartiles). A Student's t test or Mann–Whitney U test was conducted. P < 0.05 was considered to indicate a statistically significant difference.*

were significant differences in the percentages of Tregs ($CD3^+CD4^+CD25^+CD127^-$), Tfh1 ($CD3^+CD4^+CXCR5^+CXCR3^+CCR4^-$), Tfh2 ($CD3^+CD4^+CXCR5^+CXCR3$-$CCR4^+$), Tfh17 ($CD3^+CD4^+CXCR5^+CXCR3^-CCR4^-CCR6^+$), and early inhibitory NK cells ($CD3$-$CD56^+CD94^+KIR^-$) between the two groups (**Figure 1**). The RIF group exhibited a significantly lower percentages of Tregs (2.24 ± 0.91 vs. 3.17 ± 1.09; $P < 0.05$; **Figure 1A**), a higher percentage of Tfh1 cells (11.70 ± 3.34 vs. 9.58 ± 2.33; $P < 0.05$; **Figure 1B**), a lower percentage of Tfh2 cells (37.72 ± 6.57 vs. 42.72 ± 4.56; $P < 0.05$; **Figure 1B**), a higher percentage of The Th17 cells (6.52 ± 2.45 vs. 4.42 ± 1.82; $P < 0.05$; **Figure 1C**), and a lower percentage of the early inhibitory NK cells (41.79 ± 13.17 vs. 55.10 ± 14.62; $P < 0.05$; **Figure 1D**), compared with the control group.

**TABLE 5** | Logistic regression analysis of the effect of the five peripheral blood lymphocyte subsets on the RIF.

| Cell type | *P* value | HR (95% CI) |
|---|---|---|
| Early inhibitory NK cells | 0.023 | 0.900 (0.821–0.985) |
| Treg | 0.018 | 0.246 (0.077–0.785) |
| Tfh1 | 0.775 | 1.076 (0.650–1.781) |
| Tfh2 | 0.124 | 0.836 (0.666–1.050) |
| Tfh17 | 0.044 | 1.730 (1.014–2.950) |

*HR, hazard ratio; CI, confidence interval; NK, natural killer; Tregs, regulatory T cells; Tfh, T follicle helper cell.*

## Diagnostic Value of Biomarkers for RIF

In order to identify potential biomarkers for the diagnosis of RIF, logistic regression analysis and ROC curves were used to evaluate the diagnostic efficiency of single and combined biomarkers (**Figure 2**). The results showed that the AUCs of T cells, Tregs, Tfh1, Tfh2, Tfh17, NK cells, and early inhibitory NK cells were 0.746 (95% CI = 0.590–0.903, $P = 0.007$), 0.745 (95% CI = 0.595–0.896; $P = 0.007$), 0.723 (95% CI = 0.561–0.884; $P = 0.015$), 0.777 (95% CI = 0.629–0.926; $P = 0.002$), 0.750 (95% CI = 0.595–0.905; $P = 0.006$), 0.702 (95% CI = 0.540–0.864; $P = 0.027$), and 0.757 (95% CI = 0.604–0.910; $P = 0.025$), respectively. A combination diagnosis including all 3 markers revealed a significantly higher AUC of 0.900 (95% CI = 0.808–0.992; $P < 0.001$) than any marker alone. A combined diagnosis using these 3 markers had a high diagnostic value and may be able to distinguish the patients with RIF from patients with other conditions during ART.

# DISCUSSION

Previous studies have focused on the aggregation of immune cells in endometrial tissue during the window of implantation, including NK cells, macrophages, dendritic cells, and Tregs, which provide a unique immune microenvironment for embryo implantation (15, 16). The immune cells found in the peripheral blood and endometrium are heterogeneous under different

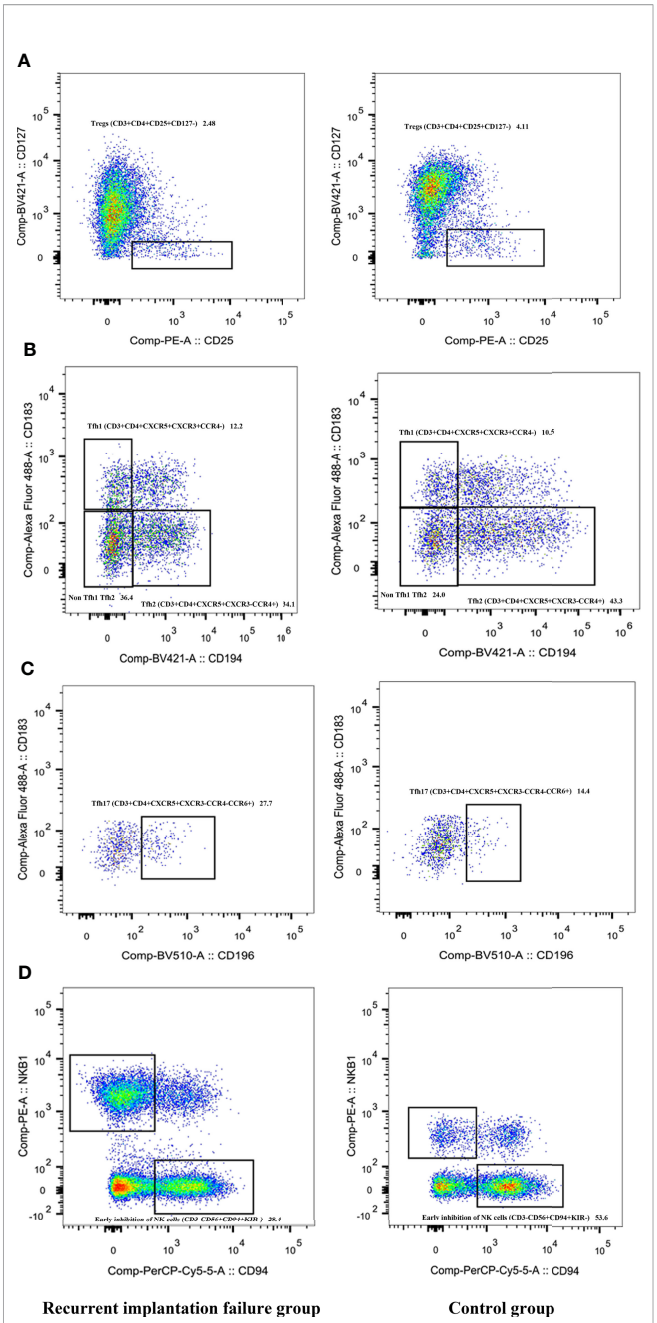



**FIGURE 1** | Comparison of Tregs **(A)**, Tfh1 **(B)**, Tfh2 **(B)**, Tfh17 **(C)**, and early inhibitory NK cells **(D)** between the RIF and control groups. RIF, recurrent implantation failure; NK, natural killer; Tregs, regulatory T; Tfh, T follicle helper.

situations (17). We therefore assumed that there may be certain special biomarkers that may become abnormal under RIF. The detection of potential biomarkers to predict RIF in the peripheral blood would be preferable to endometrial biopsies. To the best of our knowledge, no reliable peripheral blood biomarker for RIF during the window of implantation has been reported yet.

In the present study, a systematic comparison of the subsets of peripheral blood lymphocytes, including T, $CD4^+$ T, $CD8^+$ T, NK, and γδ T cells in women with RIF (RIF group) and those with successful pregnancies (control group), was performed. The results showed that the RIF group exhibited a significantly higher percentage of Tfh1, Tfh17, and NK cells, compared with the control group. On the contrary, a significantly lower percentage of T cells, Tregs, Tfh2, and early inhibitory NK cells was identified in the RIF group, as compared with that in the control group. Finally, these findings indicated that a combined diagnosis using these 7 biomarkers (T cells, Tregs, Tfh1, Tfh2, Tfh17, NK cells, and early inhibitory NK cells) has a high diagnostic value and may be able to distinguish patients with RIF from other patients during ART. These findings suggested that the use of peripheral blood samples may be a safe and reliable potential diagnostic tool for women with RIF who underwent ART.

T cells could be divided into $CD4^+$ and $CD8^+$ T cells, according to their cell surface antigens (18). T cell subsets could be further differentiated into stem cell memory, central memory, effector memory, and effector T cells, which exist in peripheral tissues and blood, based on their effector memory differentiation. Those T cells can produce effector molecules upon activation (19). Our results indicated that there may be a decreased percentage of T cells in patients with RIF compared with fertile controls. A relationship between T cell activation and Th1 has previously been reported in women with RIF or RPLs (9). In addition, Li et al. further analyzed the levels of peripheral blood T cells in women with chronic endometritis (CE) and compared them with that in a non-CE group, in which patients had undergone recurrent miscarriage (RM) and RIF. However, no statistical difference was identified between the two groups (20), suggesting that peripheral blood T cells were not involved in the regulation of inflammatory responses in either RM or RIF. In the present study, flow cytometry was first used to investigate the expression of $CD4^+$ and $CD8^+$ T cell subsets for effector memory differentiation in women with RIF. However, no significant differences were observed between these subsets.

Of note, once the antigens were stimulated, naive $CD4^+$ and $CD8^+$ T cells could be characterized by several effector subsets based on their pattern of cytokine expression. These include type 1 T helper (Th1), Th2, Th17, Tregs, and T follicle helper cells (Tfh) for $CD4^+$ T cells (21), as well as Tc1, Tc2, and Tc17 for $CD8^+$ T cells (19), all of which play critical roles in maintaining immune tolerance. Tfh cells are generally considered the dominant T cell population, which could induce B cells to help reduce inflammation (22). The programmed cell death-1 molecule has been demonstrated to regulate the positioning and function of Tfh cells (23). A previous study also revealed an association between Tfh cells and human immunodeficiency virus (HIV) infection and showed that Tfh cells may play critical roles in antimicrobial defense, cancer, and autoimmunity (24, 25). A recent study found that E2 and P4 cooperate in the humoral immune response by favoring the expansion of different cyclic Tfh cell subsets (26). No study has yet reported the relationship between Tfh cells and RIF. To the best of our knowledge, the present

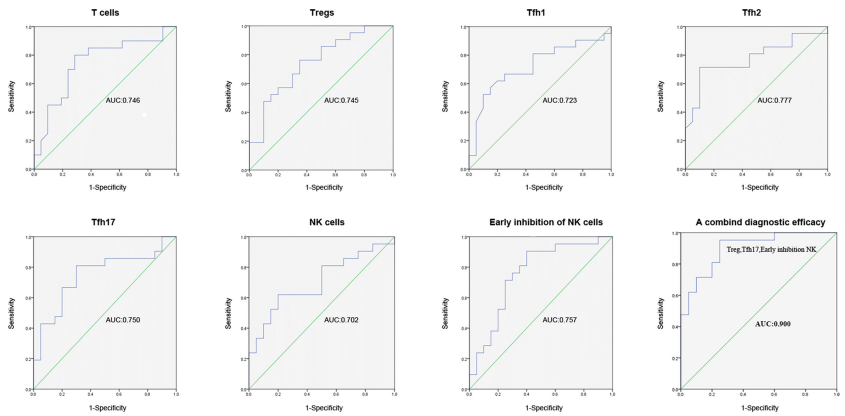



**FIGURE 2** | ROC curve for the efficiency of single (Tregs, Tfh1, Tfh2, Tfh17, and early inhibitory NK cells) and of combined biomarker diagnosis for RIF. ROC, receiver operating characteristic; RIF, recurrent implantation failure, NK, natural killer; Tregs, regulatory T; Tfh, T follicle helper.

findings were the first to suggest that Tfh1, Tfh2, and Tfh17 cells are associated with pregnancy outcomes in IVF treatment, and these types of cells may serve as novel indicators for the prediction of implantation success in patients undergoing ART.

Tregs that are distributed in peripheral blood circulation and tissues have been suggested to be necessary for the maintenance of maternal–fetal tolerance. An elevated expression of Tregs in the peripheral blood has been reported to be correlated with a favorable pregnancy outcome (27). It has also been shown that human chorionic gonadotropin could regulate the differentiation of Tregs in order to affect pregnancy outcomes in women with RIF (28). The results of certain studies focusing on human and murine models have revealed a reduction in the percentageof Tregs during RIF or unexplained infertility (29). A lower percentage of Tregs was also observed in the peripheral blood of women with RIF (30). In the present study, the levels of Tregs were significantly lower in patients with RIF, as compared with fertile controls, and these findings were consistent with previous studies. These findings provide a foundation for the use of Tregs for the detection of RIF.

NK cells constitute the dominant cell population in the endometrium, and they make contact with the extravillous trophoblast cells in the decidua during the early stage of pregnancy. Previous studies have focused on the role of NK cells in recurrent spontaneous abortion and RIF. The expression of NKP30 on cytotoxic NK cells (CD56dim CD16pos/neg) significantly increased in RIF (31). High NK cell numbers may be adisadvantage for ovarian reserve or function (32). Sacks et al. (33) reported that women with RIF had a higher NK cell activity in the peripheral blood, which was consistent with the findings of the present study. The present findings showed that patients with RIF may exhibit an increased number of NK cells. However, a study by Kolanska et al. (17) showed that peripheral blood NK cells alone were not able to reflect the risk of pregnancy failure or miscarriage, and it should therefore not be recommended for the management of RM and RIF. Nevertheless, differences in subsets of NK cells in the RIF and control groups were observed in the present study. The results showed a decrease in the percentage of early inhibitory NK cells in patients with RIF, which may provide some insights into the pathogenesis of RIF. To the best of our knowledge, no studies have investigated the early inhibition of NK cells in RIF to date. Further studies with larger samples need to be performed to re-verify these findings.

Pregnancy success or failure has been found to be correlated with the number of γδ T cells in the decidua of pregnant mice (34). Clark et al. (34) reported that γδ T cells could produce cytokines through an imbalance of Th1/2/3 cells in murine pregnancy decidua, leading to abortions. The present study focused on the relationship between γδ T cells in the peripheral blood and the success or failure of pregnancy, and the γδ T cells and subsets in the peripheral blood samples of patients with RIF were identified using flow cytometry. The results showed that there was no significant difference between groups. We therefore hypothesized that the γδ T cells in the peripheral blood and decidua were heterogeneous.

In conclusion, the present findings indicated that an increase in the percentage of Tfh1, Tfh17, and NK cells and a decrease in the percentages of Tregs, and T, Tfh2, and early inhibitory NK cells were associated with RIF. The data was strengthened by binary logistic regression modeling and the screening of three significant difference indicators: Treg, Tfh17, and early inhibitory NK cells. Combined diagnosis using these 3 molecular markers showed high diagnostic efficacy for assessing patients with RIF and could act as a novel potential biomarker for ART. We hope that our findings could provide a reference either for the clinical treatment of patients with RIF or for future large, well-designed studies.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by this research was carried out on patients from the Reproductive Medicine Center, Maternal and Child Health Hospital of Guangxi Zhuang Autonomous Region and approved by the Ethics Committee of the Maternal and Child Health Hospital of Guangxi Zhuang Autonomous Region for Reproductive Medicine. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

Designed the study: J-YCand HZ. Collected patients: Y-YT and X-HD. Performed the research: Y-JL, GL, and Y-QM. Statistical analyses: J-YC. Wrote the manuscript: J-YC. All authors contributed to the article and approved the submitted version.

## FUNDING

This research was supported by by the Natural Science Foundation of Guang Xi, China (2017GXNSFBA198150) to J-Y Cai, and the appropriate health technique development and Spreading and Application project of Guang Xi, China (S2020056) to H Zhou.

## ACKNOWLEDGMENTS

Thanks to the flow cytometry service provided by Wuhan Purui Medical Laboratory Co., Ltd.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.865807/full#supplementary-material

**Supplementary Figure 1** | Stepwise gating procedure for the flow cytometry analysis.

## REFERENCES

1. Mak JSM, Chung CHS, Chung JPW, Kong GWS, Saravelos SH, Cheung LP, et al. The Effect of Endometrial Scratch on Natural-Cycle Cryopreserved Embryo Transfer Outcomes: A Randomized Controlled Study. *Reprod BioMed Online* (2017) 35(1):28–36. doi: 10.1016/j.rbmo.2017.04.004
2. Achache H, Revel A. Endometrial Receptivity Markers, the Journey to Successful Embryo Implantation. *Hum Reprod Update* (2006) 12(6):731–46. doi: 10.1093/humupd/dml004
3. Cakiroglu Y, Tiras B. Determining Diagnostic Criteria and Cause of Recurrent Implantation Failure. *Curr Opin Obstet Gynecol* (2020) 32 (3):198–204. doi: 10.1097/GCO.0000000000000620
4. Yang F, Zheng Q, Jin L. Dynamic Function and Composition Changes of Immune Cells During Normal and Pathological Pregnancy at the Maternal-Fetal Interface. *Front Immunol* (2019) 10:2317. doi: 10.3389/fimmu.2019.02317
5. Liu S, Wei H, Li Y, Diao L, Lian R, Zhang X, et al. Characterization of Dendritic Cell (DC)-10 in Recurrent Miscarriage and Recurrent Implantation Failure. *Reproduction* (2019) 158(3):247–55. doi: 10.1530/REP-19-0172
6. Amjadi F, Zandieh Z, Mehdizadeh M, Aghajanpour S, Raoufi E, Aghamajidi A, et al. The Uterine Immunological Changes may be Responsible for Repeated Implantation Failure. *J Reprod Immunol* (2020) 138:103080. doi: 10.1016/j.jri.2020.103080
7. Fukui A, Funamizu A, Fukuhara R, Shibahara H. Expression of Natural Cytotoxicity Receptors and Cytokine Production on Endometrial Natural Killer Cells in Women With Recurrent Pregnancy Loss or Implantation Failure, and the Expression of Natural Cytotoxicity Receptors on Peripheral Blood Natural Killer Cells in Pregnant Women With a History of Recurrent Pregnancy Loss. *J Obstet Gynaecol Res* (2017) 43(11):1678–86. doi: 10.1111/jog.13448
8. Nancy P, Erlebacher A. T Cell Behavior at the Maternal-Fetal Interface. *Int J Dev Biol* (2014) 58(2-4):189–98. doi: 10.1387/ijdb.140054ae
9. Yang KM, Ntrivalas E, Cho HJ, Kim NY, Beaman K, Gilman-Sachs A, et al. Women With Multiple Implantation Failures and Recurrent Pregnancy Losses Have Increased Peripheral Blood T Cell Activation. *Am J Reprod Immunol* (2010) 63(5):370–8. doi: 10.1111/j.1600-0897.2010.00811.x
10. Yin B, Zeng Y, Wu T, Yu S, Xu J, Liu S, et al. Functional Properties of Peripheral CD8(+) T Cells in Patients With Repeated Implantation Failure. *Am J Reprod Immunol* (2017) 78(2):e12704. doi: 10.1111/aji.12704
11. Shin HJ, Kim DY, Chung J, Shin KH, Lee H, et al. Prognostic Impact of Peripheral Blood T-Cell Subsets at the Time of Diagnosis on Survival in Patients With Diffuse Large B-Cell Lymphoma. *Acta Haematol* (2021) 144 (4):71–81. doi: 10.1159/000510912
12. Wang F, Nie J, Wang H, Zhao Q, Xiong Y, Deng L, et al. Characteristics of Peripheral Lymphocyte Subset Alteration in COVID-19 Pneumonia. *J Infect Dis* (2020) 221(11):1762–9. doi: 10.1093/infdis/jiaa150
13. Gardner DK, Lane M. Culture and Selection of Viable Blastocysts: A Feasible Proposition for Human IVF? *Hum Reprod Update* (1997) 3(4):367–82. doi: 10.1093/humupd/3.4.367
14. Simon A, Laufer N. Repeated Implantation Failure: Clinical Approach. *Fertil Steril* (2012) 97(5):1039–43. doi: 10.1016/j.fertnstert.2012.03.010
15. Gellersen B, Brosens I, Brosens J. Decidualization of the Human Endometrium: Mechanisms, Functions, and Clinical Perspectives. *Semin Reprod Med* (2007) 25(6):445–53. doi: 10.1055/s-2007-991042
16. Hanna J, Goldman-Wohl D, Hamani Y, Avraham I, Greenfield C, Natanson-Yaron S, et al. Decidual NK Cells Regulate Key Developmental Processes at the Human Fetal-Maternal Interface. *Nat Med* (2006) 12(9):1065–74. doi: 10.1038/nm1452
17. Kolanska K, Suner L, Cohen J, Ben Kraiem Y, Placais L, Fain O, et al. Proportion of Cytotoxic Peripheral Blood Natural Killer Cells and T-Cell Large Granular Lymphocytes in Recurrent Miscarriage and Repeated Implantation Failure: Case-Control Study and Meta-Analysis. *Arch Immunol Ther Exp (Warsz)* (2019) 67(4):225–36. doi: 10.1007/s00005-019-00546-5
18. Brummelman J, Pilipow K, Lugli E. The Single-Cell Phenotypic Identity of Human CD8(+) and CD4(+) T Cells. *Int Rev Cell Mol Biol* (2018) 341:63–124. doi: 10.1016/bs.ircmb.2018.05.007
19. Mousset CM, Hobo W, Woestenenk R, Preijers F, Dolstra H, van der Waart AB. Comprehensive Phenotyping of T Cells Using Flow Cytometry. *Cytom Part A* (2019) 95(6):647–54. doi: 10.1002/cyto.a.23724
20. Li Y, Yu S, Huang C, Lian R, Chen C, Liu S, et al. Evaluation of Peripheral and Uterine Immune Status of Chronic Endometritis in Patients With Recurrent Reproductive Failure. *Fertil Steril* (2020) 113(1):187–96. doi: 10.1016/j.fertnstert.2019.09.001
21. Zhu J. T Helper Cell Differentiation, Heterogeneity, and Plasticity. *Cold Spring Harb Perspect Biol* (2018) 10(10):a030338. doi: 10.1101/cshperspect.a030338
22. Rao DA. T Cells That Help B Cells in Chronically Inflamed Tissues. *Front Immunol* (2018) 9:1924. doi: 10.3389/fimmu.2018.01924
23. Shi J, Hou S, Fang Q, Liu X, Liu X, Qi H, et al. PD-1 Controls Follicular T Helper Cell Positioning and Function. *Immunity* (2018) 49(2):264–74.e4. doi: 10.1016/j.immuni.2018.06.012
24. Beck SE, Veenhuis RT, Blankson JN. Does B Cell Follicle Exclusion of CD8+ T Cells Make Lymph Nodes Sanctuaries of HIV Replication? *Front Immunol* (2019) 10:2362. doi: 10.3389/fimmu.2019.02362

25. Vinuesa CG, Linterman MA, Yu D, MacLennan IC. Follicular Helper T Cells. *Annu Rev Immunol* (2016) 34:335–68. doi: 10.1146/annurev-immunol-041015-055605
26. Monteiro C, Kasahara T, Sacramento PM, Dias A, Leite S, Silva VG, et al. Human Pregnancy Levels of Estrogen and Progesterone Contribute to Humoral Immunity by Activating T(FH) /B Cell Axis. *Eur J Immunol* (2021) 51(1):167–79. doi: 10.1002/eji.202048658
27. Zhou J, Wang Z, Zhao X, Wang J, Sun H, Hu Y. An Increase of Treg Cells in the Peripheral Blood is Associated With a Better *In Vitro* Fertilization Treatment Outcome. *Am J Reprod Immunol* (2012) 68(2):100–6. doi: 10.1111/j.1600-0897.2012.01153.x
28. Diao LH, Li GG, Zhu YC, Tu WW, Huang CY, Lian RC, et al. Human Chorionic Gonadotropin Potentially Affects Pregnancy Outcome in Women With Recurrent Implantation Failure by Regulating the Homing Preference of Regulatory T Cells. *Am J Reprod Immunol* (2017) 77(3):e12618. doi: 10.1111/aji.12618
29. Royster GD, Harris JC, Nelson A, Castro Y, Weitzel RP, Tisdale J, et al. Rapamycin Corrects T Regulatory Cell Depletion and Improves Embryo Implantation and Live Birth Rates in a Murine Model. *Reprod Sci* (2019) 26 (12):1545–56. doi: 10.1177/1933719119828110
30. Ghaebi M, Abdolmohammadi-Vahid S, Ahmadi M, Eghbal-Fard S, Dolati S, Nouri M, et al. T Cell Subsets in Peripheral Blood of Women With Recurrent Implantation Failure. *J Reprod Immunol* (2019) 131:21–9. doi: 10.1016/j.jri.2018.11.002
31. Comins-Boo A, Cristóbal I, Fernández-Arquero M, Rodríguez de Frías E, Calvo Urrutia M, Pilar Suárez L, Gasca Escorial P, et al. Functional NK Surrogate Biomarkers for Inflammatory Recurrent Pregnancy Loss and Recurrent Implantation Failure. *Am J Reprod Immunol* (2021) 86(2):e13426. doi: 10.1111/aji.13426
32. Hur YJ, Yu EJ, Choe SA, Paek J, Kim YS. Peripheral Blood Natural Killer Cell Proportion and Ovarian Function in Women With Recurrent Implantation Failure. *Gynecol Endocrinol* (2020) 36(10):922–5. doi: 10.1080/09513590.2020.1725969
33. Sacks G, Yang Y, Gowen E, Smith S, Fay L, Chapman M, et al. Detailed Analysis of Peripheral Blood Natural Killer Cells in Women With Repeated IVF Failure. *Am J Reprod Immunol* (2012) 67(5):434–42. doi: 10.1111/j.1600-0897.2012.01105.x
34. Clark DA, Croitoru K. TH1/TH2,3 Imbalance Due to Cytokine-Producing NK, Gammadelta T and NK-Gammadelta T Cells in Murine Pregnancy Decidua in Success or Failure of Pregnancy. *Am J Reprod Immunol* (2001) 45(5):257–65. doi: 10.1111/j.8755-8920.2001.450501.x

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Endocrinology

ORIGINAL RESEARCH
published: 22 July 2022
doi: 10.3389/fendo.2022.899000

# An Individualized Recommendation for Controlled Ovary Stimulation Protocol in Women Who Received the GnRH Agonist Long-Acting Protocol or the GnRH Antagonist Protocol: A Retrospective Cohort Study

*Ming-Xing Chen[1,2†], Xiang-Qian Meng[3†], Zhao-Hui Zhong[1,4], Xiao-Jun Tang[4], Tian Li[5], Qian Feng[6], Enoch Appiah Adu-Gyamfi[2], Yan Jia[7], Xing-Yu Lv[3], Li-Hong Geng[7], Lin Zhu[8], Wei He[8*], Qi Wan[3*] and Yu-Bin Ding[1,2*]*

[1] Department of Obstetrics and Gynecology, Women and Children's Hospital of Chongqing Medical University, Chongqing, China, [2] Joint International Research Laboratory of Reproduction and Development of the Ministry of Education of China, School of Public Health, Chongqing, China, [3] Reproductive Medical Center,Chengdu Xinan Gynecological Hospital, Chengdu, China, [4] Department of Epidemiology, School of Public Health and Management, Research Center for Medicine and Social Development, Innovation Center for Social Risk Governance in Health, Chongqing Medical University, Chongqing, China, [5] The Department of Reproductive Medicine, The First Affiliated Hospital of Chongqing Medical University, Chongqing, China, [6] Department of Gynecology, Chongqing Hospital of Traditional Chinese Medicine, Chongqing, China, [7] Infertility and Infertility Center, Chengdu Jinjiang Hospital for Women's and Children's Health, Chengdu, China, [8] Reproductive Medical Center, Southwest Hospital, Army Medical University, Third Military Medical University, Chongqing, China

**Background:** The GnRH agonist long-acting protocol and GnRH antagonist protocol are widely used in ovarian stimulation. Which protocol eliciting higher live birth rate for IVF/ICSI patients with different ages, different ovarian reserves and different body mass index (BMI) has not been studied. However, among these protocols, the one that elicits higher live birth in IVF/ICSI patients with different ages, ovarian reserves and body mass indexes (BMI) has not been identified.

**Methods:** This was a retrospective cohort study about 8579 women who underwent the first IVF-ET from January, 2018 to August, 2021. Propensity Score Matching (PSM) was used to improve the comparability between two protocols.

**Results:** After PSM, significant higher live birth rates were found in the GnRH agonist long-acting protocol compared to GnRH antagonist protocol (44.04% vs. 38.32%) ($p<0.001$). Stratified analysis showed that for those with AMH levels between 3 ng/ml and 6 ng/ml, with BMI ≥ 24 kg/m$^2$ and were aged ≥ 30 years old, and for those women with BMI < 24kg/m$^2$ and were aged ≥30 years whose AMH levels were ≤ 3ng/ml, the GnRH agonist long-acting protocol was more likely to elicit live births [OR (95%CI), 2.13

OPEN ACCESS

***Edited by:***
*Yimin Zhu,*
*Zhejiang University, China*

***Reviewed by:***
*Yong-Jiang Zhou,*
*Hainan Medical University, China*
*Zi Yang,*
*Peking University Third Hospital, China*
*Wei Wang,*
*Second Hospital of Hebei Medical University, China*

****Correspondence:***
*Wei He*
*anyhewei@163.com*
*Qi Wan*
*wanqi123@163.com*
*Yu-Bin Ding*
*dingyb@cqmu.edu.cn*

*†These authors share first authorship*

***Specialty section:***
*This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology*

***Received:*** *18 March 2022*
***Accepted:*** *17 June 2022*
***Published:*** *22 July 2022*

***Citation:***
*Chen M-X, Meng X-Q, Zhong Z-H, Tang X-J, Li T, Feng Q, Adu-Gyamfi EA, Jia Y, Lv X-Y, Geng L-H, Zhu L, He W, Wan Q and Ding Y-B (2022) An Individualized Recommendation for Controlled Ovary Stimulation Protocol in Women Who Received the GnRH Agonist Long-Acting Protocol or the GnRH Antagonist Protocol: A Retrospective Cohort Study. Front. Endocrinol. 13:899000. doi: 10.3389/fendo.2022.899000*

(1.19,3.80)], [OR (95%CI), 1.41(1.05,1.91)]. However, among women with BMI ≥ 24kg/$m^2$ and were aged ≥30 years whose AMH levels were ≤ 3ng/ml, the GnRH agonist long-acting protocol had a lower possibility of eliciting live births [OR (95%CI), 0.54(0.32,0.90)]. Also, among women with AMH levels between 3 ng/ml and 6 ng/ml, with BMI ≥ 24 kg/$m^2$ and with age < 30 years and for those with AMH levels between 3 ng/ml and 6 ng/ml, regardless of age, and with BMI<24kg/m2,, the possibility of live births was similar between the two protocols [OR (95%CI), 1.06(0.60,1.89)], [OR (95%CI), 1.38(0.97,1.97)], [OR (95%CI), 0.99(0.72,1.37)]. Among the women with AMH levels ≤ 3 ng/ml and with were aged < 30years, regardless of BMI, the possibility of live birth was similar between the two protocols [OR (95%CI), 1.02(0.68,1.54)], [OR (95%CI), 1.43(0.68,2.98)]. Moreover, among women with AMH levels ≥ 6ng/ml, the possibility of live birth was similar between the two protocols [OR (95%CI),1.42(0.75,2.69)], [OR (95%CI),1.02 (0.19,5.35)], [OR (95%CI), 1.68(0.81,3.51)], [OR (95%CI), 0.51(0.10,2.55)].

**Conclusions:** The suitability of the GnRH agonist long-acting protocol or GnRH antagonist protocol to infertility patients is dependent on specific biological characteristics of the patients.

**Keywords: GnRH agonist long-acting protocol, GnRH antagonist protocol, live birth rate, ovarian reserve, body mass index**

## INTRODUCTION

*In vitro* fertilization and embryo transfer (IVF-ET) is the most commonly patronized treatment option for women experiencing infertility. This is attributable to the increase in pregnancy rates of patients undergoing IVF-ET. A key to the improvement in pregnancy rate is the application of the controlled ovarian stimulation (COS) protocols (1, 2). Among the COS protocols that have been developed are the gonadotropin-releasing hormone (GnRH) agonist long protocol and the GnRH antagonist protocol (2, 3). The GnRH agonist long-acting protocol is one of the mainstream protocols of COS in China because of its advantages such as effectively improving endometrial receptivity and increasing the clinical pregnancy rate of fresh IVF cycles (4, 5). The GnRH antagonist protocol, on the other hand, is widely used because of its shorter duration of stimulation and its association with a low incidence of ovarian hyperstimulation syndrome (OHSS) (5–7).

Since both protocols are advantageous to some extent, clinicians have become indecisive about which one to fully rely upon. Previous studies that compared both protocols on live birth rates yielded seemingly conflicting findings. Yang et al. reported (8) that live birth rate, clinical pregnancy rate and implantation rate of the GnRH agonist long-acting protocol are significantly higher than those of the antagonist protocol. However, Wang et al. found (9) that there is no significant difference in live birth rate between both protocols in patients with normal ovarian reserves. Li et al. (10) observed that in patients with poor ovarian response, the GnRH agonist long-acting protocol is associated with higher live birth rates than the GnRH-antagonist protocol. These seemingly conflicting reports, together with the confounding factors such as variation in the basic characteristics of women, make it difficult to decide on which of the two protocols is optimal for IVF women. Hence, it is necessary to implement individualized COS protocols in accordance with the specific characteristics of the patients.

An important clinical feature of female infertility is ovarian reserve, which is also a crucial factor used in selecting the most appropriate COS protocol (11–13). Several studies have shown that AMH is a reliable marker of ovarian reserve (14–18), and has a significant correlation with age (19, 20). Due to this, AMH, combined with age, is commonly used to evaluate ovarian reserve in clinical practice.

It has been found that increased body mass index (BMI) affects the success of IVF (21, 22) as well as live births following IVF (23). Also, it has been observed that serum AMH is positively correlated with BMI in normal weight women with normal ovarian reserve (24). However, in women with polycystic ovary syndrome (PCOS), serum AMH was observed to correlate negatively with BMI (25). These findings indicate that BMI and AMH serum levels should be taken into account when establishing an individualized COS protocol. Thus, in this study, we retrieved the data of infertile women who had been exposed to the GnRH agonist long-acting protocol or the antagonist protocol, and assessed their live birth rate by combining the basic characteristics: age, BMI and AMH levels. Our findings would provide reference for clinical guidance and treatment of female infertility.

## MATERIAL AND METHODS

### Participants

Women who had undergone their first IVF/ICSI cycles between January, 2018 and August, 2021 at the Chengdu Xinan Gynecology Hospital and Chengdu Jinjiang Hospital for

Women's and Children's Health were retrospectively identified in the institutional database. Only women who received COS with GnRH agonist long-acting protocol or the GnRH antagonist protocol and received fresh embryo transfer were included in this study. Exclusion criteria were abnormal results on parental karyotyping, missing lab data, and incomplete live birth information. Patients' flow chart detailing the whole process is shown in **Figure 1**.

## GnRH Agonist Long-Acting Protocol

Each woman received a GnRH agonist (Diphereline, 3.75mg, Beaufort-Ipson, France) on the 2nd to 4th day of menstruation (follicular phase). Serum levels of sex hormones and ultrasound assessment of developing follicles were monitored on the 28th to the 35th day after GnRH agonist administration. The following criteria were used: (a) endometrial thickness < 5mm, (b) estradiol (E2) < 50pmol/L, luteinizing hormone (LH) < 5IU/L follicle-stimulating hormone (FSH) <5 IU/L, progesterone (P) of <1 ng/ml, (c) no functional cyst, (d) follicle size 3–5 mm under ultrasound. In accordance with the patient's age, BMI, antral follicle number (AFC) and AMH levels, we determined the initial dose of gonadotropin (Gn) that could control ovulation. The dosage was adjusted continually according to the patient's ovarian reaction and follicular growth. 250 μg of recombinant human chorionic gonadotropin (rhCG, Merck Schlano, Germany) were given to each woman until two to three ovarian follicles were, at least, 17–18 mm in diameter. Oocyte retrieval was performed 36 hours post-hCG injection.

## GnRH Antagonist Protocol

In accordance with the patient's age, BMI, antral follicle number (AFC) and AMH levels, recombinant FSH 100 ~ 300 IU/d (rFSH, Gonal-F, Merck Serono S.A., Switzerland) administration was done from the 2nd to the 4th day of the menstrual cycle. This was followed by Gn administration. The Gn dosage was adjusted as the follicles developed. A daily dose of 0.25 mg GnRH-ant (Ganerik acetate, Merck Serono, Switzerland) was started either on the 6th day of rFSH stimulation until the hCG injection or when the dominant follicle's diameter was ≥ 12-14 mm. The induction of ovulation was performed by administering the women with 250 μg of rhCG (Merck Schlano, Germany) or with the 0.2 mg of Decapeptyl either alone or in combination with, 2000 IU of urinary hCG [Merck Schlano]). This was done during the period when two to three ovarian follicles were, at least, 17–18 mm in diameter. Oocyte retrieval was performed 36 hours after the ovulation induction.

## Embryo Transfer and Luteal Support

On the 3rd to 5th day after fertilization, 1 to 2 of grade I-II high-quality embryos were selectively transferred. Embryo grading was done in accordance with the proceedings of the Istanbul consensus (26). The luteal phase support was started on the day when the oocytes were retrieved with 200 mg intravaginal progesterone soft capsules for 8 hours/times. 20mg of dydrogesterone (Dupbaston, Dutch) was taken twice on each day.

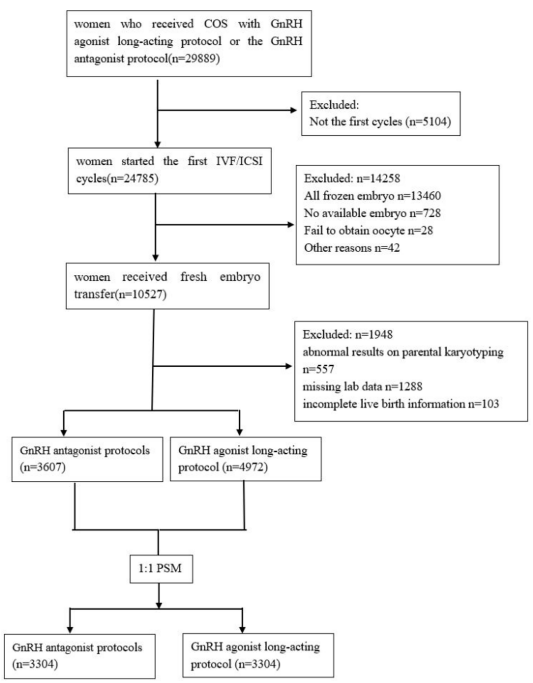



**FIGURE 1** | Flow chart.

## Outcome Measures

The primary outcome measure was the live birth which was defined as the delivery of any living baby at or after 28 weeks of pregnancy during the first embryo transfer. Live birth rate = number of live birth cycles/number of embryo transfer cycles. The secondary outcomes were biochemical pregnancy rate, clinical pregnancy rate, incidence of ovarian hyperstimulation syndrome (OHSS), number of retrieved oocytes, number of metaphase II (MII) oocytes, and number of 2 pronuclear (2PN) embryos. The biochemical pregnancy was defined as the serum β-HCG>25U/L 14 days after embryo transfer Clinical pregnancy, defined as the presence of gestational sac or fetal heart, was confirmed with transvaginal ultrasound 28 days after embryo transfer. The OHSS was defined according to the Golan et al' criteria (27).

## Statistical Analysis

Propensity Score Matching (PSM) was used in data analysis to balance the baseline and improve the comparability between GnRH agonist long-acting protocol group and GnRH antagonist protocol group. The variables in PSM model included female age, BMI, duration of infertility, type of infertility, basal sex hormone (E2, P, FSH, LH), AFC, AMH, insemination methods, the number of good quality embryos transferred and the type of embryos transferred. A 1:1 nearest neighbor matching method with caliper (0.1) was used to match data between groups.

Continuous variables are expressed as mean ± SD or median (IQR); and Categorical variables are expressed as number (n) and percentage (%). Normality was checked through Shapiro-Wilk normality test. Mann-Whitney U test or Student's t-tests were used for continuous variables and the Chi-square test was used for categorical variables.

Multivariate logistic regression analysis was performed to compare the live birth rates between the two protocols. Additional analyses were performed after stratification of the participants by age (age<30 years vs age≥30 years) (28), BMI (BMI<24 kg/m$^2$ vs BMI≥24 kg/m$^2$), AMH levels (AMH ≤ 3ng/ml vs 3ng/ml<AMH<6ng/ml vs AMH≥6ng/ml) (29) and also after combining the above three parameters. All analyses were performed using the Statistical Package for the Social Sciences (Version 25.0; SPSS, Chicago, IL). P <0.05 was used to indicate a significant statistical difference.

# RESULTS

## Demographic, Cycle Characteristics and Pregnancy Outcomes Calculated Without Specific Stratification

The demographic characteristics, cycle characteristics and pregnancy outcomes of the study participants before and after PSM are shown in **Tables 1**, **2**. Before PSM, a total of, 8579 cycles were included in this study. Significant differences in the comparison of baseline characteristics were observed between two groups in age, BMI, AMH, AFC, basal FSH, basal LH, basal E2, basal P, Gn dose, duration of Gn, number of good quality embryos transferred., 4972 of the cycles used the GnRH agonist long-acting protocol and generated 45.09% of live birth rate while, 3607 of the cycles used the GnRH antagonist protocol and generated 38.70% of live birth rate. After 1:1 matching, a total of, 6608 cycles were analyzed in this study. There were no significant differences in age, BMI, basal FSH, basal LH, and the number of good quality embryos transferred between the two groups. However, the GnRH agonist long protocol group still received a higher gonadotropin dosage (1875IU vs, 1800IU) and longer gonadotropin exposure duration (12 vs 9) than the antagonist protocol group., 3304 of the cycles used the GnRH agonist long-acting protocol and generated 44.04% of live birth rate while, 3304 of the cycles used the GnRH antagonist protocol and generated 38.32% of live birth rate. The live birth rate of the GnRH agonist long-acting protocol group was significantly higher than that of the GnRH antagonist protocol group before and after matching(P<0.001).

After matching, the number of oocytes retrieved (9.69 ± 4.22 vs 9.16 ± 4.28), the mature eggs number (8.51 ± 3.90 vs 8.05 ± 3.91), the biochemical pregnancy rate (60.90% vs 55.75%), the clinical pregnancy rate (53.03% vs 47.79%) and the incidence of OHSS (4.57% vs 1.91%) were higher in the GnRH agonist long-acting protocol group than in the antagonist protocol group. Nonetheless, the ectopic pregnancy rates (1.43% vs 4.12%) in the GnRH agonist long-acting protocol group were significantly lower than those of the GnRH antagonist protocol group. There was no significant difference in the two-Pro-Nuclei (2PN) fertilized eggs number (6.07 ± 3.27 vs 6.05 ± 3.32), early miscarriage (13.87% vs 14.06%) and late miscarriage rate (1.94% vs 2.28%) between two groups (**Table 2**).

## Live Birth Measured With Stratification Analysis Using Multivariate Logistic Regression

Before and after matching, and after adjusting for potential confounding factors (such as age, BMI, AMH, AFC, basal FSH, basal LH, basal E2, basal P, Gn dose, duration of Gn, number of good quality embryos transferred), the multivariate logistic regression analysis showed that the GnRH agonist long-acting protocol was associated with a higher possibility of having live birth than that of the GnRH antagonist protocol [OR (95%CI), 1.25 (1.01,1.53)], P<0.001; [OR (95%CI), 1.20(1.00,1.43)], P=0.002 (**Table 3**).

To find the live birth rate of the GnRH agonist long or antagonist protocols in patients with different characteristics, we carried out a further analysis by stratifying the patients according to their ages, BMIs and AMH levels. After matching, the multivariate logistic regression analysis showed a significantly higher possibility of having live births of each layer stratified by age in the GnRH agonist long protocol group than in the GnRH antagonist protocol group [OR (95%CI), 1,24(1.10,1.40)], [OR (95%CI), 1.24(1.08,1.42)]. For women with BMI <24kg/m$^2$, the GnRH agonist long-acting protocol was associated with a higher opportunity of getting live births [OR (95%CI), 1.28(1.10,1.50)]; for women with overweight (BMI≥24kg/m$^2$), the two protocols had similar live birth rates [OR (95%CI),1.17(0.90,1.52)]. When

**TABLE 1 |** Comparison of baseline parameters between the GnRH agonist long-acting protocol and GnRH antagonist protocol and after PS matching.

| | Before matching | | | after matching | | |
|---|---|---|---|---|---|---|
| | **GnRH antagonist** | **GnRH agonist** | **P value** | **GnRH antagonist** | **GnRH agonist** | **P value** |
| NO. of cycles | 3607 | 4972 | | 3304 | 3304 | |
| Female age (year) | 30.59 ± 4.18 | 30.39 ± 3.75 | 0.021* | 30.58 ± 4.17 | 30.49 ± 3.78 | 0.884 |
| BMI (kg/m2) | 22.28 ± 3.20 | 21.92 ± 3.01 | < 0.001* | 22.15 ± 3.13 | 22.10 ± 3.10 | 0.523 |
| [a]Duration of infertility (years) | 3 (2,5) | 3 (2,5) | 0.107 | 3 (2,5) | 3 (2,5) | 0.809 |
| [a]Basal FSH (MIU/mL) | 7.54 (6.49,8.77) | 7.45 (6.35,8.70) | 0.002* | 7.54 (6.49,8.77) | 7.52 (6.46,8.84) | 1 |
| [a]Basal LH (MIU/mL) | 3.96 (2.95,5.30) | 3.87 (2.84,5.17) | 0.001* | 3.88 (2.89,5.12) | 3.92 (2.90,5.27) | 0.389 |
| [a]Basal E2 (p g/mL) | 44 (34,57) | 47 (36,62) | < 0.001* | 44 (34,57) | 47 (35,61) | < 0.001* |
| [a]Basal P (ng/mL) | 0.56 (0.38,0.80) | 0.58 (0.39,0.84) | < 0.001* | 0.56 (0.38,0.80) | 0.58 (0.39,0.84) | < 0.001* |
| [a]AFC | 15 (10,21) | 14 (11,18) | < 0.001* | 14 (10,20) | 15 (11,19) | 0.002* |
| [a]AMH (ng/mL) | 3.28 (1.98,5.21) | 3.11 (2.31,4.17) | < 0.001* | 3.04 (1.90,4.82) | 3.25 (2.38,4.38) | < 0.001* |
| [a]Total dose of Gn (IU) | 1800 (1425,2100) | 1875 (1500,2325) | < 0.001* | 1800 (1488,2175) | 1875 (1500,2325) | < 0.001* |
| [a]Duration of Gn (d) | 9 (8,10) | 12 (11,13) | < 0.001* | 9 (8,10) | 12 (10,13) | < 0.001* |
| Cause of infertility | | | 0.872 | | | 0.755 |
| Tubal factor | 2036 (56.45%) | 2868 (57.68%) | | 1863 (56.39%) | 1894 (57.32%) | |
| Pelvic and uterine factor | 309 (8.57%) | 407 (8.19%) | | 290(8.78%) | 266 (8.05%) | |
| PCOS | 200 (5.54%) | 256 (5.15%) | | 190 (5.75%) | 176 (5.33%) | |
| male factor | 593 (16.44%) | 815 (16.39%) | | 531 (16.07%) | 549 (16.62%) | |
| female and male factors | 185 (5.13%) | 245 (4.93%) | | 174 (5.27%) | 161 (4.87%) | |
| Other causes | 284 (7.87%) | 381 (7.66%) | | 256 (7.75%) | 258 (7.81%) | |
| Infertility type (n, %) | | | 0.592 | | | 1 |
| Primary infertility | 1837 (50.93%) | 2503 (50.34%) | | 1665 (50.39%) | 1665 (50.39%) | |
| Secondary infertility | 1770 (49.07%) | 2469 (49.66%) | | 1639 (49.61%) | 1639 (49.61%) | |
| Fertilization method (n, %) | | | 0.572 | | | 0.899 |
| IVF | 2931 (81.26%) | 4064 (81.74%) | | 2689 (81.39%) | 2693 (81.51%) | |
| ICSI | 676 (18.74%) | 908 (18.26%) | | 615 (18.61%) | 611 (18.49%) | |
| No. of embryos transferred (n, %) | | | 0.866 | | | 0.734 |
| 1 | 725 (20.10%) | 992 (19.95%) | | 658 (19.92%) | 647 (19.58%) | |
| 2 | 2882 (79.90%) | 3980 (80.05%) | | 2646 (80.08%) | 2657 (80.42%) | |
| Embryo type (n, %) | | | 0.301 | | | 0.933 |
| Day3 | 2648 (73.41%) | 3600(72.41%) | | 2429 (73.52%) | 2432 (73.61%) | |
| Day5 | 959 (26.59%) | 1372 (27.59%) | | 875 (26.48%) | 872 (26.39%) | |
| No. of good quality embryos transferred (n, %) | | | 0.001* | | | 0.936 |
| 0 | 1118 (31.00%) | 1371 (27.57%) | | 987 (29.87%) | 976 (29.54%) | |
| 1 | 1153 (31.97%) | 1732 (34.84%) | | 1087 (32.90%) | 1085 (32.84%) | |
| 2 | 1336 (37.04%) | 1869 (37.59%) | | 1230 (37.23%) | 1243 (37.62%) | |

*BMI, body mass index; AFC, antral follicular count; AMH, anti-Müllerian hormone; FSH, follicle stimulating hormone; LH, luteinizing hormone; E2, estradiol; P, Progesterone; Gn, Gonadotropin; ICSI, intracytoplasmic single sperm injection; IVF, in vitro fertilization;*

*Data are presented as mean ± SD, median (IQR) and n (%).*

*Chi-square test, Mann-Whitney U test and Student's t-tests were used for the preliminary comparison between the two groups.*

*[a]Cited as median (IQR).*

**Statistically significant (P < 0.05).*

**TABLE 2 |** Comparison of clinical outcomes between the GnRH agonist long-acting protocol and GnRH antagonist protocol and after PS matching.

| | Before matching | | | after matching | | |
|---|---|---|---|---|---|---|
| | **GnRH antagonist** | **GnRH agonist** | **P value** | **GnRH antagonist** | **GnRH agonist** | **P value** |
| Number of retrieved oocytes | 9.30 ± 4.37 | 9.73 ± 4.22 | <0.001* | 9.16 ± 4.28 | 9.69 ± 4.22 | <0.001* |
| Number of MII oocytes | 8.17 ± 3.99 | 8.57 ± 3.90 | <0.001* | 8.05 ± 3.91 | 8.51 ± 3.90 | <0.001* |
| Number of 2PN embryos | 6.13 ± 3.37 | 6.14 ± 3.29 | 0.941 | 6.05 ± 3.32 | 6.07 ± 3.27 | 0.802 |
| [b] OHSS rate | 2.13% (77/3607) | 4.42% (220/4972) | <0.001* | 1.91% (63/3304) | 4.57% (151/3304) | <0.001* |
| [b] Live birth | 38.70% (1396/3607) | 45.09% (2242/4972) | <0.001* | 38.32% (1266/3304) | 44.04% (1455/3304) | <0.001* |
| [b] biochemical pregnancy | 55.78% (2012/3607) | 61.38% (3052/4972) | <0.001* | 55.75% (1842/3304) | 60.90% (2012/3304) | <0.001* |
| [b] Clinical pregnancy | 47.91% (1728/3607) | 53.74% (2672/4972) | <0.001* | 47.79% (1579/3304) | 53.03% (1752/3304) | <0.001* |
| [b] ectopic pregnancy | 3.99% (69/1728) | 1.46% (39/2672) | <0.001* | 4.12% (65/1579) | 1.43% (25/1752) | <0.001* |
| [b] Early Miscarriage | 13.54% (234/1728) | 13.14% (351/2672) | 0.699 | 14.06% (222/1579) | 13.87 (243/1752) | 0.875 |
| [b] Late Miscarriage | 2.26% (39/1728) | 1.72% (46/2672) | 0.208 | 2.28% (36/1579) | 1.94% (34/1752) | 0.495 |

*MII, metaphase II; 2PN, 2 pronuclear; OHSS, ovarian hyperstimulation syndrome;*

*Data are presented as mean ± SD and n (%).*

*Student's t-tests and Chi-square test were used for comparison of clinical outcomes between the two groups.*

**Statistically significant (P < 0.05).*

the population was stratified by AMH, for women with normal ovarian reserves (3ng/ml<AMH<6ng/ml), we found a significantly higher possibility of live birth in the GnRH agonist long protocol group than in the GnRH antagonist protocol group [OR (95%CI), 1.24(1.02,1.52)]; Among women with AMH≥ 3ng/ml or AMH≥ 6ng/ml, the chances of getting live births were similar between the two groups. [OR (95%CI),1.12(0.92,1.38)], [OR (95%CI), 1.41(0.92,2.15)] (**Table 3**).

After matching, the study population was divided into 12 groups according to the combination of AMH levels, age and BMI (**Table 4**). The multivariate logistic regression analysis showed that for younger women (age<30 years old), regardless of their BMI and ovarian reserves, the GnRH agonist long-acting protocol was more likely to elicit live births than the antagonist protocol, although the difference was not statistically significant. However, among women who were above 30 years old and who had normal ovarian reserves (3ng/ml<AMH<6ng/ml) and variable BMI, the abilities of the two protocols to elicit live births may differ significantly. For women who had AMH levels from 3ng/ml to 6ng/ml (3ng/ml<AMH<6ng/ml), were aged ≥ 30 years old and had BMI ≥ 24kg/m$^2$, the GnRH agonist long-acting protocol was more likely to have live births than the antagonist protocol [OR (95%CI), 2.13(1.19,3.80)]; while among the women with normal ovarian reserves, were aged ≥30 years old and had BMI < 24kg/m$^2$, the chances to have live births were similar between the two protocol groups [OR (95%CI),0.99(0.72,1.37)]. Among women with AMH ≤ 3ng/ml, aged ≥ 30 years old and with BMI < 24kg/m$^2$, the GnRH agonist long-acting protocol had a higher possibility to live births than the antagonist protocol [OR (95%CI), 1.41(1.05,1.91)]. Interestingly, for women with AMH ≤ 3ng/ml, age ≥ 30 years old and BMI≥ 24kg/m$^2$, the GnRH agonist long-acting protocol had a lower possibility of live births the antagonist protocol [OR (95%CI), 0.54(0.32,0.90)]. Among the women who had AMH level ≥ 6ng/ml, aged ≥ 30 years old and had BMI < 24kg/m$^2$, the possibilities to have live births were similar between the two protocols [OR (95%CI), 1.68(0.81,3.51)] However, among the women who had AMH level ≥ 6ng/ml, aged ≥ 30 years old and with BMI≥ 24kg/m$^2$, the GnRH agonist long-acting protocol had a lower possibility of eliciting live birth than the antagonist protocol [OR (95%CI), 0.51(0.10,2.55)]. Before matching, and after adjusting potential confounding factors, the multivariate logistic regression analysis showed that for younger women (age<30 years old), who had normal ovarian reserves and with BMI < 24kg/m$^2$, the GnRH agonist long-acting protocol was more likely to elicit live births than the antagonist protocol [OR (95%CI),1.58(1.16, 2.16)] (**Supplemental Table 1**).

## DISCUSSION

Providing an individualized IVF-ET protocol, *via* individual characteristics, so as to maximize the rate of pregnancy and live births while reducing OHSS and adverse pregnancy outcomes, is still a big challenge in clinical medicine. In this study, we first analyzed the variables of the participants without any special stratification; and observed that the GnRH agonist long-acting protocol group had higher live birth rates, biochemical pregnancy rates and clinical pregnancy rates than the antagonist protocol group (**Tables 2**, **3**). This is consistent with the findings of other studies (4, 30) which showed that in the fresh cycle, the GnRH agonist long-acting protocol group had a higher clinical pregnancy rate and implantation rate than the GnRH antagonist protocol group. The mRNA and protein levels of HOXA10, MEIS1 and LIF, which are markers of uterine development and endometrial receptivity (31, 32), were found to be higher in the GnRH agonist long-acting protocol group than in the antagonist protocol group. This indicates that the GnRH agonist long-acting protocol, unlike the antagonist protocol, may have a less association with the impairment of the patients' endometrial receptivity. In addition, we found that the GnRH agonist long-acting protocol was associated with a higher risk of OHSS (4.57% vs 1.91%), which is consistent with Toftager et al's results (33). These findings indicate that the GnRH agonist long-acting protocol, rather than the GnRH antagonist protocol, may be more beneficial to women who undergo ART therapy.

To date, there is no single COS solution that works for all infertile women. Zhang et al. (34) indicated that the choice of COS protocol is highly dependent on ovarian reserve and age. Marci et al. (35) reported that high BMI could impair the ovarian response to exogenous gonadotropins. However, it is not a common practice to combine these factors to select a COS protocol for infertile women. Therefore, to explore whether women with different characteristics are more suitable for any protocol, we divided the study population into several groups according to the ages, AMH levels and BMI of the study participants. We found that among women with normal ovarian reserve, BMI < 24kg/m$^2$ and age <30 years old, the GnRH agonist long-acting protocol was associated with a higher possibility of having live birth than that of the GnRH antagonist protocol [OR (95%CI),1.58(1.16,2.16)] (**Supplemental Table 1**). Grow et al. (36) reported that good-prognosis patients had higher live birth rate with the GnRH agonist long-acting protocol than with the antagonist protocol [OR (95%CI),1.13 (1.03,1.25)]. The results of this study are consistent with our findings. Additionally, in overweight women (BMI ≥ 24 kg/m2) with normal ovarian reserve, the women aged ≥ 30 years old had higher live birth rates with the GnRH agonist long-acting protocol than with the antagonist protocol [OR (95%CI), 2.13 (1.19,3.80)]. Also, our results showed that a higher number of oocytes was retrieved in the GnRH agonist long-acting protocol group than in the antagonist protocol group. Since a decline in the number of oocyte as well as the increase of age, old age (37) and embryo aneuploidy (38) are crucial factors of infertility, the GnRH agonist long-acting protocol is recommended for infertile women with normal ovarian reserve, who have BMI<24kg/m2 and are aged <30 years old as well as those who have normal ovarian reserve have BMI ≥ 24kg/m2 and are aged ≥ 30 years.

Further, in women with normal ovarian reserve (3ng/ml < AMH < 6ng/ml), with BMI < 24kg/m2 and are aged ≥30 years old or with BMI ≥ 24 kg/m2 and with ages < 30 years old, the possibilities to have live births were similar between the two protocols [OR (95%CI), 0.99(0.72,1.37)], [OR (95%CI), 1.06 (0.60,1.89)]. Our results are consistent with that of a meta-

**TABLE 3 |** Comparison of live birth rate of the GnRH agonist long-acting protocol and GnRH antagonist protocol using multivariable logistic regression analysis in subgroup women with different BMI, AMH and age and after PS matching. (the GnRH antagonist protocol as a reference).

| | Before matching | | after matching | |
|---|---|---|---|---|
| | **Adjusted OR (95% CI)** | **P** | **Adjusted OR (95% CI)** | **P** |
| Total | 1.24 (1.10,1.40) | <0.001 | 1.24 (1.08,1.42) | 0.002 |
| Age (year) | | | | |
| <30 | 1.35 (1.12,1.61) | 0.001* | 1.25 (1.01,1.53) | 0.036* |
| ≥30 | 1.14 (0.973,1.33) | 0.105 | 1.20 (1.00,1.43) | 0.047* |
| BMI (kg/m2) | | | | |
| <24.0 | 1.30 (1.13,1.49) | <0.001* | 1.28 (1.10,1.50) | 0.002* |
| ≥24.0 | 1.11 (0.88,1.41) | 0.382 | 1.17 (0.90,1.52) | 0.249 |
| AMH (ng/ml) | | | | |
| AMH ≤ 3 | 1.12 (0.94,1.34) | 0.205 | 1.12 (0.92,1.38) | 0.264 |
| 3 <AMH<6 | 1.31 (1.09,1.57) | 0.004* | 1.24 (1.02,1.52) | 0.035* |
| AMH≥6 | 1.21 (0.83,1.76) | 0.314 | 1.41 (0.92,2.15) | 0.115 |

*CI, confidence interval*

*adjusting for confounders of female age, female BMI, AMH, AFC, basal E2, basal FSH, basal LH, basal P, number of good quality embryos, total dose of Gn, duration of Gn.*

**Statistically significant (P < 0.05).*

analysis (9) which showed no difference between the agonist protocol group and the antagonist protocol group of women with normal ovarian reserves (OR [95% CI] = 0.95 [0.74, 1.09], P = 0.27). Al-Inany et al. [35] found that compared to the GnRH agonist long-acting protocol, the antagonist protocol significantly reduced the incidence of any grade of OHSS (OR 0.61, 95% C 0.51 to 0.72; 36 RCTs, n = 7944, I2 = 31%, moderate quality evidence) without affecting the live birth rate (OR 1.02, 95% CI 0.85 to 1.23; 12 RCTs, n = 2303, I2 = 27%, moderate quality evidence). Therefore, the antagonist protocol is recommended for infertile women with normal ovarian reserve, with BMI < 24kg/m$^2$ and with ages ≥30 years or with BMI ≥ 24kg/m$^2$ and with ages < 30 years.

Other studies (33, 39–41) have reported that the GnRH antagonist protocol is safer for women with a low and high ovarian reserve, just that live birth rates are similar in both protocols. Our study with larger sample size further revealed that, regardless of age and BMI, among women with relatively high ovarian reserve (AMH ≥ 6 ng/ml), the two protocols had similar live birth rates. Particularly, in women with relatively high ovarian reserve (AMH ≥ 6 ng/ml), with BMI ≥ 24kg/m2 and have ages ≥30 years, the possibility of getting live birth in the GnRH agonist protocol was lower although the difference was not significant [OR (95%CI), 0.54(0.32,0.90)]. Moreover, among younger (age <30 years) women with relatively low ovarian reserve (AMH≤ 3ng/ml), regardless of BMI, the live birth rate was similar in the two protocols. Therefore, the GnRH antagonist protocol is strongly recommended for women with the above characteristics.

Li et al. (10) reported that among women in POSEIDON group 4 of advanced age and have diminished ovarian reserves, the GnRH agonist long-acting protocol and the antagonist protocol achieved comparable live birth rates. However, our study found that among the women with relatively low ovarian reserve (AMH ≤ 3ng/ml), with ages ≥ 30 years old and with BMI < 24kg/m2, the GnRH agonist long-acting protocol was more likely to have live births than the antagonist protocol [OR (95% CI), 1.41(1.05,1.91)]; while among women with relatively low ovarian reserve (AMH ≤ 3ng/ml), with age ≥ 30 years old and with BMI ≥ 24kg/m2, the GnRH agonist long-acting protocol had a lower possibility of live birth than the antagonist protocol [OR (95%CI), 0.54(0.32,0.90)]. These indicate that BMI is a vital

**TABLE 4 |** Multivariable logistic regression analysis of live birth rate of the GnRH agonist long-acting protocol and GnRH antagonist protocol for women with different AMH, Age and BMI (after PS matching) (the GnRH antagonist protocol group as a reference).

| | | after matching | | | |
|---|---|---|---|---|---|
| | | **Adjusted OR (95% CI) BMI<24.0kg/m2** | **P** | **Adjusted OR (95% CI) BMI≥24.0kg/m2** | **P** |
| age<30year | | | | | |
| | AMH ≤ 3ng/ml | 1.02 (0.68,1.54) | 0.909 | 1.43 (0.68,2.98) | 0.342 |
| | 3ng/ml <AMH<6ng/ml | 1.38 (0.97,1.97) | 0.072 | 1.06 (0.60,1.89) | 0.842 |
| | AMH≥6ng/ml | 1.42 (0.75,2.69) | 0.286 | 1.02 (0.19,5.35) | 0.985 |
| age≥30year | | | | | |
| | AMH ≤ 3ng/ml | 1.41 (1.05,1.91) | 0.024* | 0.54 (0.32,0.90) | 0.018* |
| | 3ng/ml <AMH<6ng/ml | 0.99 (0.72,1.37) | 0.964 | 2.13 (1.19,3.80) | 0.011* |
| | AMH≥6ng/ml | 1.68 (0.81,3.51) | 0.164 | 0.51 (0.10,2.55) | 0.413 |

*adjusting for confounders of female age, BMI, AMH, AFC, E2, FSH, LH, P, number of good quality embryos, total dose of Gn, duration of Gn.*

**Statistically significant (P < 0.05).*

factor to be considered in a personalized COS protocol. Unfortunately, to the best of our knowledge, there have been no studies comparing the GnRH agonist long-acting protocol and the GnRH antagonist protocol in women who have low ovarian reserve and who have different BMIs. Rabinson et al. (42) showed that in general women with BMI < 25kg/m2, the GnRH agonist protocol had a higher pregnancy rate. Although the ovarian reserve of women included in the study was not selected, the trend of their results was consistent with ours. These findings show that the GnRH agonist long-acting protocol may be more suitable for women with relatively low ovarian reserve (AMH ≤ 3ng/ml), with ages ≥ 30 years old and with BMI < 24kg/m2. Nevertheless, among women with relatively low ovarian reserve (AMH ≤ 3ng/ml), with age ≥ 30 years old and with BMI ≥ 24kg/$m^2$, the GnRH antagonist protocol is recommended since it can help avoid the excessive suppression of the pituitary-gonadal axis and the concentrations of endogenous FSH and LH (43).

To our knowledge, this is the first study to compare the live birth rates of the GnRH agonist long-acting protocol and antagonist protocol in women with different characteristics by combining BMI with ovarian reserve markers. In spite of all the efforts to control bias, this study is inherently limited by the review of a retrospectively collected data set. In addition, this study did not follow up to the frozen embryo cycle, and could not provide relevant indicators such as cumulative live birth rate.

## CONCLUSION

Among infertile women who receive fresh embryo transfer after the first IVF treatment, the GnRH agonist long-acting protocol is recommended for women with normal ovarian reserve (3ng/ml < AMH < 6ng/ml), with BMI<24 kg/$m^2$ and with ages<30 years, and for those with normal ovarian reserve (3ng/ml < AMH < 6ng/ml), with BMI ≥ 24 kg/$m^2$ and are aged above 30 years. It is also recommended for women with BMI < 24kg/$m^2$ and with ages<30 years whose AMH levels are ≤ 3ng/ml. However, among the remaining infertile women in the cohort, the antagonist protocol may suite them because of the lower incidence of ovarian hyperstimulation syndrome, duration and dosage of Gn. Taken together, our results may provide a personalized recommendation in COS protocol selection. The recommendation of two protocols for women in different characters is shown in **Supplemental Table 2**.

## DATA AVAILABILITY STATEMENT

The data used in this article were obtained from the Chengdu Xinan Gynecology Hospital and Chengdu Jinjiang Hospital for Women's and Children's Health by request. Upon data request, the corresponding author would obtain permission from the Chengdu Xinan Gynecology Hospital and Chengdu Jinjiang Hospital for Women's and Children's Health before sharing them.

## AUTHOR CONTRIBUTIONS

M-XC contributed to study design, data collection, statistical analysis and drafting of the manuscript. QW assisted with data collection and interpretation. X-JT reviewed the analyzed results. Z-HZ reviewed the analyzed results and revised the manuscript. X-QM, TL, QF, YJ, X-YL, L-HG, LZ and QW provided ART-related clinical theory and technical support. Y-BD and Enoch Appiah Adu-Gyamfi critically revised the manuscript. All authors contributed to the article and approved the submitted version.

## FUNDING

This study was funded by the the National Natural Science Foundation of China (Grant No. 81971391), National Key Research and Development Program of China (2017YFC1002001), and the Chongqing Science and Technology Bureau (Grant No. cstc2019jxjl130030).

## ACKNOWLEDGMENTS

The authors are grateful to all the physicians, nurses and laboratory staff (at the Chengdu Xinan Gynecology Hospital and Chengdu Jinjiang Hospital for Women's and Children's Health) who contributed to this work.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.899000/full#supplementary-material

## REFERENCES

1. Bosch E, Ezcurra D. Individualised Controlled Ovarian Stimulation (Icos): Maximising Success Rates for Assisted Reproductive Technology Patients. *Reprod Biol Endocrinol* (2011) 9:82. doi: 10.1186/1477-7827-9-82
2. Racca A, Drakopoulos P, Neves AR, Polyzos NP. Current Therapeutic Options for Controlled Ovarian Stimulation in Assisted Reproductive Technology. *Drugs* (2020) 80(10):973–94. doi: 10.1007/s40265-020-01324-w
3. Pacchiarotti A, Selman H, Valeri C, Napoletano S, Sbracia M, Antonini G, et al. Ovarian Stimulation Protocol in Ivf: An Up-to-Date Review of the Literature. *Curr Pharm Biotechnol* (2016) 17(4):303–15. doi: 10.2174/1389201017666160118103147
4. Geng Y, Xun Y, Hu S, Lai Q, Jin L. Gnrh Antagonist Versus Follicular-Phase Single-Dose Gnrh Agonist Protocol in Patients of Normal Ovarian Responses During Controlled Ovarian Stimulation. *Gynecol Endocrinol* (2019) 35(4):309–13. doi: 10.1080/09513590.2018.1528221
5. Depalo R, Jayakrishan K, Garruti G, Totaro I, Panzarino M, Giorgino F, et al. Gnrh Agonist Versus Gnrh Antagonist in *In Vitro* Fertilization and Embryo Transfer (Ivf/Et). *Reprod Biol Endocrinol* (2012) 10:26. doi: 10.1186/1477-7827-10-26
6. Al-Inany HG, Abou-Setta AM, Aboulghar M. Gonadotrophin-Releasing Hormone Antagonists for Assisted Conception: A Cochrane Review. *Reprod Biomedicine Online* (2007) 14(5):640–9. doi: 10.1016/s1472-6483(10)61059-0

7. Zhang Y, Liu L, Qin J, Huang H, Xue L, Wang S, et al. Evaluation of Gnrh Antagonist Pretreatment Before Ovarian Stimulation in a Gnrh Antagonist Protocol in Normal Ovulatory Women Undergoing Ivf/Icsi: A Randomized Controlled Trial. *Reprod Biol Endocrinol* (2021) 19(1):158. doi: 10.1186/s12958-021-00836-8
8. Yang R, Guan Y, Perrot V, Ma J, Li R. Comparison of the Long-Acting Gnrh Agonist Follicular Protocol With the Gnrh Antagonist Protocol in Women Undergoing *In Vitro* Fertilization: A Systematic Review and Meta-Analysis. *Adv Ther* (2021) 38(5):2027–37. doi: 10.1007/s12325-020-01612-7
9. Wang R, Lin S, Wang Y, Qian W, Zhou L. Comparisons of Gnrh Antagonist Protocol Versus Gnrh Agonist Long Protocol in Patients With Normal Ovarian Reserve: A Systematic Review and Meta-Analysis. *PLoS One* (2017) 12(4):e0175985. doi: 10.1371/journal.pone.0175985
10. Li F, Ye T, Kong H, Li J, Hu L, Jin H, et al. Efficacies of Different Ovarian Hyperstimulation Protocols in Poor Ovarian Responders Classified by the Poseidon Criteria. *Aging* (2020) 12(10):9354–64. doi: 10.18632/aging.103210
11. Haahr T, Esteves SC, Humaidan P. Individualized Controlled Ovarian Stimulation in Expected Poor-Responders: An Update. *Reprod Biol Endocrinol* (2018) 16(1):20. doi: 10.1186/s12958-018-0342-1
12. Pilsgaard F, Grynnerup AG, Lossl K, Bungum L, Pinborg A. The Use of Anti-Mullerian Hormone for Controlled Ovarian Stimulation in Assisted Reproductive Technology, Fertility Assessment and -Counseling. *Acta Obstet Gynecol Scand* (2018) 97(9):1105–13. doi: 10.1111/aogs.13334
13. Vazquez AC, Rodriguez J, Algara ALC, Garcia JDM. Correlation Between Biochemical, Ultrasonographic and Demographic Parameters With Ovarian Response to Ivf/Icsi Treatments in Mexican Women. *JBRA Assist Reprod* (2021) 25(1):4–9. doi: 10.5935/1518-0557.20200040
14. Alipour F, Rasekhjahromi A, Maalhagh M, Sobhanian S, Hosseinpoor M. Comparison of Specificity and Sensitivity of Amh and Fsh in Diagnosis of Premature Ovarian Failure. *Dis Markers* (2015) 2015:585604. doi: 10.1155/2015/585604
15. Elgindy EA, El-Haieg DO, El-Sebaey A. Anti-Mullerian Hormone: Correlation of Early Follicular, Ovulatory and Midluteal Levels With Ovarian Response and Cycle Outcome in Intracytoplasmic Sperm Injection Patients. *Fertility sterility* (2008) 89(6):1670–6. doi: 10.1016/j.fertnstert.2007.05.040
16. Kotlyar AM, Seifer DB. Ethnicity/Race and Age-Specific Variations of Serum Amh in Women-A Review. *Front Endocrinol* (2020) 11. doi: 10.3389/fendo.2020.593216
17. Celik H, Bildircin D, Guven D, Cetinkaya MB, Alper T, Batuoglu AS. Random Anti-Mullerian Hormone Predicts Ovarian Response in Women With High Baseline Follicle-Stimulating Hormone Levels : Anti-Mullerian Hormone in Poor Responders in Assisted Reproductive Treatment. *J assisted Reprod Genet* (2012) 29(8):797–802. doi: 10.1007/s10815-012-9794-y
18. Tal R, Seifer DB. Ovarian Reserve Testing: A User's Guide. *Am J Obstet Gynecol* (2017) 217(2):129–40. doi: 10.1016/j.ajog.2017.02.027
19. Cui L, Qin Y, Gao X, Lu J, Geng L, Ding L, et al. Antimullerian Hormone: Correlation With Age and Androgenic and Metabolic Factors in Women From Birth to Postmenopause. *Fertility Sterility* (2016) 105(2):481–5.e1. doi: 10.1016/j.fertnstert.2015.10.017
20. Kedem A, Yung Y, Yerushalmi GM, Haas J, Maman E, Hanochi M, et al. Anti Müllerian Hormone (Amh) Level and Expression in Mural and Cumulus Cells in Relation to Age. *J Ovarian Res* (2014) 7:113. doi: 10.1186/s13048-014-0113-3
21. Koning AM, Kuchenbecker WK, Groen H, Hoek A, Land JA, Khan KS, et al. Economic Consequences of Overweight and Obesity in Infertility: A Framework for Evaluating the Costs and Outcomes of Fertility Care. *Hum Reprod Update* (2010) 16(3):246–54. doi: 10.1093/humupd/dmp053
22. Chen R, Chen S, Liu M, He H, Xu H, Liu H, et al. Pregnancy Outcomes of Pcos Overweight/Obese Patients After Controlled Ovarian Stimulation With the Gnrh Antagonist Protocol and Frozen Embryo Transfer. *Reprod Biol Endocrinol* (2018) 16(1):36. doi: 10.1186/s12958-018-0352-z
23. Sermondade N, Huberlant S, Bourhis-Lefebvre V, Arbo E, Gallot V, Colombani M, et al. Female Obesity Is Negatively Associated With Live Birth Rate Following Ivf: A Systematic Review and Meta-Analysis. *Hum Reprod Update* (2019) 25(4):439–51. doi: 10.1093/humupd/dmz011
24. Albu D, Albu A. The Relationship Between Anti-Mullerian Hormone Serum Level and Body Mass Index in a Large Cohort of Infertile Patients. *Endocrine* (2019) 63(1):157–63. doi: 10.1007/s12020-018-1756-4
25. Kriseman M, Mills C, Kovanci E, Sangi-Haghpeykar H, Gibbons W. Antimullerian Hormone Levels Are Inversely Associated With Body Mass Index (Bmi) in Women With Polycystic Ovary Syndrome. *J assisted Reprod Genet* (2015) 32(9):1313–6. doi: 10.1007/s10815-015-0540-0
26. Alpha Scientists in Reproductive M and Embryology ESIGo. The Istanbul Consensus Workshop on Embryo Assessment: Proceedings of an Expert Meeting. *Hum Reprod (Oxford England)* (2011) 26(6):1270–83. doi: 10.1093/humrep/der037
27. Golan A, Ron-el R, Herman A, Soffer Y, Weinraub Z, Caspi E. Ovarian Hyperstimulation Syndrome: An Update Review. *Obstet Gynecol Surv* (1989) 44(6):430–40. doi: 10.1097/00006254-198906000-00004
28. Rosen MP, Johnstone E, McCulloch CE, Schuh-Huerta SM, Sternfeld B, Reijo-Pera RA, et al. A Characterization of the Relationship of Ovarian Reserve Markers With Age. *Fertility Sterility* (2012) 97(1):238–43. doi: 10.1016/j.fertnstert.2011.10.031
29. Keane K, Cruzat VF, Wagle S, Chaudhary N, Newsholme P, Yovich J. Specific Ranges of Anti-Mullerian Hormone and Antral Follicle Count Correlate to Provide a Prognostic Indicator for Ivf Outcome. *Reprod Biol* (2017) 17(1):51–9. doi: 10.1016/j.repbio.2016.12.002
30. Xia M, Zheng J. Comparison of Clinical Outcomes Between the Depot Gonadotrophin-Releasing Hormone Agonist Protocol and Gonadotrophin-Releasing Hormone Antagonist Protocol in Normal Ovarian Responders. *BMC Pregnancy Childbirth* (2021) 21(1):372. doi: 10.1186/s12884-021-03849-8
31. Xu B, Zhou M, Wang J, Zhang D, Guo F, Si C, et al. Increased Aif-1-Mediated Tnf-Alpha Expression During Implantation Phase in Ivf Cycles With Gnrh Antagonist Protocol. *Hum Reprod (Oxford England)* (2018) 33(7):1270–80. doi: 10.1093/humrep/dey119
32. Li F, Zhang M, Zhang Y, Liu T, Qu X. Gnrh Analogues May Increase Endometrial Hoxa10 Promoter Methylation and Affect Endometrial Receptivity. *Mol Med Rep* (2015) 11(1):509–14. doi: 10.3892/mmr.2014.2680
33. Toftager M, Bogstad J, Bryndorf T, Lossl K, Roskaer J, Holland T, et al. Risk of Severe Ovarian Hyperstimulation Syndrome in Gnrh Antagonist Versus Gnrh Agonist Protocol: Rct Including 1050 First Ivf/Icsi Cycles. *Hum Reprod (Oxford England)* (2016) 31(6):1253–64. doi: 10.1093/humrep/dew051
34. Zhang W, Xie D, Zhang H, Huang J, Xiao X, Wang B, et al. Cumulative Live Birth Rates After the First Art Cycle Using Flexible Gnrh Antagonist Protocol Vs. Standard Long Gnrh Agonist Protocol: A Retrospective Cohort Study in Women of Different Ages and Various Ovarian Reserve. *Front Endocrinol* (2020) 11. doi: 10.3389/fendo.2020.00287
35. Marci R, Lisi F, Soave I, Lo Monte G, Patella A, Caserta D, et al. Ovarian Stimulation in Women With High and Normal Body Mass Index: Gnrh Agonist Versus Gnrh Antagonist. *Gynecol Endocrinol* (2012) 28(10):792–5. doi: 10.3109/09513590.2012.664192
36. Grow D, Kawwass JF, Kulkarni AD, Durant T, Jamieson DJ, Macaluso M. Gnrh Agonist and Gnrh Antagonist Protocols: Comparison of Outcomes Among Good-Prognosis Patients Using National Surveillance Data. *Reprod Biomedicine Online* (2014) 29(3):299–304. doi: 10.1016/j.rbmo.2014.05.007
37. Steiner AZ, Jukic AM. Impact of Female Age and Nulligravidity on Fecundity in an Older Reproductive Age Cohort. *Fertility Sterility* (2016) 105(6):1584–8.e1. doi: 10.1016/j.fertnstert.2016.02.028
38. Fritz R, Jindal S. Reproductive Aging and Elective Fertility Preservation. *J Ovarian Res* (2018) 11(1):66. doi: 10.1186/s13048-018-0438-4
39. Nardo LG, Bosch E, Lambalk CB, Gelbaya TA. Controlled Ovarian Hyperstimulation Regimens: A Review of the Available Evidence for Clinical Practice. Produced on Behalf of the Bfs Policy and Practice Committee. *Hum Fertil (Camb)* (2013) 16(3):144–50. doi: 10.3109/14647273.2013.795385
40. Oudshoorn SC, van Tilborg TC, Eijkemans MJC, Oosterhuis GJE, Friederich J, van Hooff MHA, et al. Individualized Versus Standard Fsh Dosing in Women Starting Ivf/Icsi: An Rct. Part 2: The Predicted Hyper Responder. *Hum Reprod (Oxford England)* (2017) 32(12):2506–14. doi: 10.1093/humrep/dex319
41. Ovarian Stimulation T, Bosch E, Broer S, Griesinger G, Grynberg M, Humaidan P, et al. Eshre Guideline: Ovarian Stimulation for Ivf/Icsi(Dagger). *Hum Reprod Open* (2020) 2020(2):hoaa009. doi: 10.1093/hropen/hoaa009
42. Rabinson J, Meltcer S, Zohav E, Gemer O, Anteby EY, Orvieto R. Gnrh Agonist Versus Gnrh Antagonist in Ovarian Stimulation: The Influence of

Body Mass Index on *In Vitro* Fertilization Outcome. *Fertility sterility* (2008) 89(2):472–4. doi: 10.1016/j.fertnstert.2007.03.007

43. Kumar P, Sharma A. Gonadotropin-Releasing Hormone Analogs: Understanding Advantages and Limitations. *J Hum Reprod Sci* (2014) 7 (3):170–4. doi: 10.4103/0974-1208.142476

**Conflict of Interest:** The authors have no conflict of interest to declare.

**Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Endocrinology

ORIGINAL RESEARCH
published: 12 August 2022
doi: 10.3389/fendo.2022.878929

# "Double Frozen Transfer" Could Influence the Perinatal and Children's Growth: A Nested Case-Control Study of 6705 Live Birth Cycles

*Jie Gao*[1,2,3,4†], *Yiyuan Zhang*[1,2,3,4†], *Linlin Cui*[1,2,3,4], *Tao Zhang*[5], *Bingjie Wu*[5], *Shanshan Gao*[1,3*] and *Zi-Jiang Chen*[1,2,3,4,6,7]

[1] Center for Reproductive Medicine, The Second Hospital, Cheeloo College of Medicine, Shandong University, Jinan, China, [2] Research Unit of Gametogenesis and Health of ART-Offspring, Chinese Academy of Medical Sciences, Jinan, China, [3] Key laboratory for Reproductive Endocrinology, Ministry of Education, Shandong University, Jinan, China, [4] Shandong Provincial Clinical Medicine Research Center for Reproductive Health, Jinan, China, [5] Department of Biostatistics, School of Public Health, Shandong University, Jinan, China, [6] Center for Reproductive Medicine, Renji Hospital, Shanghai JiaoTong University School of Medicine, Shanghai, China, [7] Shanghai Key Laboratory for Assisted Reproduction and Reproductive Genetics, Shanghai, China

OPEN ACCESS

**Edited by:**
*Yimin Zhu,*
*Zhejiang University, China*

**Reviewed by:**
*Zhexin Nl,*
*Second Military Medical University, China*
*Qianqian Zhu,*
*Shanghai Jiao Tong University, China*
*Xitong Liu,*
*Northwest Women's and Children's Hospital, China*

***Correspondence:**
*Shanshan Gao*
*sdszgaoshanshan@163.com*

*†These authors share first authorship*

**Specialty section:**
*This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology*

**Received:** *18 February 2022*
**Accepted:** *12 May 2022*
**Published:** *12 August 2022*

**Citation:**
*Gao J, Zhang Y, Cui L, Zhang T, Wu B, Gao S and Chen Z-J (2022) "Double Frozen Transfer" Could Influence the Perinatal and Children's Growth: A Nested Case-Control Study of 6705 Live Birth Cycles. Front. Endocrinol. 13:878929. doi: 10.3389/fendo.2022.878929*

**Objective:** This study aims to evaluate neonatal and children growth outcomes of cryotransfer of embryos developed from frozen gametes [double frozen transfer (DFT)].

**Methods:** This nested case-control study included 6,705 women who had a singleton live birth after embryo transfer at the Center for Reproductive Medicine, Shandong University, from 2008 to 2020. Of these, 745 women underwent frozen embryo transfer (FET) using embryos developed from frozen gametes (DFT). Propensity score methodology was used to balance the two groups by maternal age and body mass index (BMI) before evaluating outcomes. After age and BMI were matched using the propensity score methodology in a ratio of 1:4, the control groups enrolled 2,980 women who underwent fresh embryo transfer (ET) and 2,980 women underwent FET from fresh gametes. The children born were followed to at least 5 years of age, and some were followed up to 10 years. Neonatal outcomes and childhood growth measurements were compared among the three groups.

**Results:** The average birth weight of the DFT group (3,462 g) was significantly higher than the FET group (3,458 g) and ET group (3,412 g). The rate of large for gestational age (LGA) babies in the DFT and FET group was higher than that for the ET group (30.9% vs. 24.8%; 29.4% vs. 24.8%, respectively). After adjusting for different confounder combinations in the three models, the birth weight and risk of LGA in the DFT and FET groups were still higher than in the ET group, and the values group of *P* for trend in the models were significant. In multiple linear regression analysis of the children's development, the height Z-score of children born from the DFT and FET group was higher than that for children from the ET group (β = 0.21, 95% CI 0.07–0.35; b = 0.17, 95% CI 0.05–0.28, respectively). However, childhood growth measurements including body weight Z-score and BMI Z-score were not significantly different among the three groups. In addition, the proportion of male children born from DET was higher than that from ET.

**Conclusions:** There is an increased risk of LGA babies associated with pregnancies conceived from DFT. Children are inclined to be taller in the future in this group than after FET. The related etiology and pathophysiology mechanisms still need to be revealed. In the future, well-designed, observational studies with in-depth collection of patients' characteristics may shed more light on this issue.

**Keywords: frozen embryo transfer (FET), double frozen transfer, gamete cryopreservation, fresh embryo transfer (ET), neonatal outcome, children growth**

## INTRODUCTION

Worldwide, more than eight million children have been conceived after assisted reproductive technology (ART) (1). However, studies have shown that pregnancies and deliveries resulting from ART are generally associated with adverse obstetric and perinatal outcomes when compared to spontaneously conceived (SC) pregnancies (2, 3). Concerns about the safety of ART are increasing, and frozen embryo transfer (FET) and gamete cryopreservation, as important components of ART, have recently focused on perinatal and neonatal outcomes (4, 5).

Literature shows that FET is related to a decrease in the incidence of low birth weight, small for gestational age (SGA), preterm birth, placenta previa, and placental abruption compared with fresh embryo transfer (ET). However, evidence from two recent meta-analyses shows some adverse obstetrics and perinatal outcomes after FET including pregnancy-induced hypertension (PIH), large for gestational age (LGA), and postpartum hemorrhage (4, 6). There are also studies on the perinatal and neonatal outcomes from gamete cryopreservation applied in ART. Most cohort studies show no increased risk of adverse perinatal outcomes following donor sperm compared with partner sperm *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI) treatment (7–9). A recent systematic review regarding the impact of oocyte vitrification on offspring shows that vitrification seems to be a safe method for oocyte cryopreservation and child health, at least in the short term (10).

However, to the best of our knowledge, almost no studies have focused on the obstetric and offspring outcome from the cryotransfer of embryos developed from frozen gametes. It is interesting to speculate that there may be an accumulating effect on the offspring after double frozen transfer (DFT). The present study aimed to evaluate the effect of DFT on the outcomes of neonatal and children growth by comparing it with "single frozen transfer" FET and fresh ET in a nested case-control study.

## MATERIALS AND METHODS

### Study Design and Oversight

To determine whether double freezing-thawing procedures influence the short- and long-term health of offspring, we assess perinatal and neonatal outcome along with children growth of DFTs, FETs, and ETs. We conducted a single-center, retrospective, nested case-control analysis at the Center for Reproductive Medicine Affiliated to Shandong University. The study was approved by the Institutional Review Board of the Second Hospital, Cheeloo College of Medicine, Shandong University. The ethics approval document number is 2022(37). Written informed consent was obtained from patients and parents or guardians of all participants.

### Study Population

This was a matched case-control retrospect analysis including DFT, FET and ET during April 2008 and May 2020 (**Supplementary Figure 1**). A total of 6705 patients were included in this study. The 745 patients in the DFT group underwent cryotransfer of embryos developed from frozen gametes; of which, 721 cycles involved sperm cryopreservation and 24 cycles involved oocyte vitrification. After maternal age and body mass index (BMI) were matched with the propensity score methodology in a ratio of 1:4, the control group enrolled 2980 cycles that underwent FET and 2980 cycles that underwent ET. All embryos transferred resulted in a singleton birth. Children were followed from birth to at least 5 years to assess growth information including height, weight, and BMI; some children were followed to 10 years of age. Patients were excluded if they were multi-gestation or had been delivered before 28 weeks of pregnancy or stillbirth.

### Study Procedures

After routine ovarian stimulation protocols, as previously described (11, 12), transvaginal ultrasound-guided oocyte retrieval was carried out 34–38 h after Human chorionic gonadotropin (HCG) administration. Oocyte fertilization was achieved by IVF or ICSI based on the male partner's sperm quality. High-quality embryos were selected for transfer at the cleavage-stage or blastocyst stage, and a maximum of two embryos were transferred. Fresh embryos are preferentially transferred at cleavage stage, whereas FET tends to transfer blastocyst embryos. Surplus or all blastocysts were vitrified on day 5 or day 6, based on embryo development, for future transfer. Sperm cryopreservation was used in two situations: the first was autologous sperm cryopreservation as a backup sperm source and the second was cryopreserved donor semen. Oocyte vitrification was used in clinical scenarios such as the unavailability of sperm at the time of egg retrieval or for couples who did not wish to cryopreserve supernumerary embryos in cases where plenty of oocytes were retrieved. Another indication for oocyte vitrification that has now become a reality is the establishment of donor oocyte banks.

## Pregnancy Assessment and Follow-Up

Endometrial preparation for FET is described in detail elsewhere (13). Luteal support continued until 11–12 weeks of gestation. Clinical pregnancy was determined through transvaginal ultrasonography by detecting one or more gestational sacs. Early miscarriage was defined as the spontaneous loss of clinical pregnancy within the first 13 weeks of gestation. Subsequently, each patient would receive a telephone survey and standardized questionnaires delivered by trained nurses. Information would be collected including perinatal complications, gestational weeks, birth date, delivery mode, newborn gender and birth weight, neonatal diseases, treatment, and prognosis. All follow-up information was recorded in the electronic medical records (14).

A live birth was defined as the delivery of a viable infant after 28 weeks of gestational age. Low birth weight was defined as a newborn baby weighing below 2,500 g. small for gestational age (SGA) was defined as a birth weight below the 10th percentile for gender and gestational age according to the reference population. Birth weight for gender- and gestational age–specific standard score (z-score) was calculated on the basis of a Chinese reference chart (15). Z-score was calculated according to the following formula: (weight of an individual infant at a given gestational age – mean weight of the reference population at the same gestational age)/standard deviation (SD) in the reference population. Pediatric growth parameters included height in centimeters, weight (kg), and BMI (kg/m$^2$). Data recorded also included whether or not the infant was breastfed.

## Statistical Methods

All data analyses were performed using SPSS statistical software v26 and R v4.0.2. Propensity score matching was used to balance the baseline maternal characteristics among the three groups. Patients of DFT, FET and ET groups were evaluated using the propensity score methodology with nearest neighbor matching (caliper 0.2). The matching ratio was 1:4 with the matching factors referring to maternal age and BMI.

Confounders were enrolled according to clinical experience and up-to-date literatures. Continuous variables were presented as mean ± standard deviation with one-way analysis of variance (ANOVA) for between-group differences. Categorical variables were expressed as frequencies and percentages, and the distribution among groups was analyzed by the chi-square test or the Fisher's exact test. We considered P-values of <0.05 to be statistically significant. Multiple logistics and linear regression analysis were used to adjust confounders. Different regression models were adjusted for different confounder combinations (see Results). All confounders adjusted in multiple regression analysis for obstetric and perinatal parameters fertilization methods, stage of the embryo, fertilization rate, number of embryos transferred, endometrial thickness before transplanting, type of infertility, weight gain during pregnancy,parity,preterm birth, fetal gender, birth weight, gestational diabetes mellitus, (GDM), and PIH hypertensive disorder of pregnancy (HDP). All confounders were adjusted in multiple regression analysis for the height, weight, and BMI of children, including gender, age, weight gain during pregnancy, parity, endometrial thickness, before transplanting, fertilization methods, stage of the embryo, fertilization rate, type of infertility, number of embryos transferred, breastfeeding, GDM, HDP, preterm birth socio-economic status (highest education, job occupation, and income per month), maternal height, and maternal weight.

# RESULTS

There were 6,705 patients enrolled in this study. Among these, 745 patients were in the DFT group, and 2,980 patients were enrolled separately in FET and ET groups (**Table 1**). Women in the DFT group gained the least weight compared with ET group and FET group. More women in the ET group were experiencing their first delivery than in the FET and DFT groups. The type of infertility and cause of infertility in the DFT group were different to the other two groups. More blastocyst transfers were carried out in the DFT and FET groups (96.4% and 96.7%, respectively) than in the ET group (24.0%; **Table 1**).

**TABLE 1 |** Demographic and clinical characteristics of the patients at the baseline.

| | DFT (N = 745) | FET (N = 2,980) | ETs (N = 2,980) | P-value |
|---|---|---|---|---|
| Maternal characters | | | | |
| Maternal age (y) | 30.8 ± 4.0 | 30.8 ± 3.9 | 31.0 ± 3.9 | 0.134 |
| BMI (kg/m$^2$) | 23.8 ± 3.9 | 23.8 ± 3.8 | 23.6 ± 3.8 | 0.252 |
| Weight gain during pregnancy (kg) | 13.9 ± 8.0 | 14.9 ± 7.6 | 14.9 ± 7.0 | 0.003* |
| Parity n (%) | | | | 0.035* |
| First | 600 (80.5) | 2464 (82.7) | 2529 (84.9) | |
| Second | 141 (18.9) | 501 (16.8) | 442 (14.8) | |
| Third or more | 4 (0.5) | 15 (0.5) | 9 (0.3) | |
| Type of infertility n (%) | | | | <0.001* |
| Primary infertility | 589 (79.1) | 1774 (59.5) | 1664(55.8) | |
| Secondary infertility | 156 (20.9) | 1206 (40.5) | 1316 (44.2) | |
| Cause of infertility n (%) | | | | |
| Male factor | 717 (96.2) | 1536 (51.5) | 2588 (86.8) | <0.001* |
| Ovulation disorder | 115 (15.4) | 718 (24.1) | 481 (16.1) | <0.001* |
| Tubal factor | 561 (75.3) | 2222 (74.6) | 2312 (77.6) | 0.022* |
| Endometriosis | 57 (7.7) | 108 (3.6) | 111 (3.7) | <0.001* |
| Unexplained | 1 (0.1) | 33 (1.1) | 28 (0.9) | <0.001* |
| Others | 363 (49.4) | 122 (4.1) | 64 (2.1) | <0.001* |
| Embryo characters n (%) | | | | |
| No. of embryos transferred | 1.1 ± 0.4 | 1.2 ± 0.4 | 1.7 ± 0.5 | <0.001* |
| Endometrial thickness (cm) | 1.0 ± 0.2 | 1.0 ± 0.2 | 1.1 ± 0.2 | <0.001* |
| Fertilization rate | 551 (93.4) | 2400 (95.0) | 2898 (97.3) | <0.001* |
| Stage at ET (%) | | | | <0.001* |
| Cleavage | 27 (3.6) | 98 (3.3) | 2264 (76.0) | |
| Blastocyst | 716 (96.4) | 2882 (96.7) | 716 (24.0) | |
| Fertilization methods (%) | | | | <0.001* |
| IVF | 657 (88.2) | 1878 (63.0) | 2130 (71.5) | |
| ICSI | 77 (10.3) | 834 (28.0) | 826 (27.7) | |
| PGT | 11 (1.5) | 268 (9.0) | 24 (0.8) | |

*Presented as n (%) for categoric variables and mean ± SD for continuous variables. DFT, double frozen embryo transfer; FET, single frozen embryo transfer; ET, fresh embryo transfer; BMI, body mass index; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; PGT, preimplantation genetic diagnosis.*

**means that the p value was statistically significant.*

**TABLE 2** | Obstetric and neonatal outcomes.

| Variable | DFT (N = 745) | FET (N = 2980) | ET (N = 2980) | P-value | DFT vs. FET | DFT vs. ET | FET vs. ET |
|---|---|---|---|---|---|---|---|
| Gestational age (week) | 39.17 ± 1.75 | 39.10 ± 1.71 | 39.23 ± 1.60 | 0.011* | 0.302 | 0.381 | 0.003* |
| Gender | | | | 0.014* | 0.069 | 0.005* | 0.126 |
| Male (%) | 424 (56.9) | 1583 (53.1) | 1523(51.1) | | | | |
| Female (%) | 321 (43.1) | 1397 (46.9) | 1457 (48.9) | | | | |
| Birth weight (g) | 3462.4 ± 530.5 | 3457.9 ± 534.3 | 3411.6 ± 517.4 | 0.001* | 0.834 | 0.019 | 0.001* |
| Weight | | | | | | | |
| ≤1500 | 7(0.9) | 22(0.7) | 13(0.4) | 0.173 | – | – | – |
| 1500–2500 | 19 (2.6) | 99 (3.3) | 103 (3.5) | 0.462 | – | – | – |
| 4000–4500 | 96 (12.9) | 372 (12.5) | 343 (11.5) | 0.403 | – | – | – |
| ≥4500 | 15 (2.0) | 73 (2.4) | 53 (1.8) | 0.193 | – | – | – |
| Body length (cm) | 50.3 ± 2.1 | 50.3 ± 2.1 | 50.3 ± 1.9 | 0.934 | – | – | – |
| Mode of | | | | <0.001* | 0.013* | <0.001* | <0.001* |
| Vaginal (%) | 237 (31.8) | 811 (27.2) | 1162 (39.0) | | | | |
| Cesarean (%) | 508 (68.2) | 2,169 (72.8) | 1818 (61.0) | | | | |
| GDM (%) | 45 (6.0) | 244 (8.2) | 238 (8.0) | 0.141 | – | – | – |
| HDP (%) | 44 (5.9) | 204 (6.8) | 124 (4.2) | <0.001* | 0.357 | 0.040 | <0.001* |
| Breastfeeding (%) | 722 (98.1) | 2,894 (98.1) | 2885 (97.9) | 0.914 | – | – | – |
| SGA (%) | 34 (4.6) | 115 (3.9) | 150 (5.0) | 0.089 | – | – | – |
| LGA (%) | 230 (30.9) | 877 (29.4) | 240 (24.8) | <0.001* | 0.446 | 0.001* | <0.001* |
| Preterm birth (%) | 47 (6.3) | 204 (6.8) | 186 (6.2) | 0.621 | – | – | – |
| Perinatal death (%) | 1 (0.1) | 2 (0.1) | 1 (0.0) | 0.588 | – | – | – |
| Neonatal mortality (%) | 1 (0.1) | 2 (0.1) | 2 (0.1) | 0.819 | – | – | – |
| Polyhydramnios (%) | 2 (0.3) | 21 (0.7) | 16 (0.5) | 0.362 | - | - | - |
| Oligohydramnios (%) | 32 (4.3) | 132 (4.4) | 97 (3.3) | 0.054 | - | - | - |
| Placental deformity (%) | 13 (1.7) | 73 (2.4) | 67 (2.2) | 0.514 | – | – | – |
| Placenta implantation (%) | 1 (0.1) | 8 (0.3) | 3 (0.1) | 0.345 | | | |
| Placenta previa (%) | 2 (0.3) | 12 (0.4) | 8 (0.3) | 0.721 | – | – | – |
| Placental abruption (%) | 1 (0.1) | 2 (0.1) | 4 (0.1) | 0.640 | – | | |
| Abnormal umbilical cord (%) | 146 (0.3) | 623 (20.9) | 571 (19.2) | 0.233 | - | - | - |
| Neonatal disease (%) | 18 (2.4) | 143 (4.8) | 84 (2.8) | <0.001* | 0.005* | 0.617 | <0.001* |
| Birth defects (%) | 13 (1.7) | 82 (2.8) | 33 (1.1) | <0.001* | 0.152 | 0.192 | <0.001* |

*DFT, double frozen embryo transfer; FET, single frozen embryo transfer; ET, fresh embryo transfer; SGA, small for gestational age; LGA. large for gestational age; NICU, neonatal intensive care unit; GDM, gestational diabetes mellitus; HDP, hypertensive disorder of pregnancy; *means p value was statistically significant.*

**Table 2** presents perinatal and neonatal outcomes. The proportion of males born in the DFT group was higher than in the ET group (56.9% vs. 51.1%). The birth weight in both DFT and FET groups were heavier than that for ET (3462 g vs. 3412 g; 3458 g vs. 3412 g). The birth weight and risk of LGA tend to increase as the times of freezing increased (*P*-value for trend <0.001). The FET group had the lowest rate of vaginal delivery (27.2%) compared with DFT (31.8%) and ET (39%). The ratio of LGA was highest in the DFT group, and the FET group showed a higher LGA ratio than the ET group. Both neonatal disease and birth defect ratio were highest in the FET group.

**Tables 3** and **4** show the multiple regression analysis for obstetrics and neonatal and child development. The ET group was set as the reference. After adjustment for different confounder combinations in the three models, the difference of birth weight, LGA, and neonatal diseases was still significant among groups (**Table 3**). In multiple linear regression analysis of child development, the height Z-score of children born from the DFT and FET groups was higher than that for children from the ET group (β = 0.21, 95% CI 0.07–0.35; b = 0.17, 95% CI 0.05–0.28, respectively). Increasing height was also associated with times of freezing procedures increased ((P-value for trend = 0.003). However, body weight Z-score and BMI Z-score were not significantly different among the three groups (**Table 4**).

For the high ratio of donor sperm in the DFT group, a subgroup analysis, excluding the male partners age >35 years and with severe sperm deficiency or azoospermia, was carried out. The subgroup outcomes were consistent with overall outcomes (see **Supplementary Tables 1** and **2**).

## DISCUSSION

This nested case-control study included 6,705 women who had a singleton live birth after embryo transplant. We found that the birth weight and LGA proportion in the DFT and FET groups were significantly higher compared with that in the ET group. In addition, the test for trend showed that the birth weight and risk of LGA tended to increase as the times of freezing increased. In the comparison of children's development, the height Z-score of children in the DFT group was greater than in the ET group and the trend test also was significant. However, there was no significant difference in body weight and BMI Z-scores of children born from DFT group than that from FET and ET group after adjustment.

Embryo cryopreservation methods especially for blastocysts have changed from slow freezing to vitrification according to safety and efficacy of the reports over the past decade (16–18). Vitrification is an ultrarapid cryopreservation method with a

**TABLE 3** | Multivariate regression models for obstetrics and neonatal outcomes.

| | Model 1 (OR/β, 95% CI) | Model 2 (OR/β, 95% CI) | Model 3 (OR/β, 95% CI) |
|---|---|---|---|
| Birth weight | | | |
| ET | Ref. | Ref. | Ref. |
| FET | **61.77 (18.70, 102.84)** | **61.28 (18.42, 104.13)** | **56.55 (19.99, 93.12)** |
| DFT | **84.11 (28.41, 139.81)** | **81.64 (26.22, 137.05)** | **72.22 (23.31, 121.12)** |
| $P_{trend}$ [b] | **<0.001** | **<0.001** | **0.002** |
| Gestational age | | | |
| ET | Ref. | Ref. | Ref. |
| FET | 0.05 (–0.08, 0.19) | 0.05 (–0.08, 0.19) | 0 (–0.09, 0.09) |
| DFT | 0.10 (–0.08, 0.27) | 0.10 (–0.07, 0.28) | 0.05 (–0.07, 0.17) |
| $P_{trend}$ [b] | 0.27 | 0.24 | 0.48 |
| Birth weight Z-score | | | |
| ET | Ref. | Ref. | Ref. |
| FET | **0.14 (0.05, 0.23)** | **0.14 (0.05, 0.23)** | **0.15 (0.06, 0.24)** |
| DFT | **0.18 (0.06, 0.30)** | **0.18 (0.06, 0.30)** | **0.18 (0.06, 0.30)** |
| $P_{trend}$ [b] | **0.002** | **0.002** | **0.001** |
| PIH | | | |
| ET | Ref. | Ref. | Ref. |
| FET | 1.21 (0.82, 1.77) | 1.21 (0.82, 1.77) | 1.17 (0.79, 1.74) |
| DFT | 1.21 (0.75, 1.97) | 1.22 (0.75, 1.98) | 1.21 (0.72, 2.03) |
| LGA | | | |
| ET | **Ref.** | **Ref.** | **Ref.** |
| FET | 1.16 (0.99, 1.38) | 1.16 (0.99, 1.38) | **1.28(1.07, 1.55)**) |
| DFT | **1.31 (1.07, 1.62)** | **1.31 (1.07, 1.62)** | **1.53(1.19, 1.97))** |
| $P_{trend}$ [b] | **<0.001** | **<0.001** | **0.001** |
| Birth defect | | | |
| ET | Ref. | Ref. | Ref. |
| FFT | 1.98 (0.95, 4.12) | 2.03 (0.97, 4.25) | **2.66 (1.25, 5.64)**) |
| DET | 1.63 (0.68, 3.91) | 1.67 (0.69, 4.07) | 1.97 (0.77, 5.03) |
| Neonatal disease | | | |
| ET | Ref. | **Ref.** | **Ref.** |
| FFT | **2.41 (1.46, 3.96)** | **2.41 (1.46, 3.98)** | **2.77 (1.62, 4.74)** |
| DET | 0.87 (0.43, 1.76) | 0.87 (0.43, 1.78) | 1.29 (0.60, 2.76) |

*Model 1: weight gain during pregnancy, parity, type of infertility, male factor, ovulation disorder, tubal factor, endometriosis, unexplained, fertilization rate, stage at ET, fertilizationmethods, no. of embryos transferred, Endometrial thickness. Model 2: model 1 + fetal gender. Model 3: model 1 + GDM + HDP + fetal gender + premature birth. Bold numbers indicate statistical significance (P < 0.05) : P trend[b] means p for trend.*

high concentration of permeable cryoprotectants, which have raised concerns about possible "toxicity" to the embryos and even to the offspring (19). Studies have observed reduced risks of preterm birth and low birth weight in FET cycles compared with that in fresh ETs (6). However, in a large cumulative meta-analysis, singletons born after FET were found to have an increased risk of being born LGA and having a heavier birth weight; there was also an increased risk of HDP (4, 5). We demonstrated similar effects in the present study. The birth weight and LGA rate in the DFT and FET groups were both significantly higher than that in ET group. After adjusting confounders by multiple regression analysis, we found that the birth weight was still higher in the FET and DFT groups. In terms of LGA rate, there was still a significant difference between the DFT and ET groups after adjustment, but the significance was no longer present in a comparison between FET and ET group. Moreover, it is important to realize that there was an increased trend among three groups in birth weight, birth weight Z-score, and LGA rates (showed by the *P*-values for trend), when the ET group was set as the reference in multiple regression analysis. This situation continued in the multiple regression analysis of height Z-score in the results of child development. All these outcomes demonstrated a cumulative effect of gamete cryopreservation and embryo cryopreservation.

Several pathophysiological processes may play roles in the low risk of SGA and higher risk of LGA in FET cycles than that in fresh ET cycles. The first one is that increased hormone blood levels, especially high estrogen levels, might alter the timing of endometrial receptivity and exert a detrimental effect on spiral artery remodeling by the trophoblast (20, 21). A potential role in placental function dysregulation for elevated estrogen exposure has been associated with higher rates of low birth weight and fetal growth restriction. (22). The second explanation proposed for the increased risk of LGA with frozen cycles is the epigenetic changes during freezing and thawing. The cryopreservation technique may cause epigenetic changes within the embryos, such as DNA methylation and histone modification (23, 24). In the present study, DFT and FET group were mostly at the blastocyst stage. It has also been shown that higher birth weight, and higher risk of LGA and VLGA are found in blastocyst vs. cleavage stage transfer, which is related to the greater number of epigenetic changes during extended culture (25–27). However, the variable of embryo stage at transfer was adjusted by multiple regression analysis, and the higher risk of LGA still existed in the DFT group compared with that in the ET group. Therefore, DFT group showed an increased trend in birth weight and LGA rate compared with FET group; this might possibly be related to epigenetic changes, as the DFT group had all the same parameters as FET group except for one additional gamete cryopreservation procedure. The freezing and thawing procedures performed in gametes and embryo stages might induce cumulus epigenetic changes and stress reactions. Moreover, the results of the long-term follow-up supported the theory that an epigenetic programming of metabolism during prenatal and postnatal periods, as a response to imprinting alterations, occurred during early embryonic development (28, 29).

However, as we mentioned previously, most studies show no increased risk of adverse perinatal outcome following the use of cryopreserved sperm or oocytes. In the present study, DFT group mostly involved cryotransfer of embryos from cryopreserved donor sperm. So why does gamete cryopreservation alone not exhibit an influence on perinatal outcomes, whereas the combination of gametes and embryo cryopreservation shows different outcomes from embryo cryopreservation alone? There might be a threshold for the epigenetic changes or the remodeling of epigenetics during meiosis and early embryo development (30) that covers the epigenetic changes during gamete freezing and thawing. The clear etiological and pathophysiological mechanisms need to be revealed.

Some studies related to double frozen procedures include repeated cryopreservation of embryos. One situation when this may take place is when a surplus of zygotes or day 3 embryos are

**TABLE 4** | Height, weight, and BMI z scores and their coefficients and 95% CIs from unadjusted and adjusted regression models.

| | Unadjusted (β, 95% CI) | Model 1 (β, 95% CI) | Model 2 (β, 95% CI) | Model 3 (β, 95% CI) |
|---|---|---|---|---|
| Height Z-score | | | | |
| ET | Ref. | Ref. | Ref. | Ref. |
| FET | 0.01 (–0.07, 0.08) | **0.12 (0.00, 0.24)** | **0.09 (0.02, 0.16)** | **0.17 (0.05, 0.28)** |
| DFT | 0.10 (–0.02, 0.21) | **0.15 (0.00, 0.29)** | **0.20 (0.08, 0.31)** | **0.21 (0.07, 0.35)** |
| $P_{trend}$ [b] | – | **0.048** | **0.001** | **0.003** |
| Weight Z-score | | | | |
| ET | Ref. | Ref. | Ref. | Ref. |
| FET | **–0.14 (–0.27, –0.00)** | –0.04 (–0.25, 0.18) | **–0.21 (–0.35, –0.07)** | –0.12 (–0.33, 0.11) |
| DFT | –0.05 (–0.26, 0.17) | 0.06 (–0.22, 0.33) | 0.08 (–0.14, 0.30) | 0.14 (–0.14, 0.41) |
| $P_{trend}$ [b] | – | 0.74 | 0.42 | 0.42 |
| BMI Z-score | | | | |
| ET | Ref. | Ref. | Ref. | Ref. |
| FET | 0.07 (–0.02, 0.16) | 0.04 (–0.10, 0.18) | –0.03 (–0.12, 0.06) | –0.05 (–0.19, 0.10) |
| DFT | 0.02 (–0.12, 0.17) | 0.06 (–0.12, 0.24) | 0.08 (–0.06, 0.23) | 0.08 (–0.10, 0.27) |
| $P_{trend}$ [b] | – | 0.56 | 0.54 | 0.44 |

*Ref., Reference; Model 1: adjusted for weight gain during pregnancy, parity, endometrial thickness before transplanting, fertilization methods, stage of the embryo, fertilization rate, number of embryos transferred, type of infertility, male factor, ovulation disorder, tubal factor, endometriosis, and unexplained. Model 2: weight gain during pregnancy, breastfeeding, GDM, the highest education, income in a month,PIH, occupation, maternal height (height Z-score), maternal weight (weight Z-score), or maternal BMI (BMI Z-score). Model 3: model 1 + model 2 + premature birth. Bold numbers indicate statistical significance (P < 0.05). Data were analyzed by multiple mixed linear model through R.4.0.*

warmed and cultured for blastocyst development (31, 32). When more blastocysts are formed than required for transfer, repeated cryopreservation may be considered. Another scenario is the repeated vitrification and warming of blastocysts for preimplantation genetic diagnosis (PGD) (33); in such repeated embryo cryopreservation, the clinical pregnancy rate and live birth rate were found to be decreased (31–33). However, limited data regarding perinatal outcomes and long-term follow up have been reported.

Interpretation of associations from observational studies is always challenging. Although we conducted a strict nested case-control study with a large sample size and adjusted for many confounders, several limitations of this study should still be noted. First, most gamete cryopreservation was of donor frozen sperm. As sperm donors are relatively young and have normal semen, there was a selection bias in the DFT population. Therefore, a subgroup analysis, excluding those male partners age >35 years and with severe sperm deficiency or azoospermia, was carried out, and the subgroup outcomes were consistent with the overall findings. Second, not all confounders were taken into accounts, owing to the retrospective nature of this study. Third, all children conceived by ART in this study were from a single medical center in Shandong, China; therefore, caution should be taken in generalizing these findings.

## CONCLUSIONS AND PERSPECTIVES

In conclusion, there is an increased risk of LGA babies associated with pregnancies conceived from DFT. Furthermore, the children are inclined to be taller in the future in this group compared with offspring following FET. The related etiology and pathophysiology mechanisms still need to be revealed. In the future, well-designed, observational studies with an in-depth collection of patient characteristics may shed more light on this issue.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of the Second Hospital, Cheeloo College of Medicine, Shandong University. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

SG and LC contributed to the study concept and design. JG, YZ, and BW analyzed data and drafted the paper. TZ, LC, and Z-JC contributed to the review and the revision of the manuscript. All authors contributed to the article and approved the submitted version.

## FUNDING

The National Natural Science Foundation of China (82171692), Research Unit of Gametogenesis and Health of ART-Offspring, Chinese Academy of Medical Sciences (2020RU001), Taishan Scholars Program for Young Experts of Shandong Province (tsqn201909195), and Natural Science Foundation of Shandong Province (ZR2021QH136).

## ACKNOWLEDGMENTS

The authors thank the medical workers in the research group at the Reproductive Hospital of Shandong University and the information engineer for assembling the data.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.878929/full#supplementary-material

**Supplementary Figure 1 |** Flow Chart. DFT, double frozen embryo transfer; FET, single frozen embryo transfer; ET, fresh embryo transfer.

**Supplementary Table 1 |** Sensitivity analysis of obstetrics and neonatal outcomes. DFT, double frozen embryo transfer; FET, single frozen embryo transfer; ET, fresh embryo transfer; LGA, large for gestational age; Ref., reference. weight gain during pregnancy, parity, type of infertility, male factors, ovulation disorder, tubal factor, endometriosis, unexplained, fertilization rate, stage at ET, fertilization methods, no. of embryos transferred, Endometrial thickness, GDM, HDP, fetal gender, and premature birth. ($P < 0.05$).

**Supplementary Table 2 |** Sensitivity analysis of children development. DFT, double frozen embryo transfer; FET, single frozen embryo transfer; ET, fresh embryo transfer; Ref., reference. weight gain during pregnancy, parity, endometrial thickness before transplanting, fertilization methods, stage of the embryo, embryo fertilization level, no. of embryos transferred, type of infertility, male factor, ovulation disorder, tubal factor, endometriosis, unexplained, breastfeeding, GDM, the highest education, income in a month, occupation, maternal height (height Z-score), maternal weight (weight Z-score), or maternal BMI (BMI Z-score), and premature birth.

## REFERENCES

1. Adamsson G, Dyer S, Chambers G, Ishihara O, Mansour R, Banker M, et al. International Committee for Monitoring Assisted Reproductive Technologies (ICMART) Preliminary World Report on ART, 2015. In: *Abstract ESHRE*. Vienna (2019).
2. Petersen SH, Bergh C, Gissler M, Åsvold BO, Romundstad LB, Tiitinen A, et al. Time Trends in Placenta-MediatedPregnancy Complications After Assisted Reproductive Technology in the Nordic Countries. *Am J Obstet Gynecol* (2020) 223(2):226 e1–e19. doi: 10.1016/j.ajog.2020.02.030
3. Sunkara SK, Antonisamy B, Redla AC, Kamath MS. Female Causes of Infertility Are Associated With Higher Risk of Preterm Birth and Low Birth Weight: Analysis of 117 401 Singleton Live Births Following IVF. *Hum Reprod* (2021) 36:676–82. doi: 10.1093/humrep/deaa283
4. Sha T, Yin X, Cheng W, Massey IY. Pregnancy-Related Complications and Perinatal Outcomes Resulting From Transfer of Cryopreserved Versus Fresh Embryos *In Vitro* Fertilization: A Meta-Analysis. *Fertil Steril* (2018) 109 (2):330–42.e9. doi: 10.1016/j.fertnstert.2017.10.019
5. Saito K, Kuwahara A, Ishikawa T, Morisaki N, Miyado M, Miyado K, et al. Endometrial Preparation Methods for Frozen-Thawed Embryo Transfer are Associated With Altered Risks of Hypertensive Disorders of Pregnancy, Placenta Accreta, and Gestational Diabetes Mellitus. *Hum Reprod* (2019) 34:1567–75. doi: 10.1093/humrep/dez079
6. Maheshwari A, Pandey S, Amalraj Raja E, Shetty A, Hamilton M, Bhattacharya S. Is Frozen Embryo Transfer Better for Mothers and Babies? Can Cumulative Meta-Analysis Provide a Definitive Answer? *Hum Reprod Update* (2018) 24(1):35–58. doi: 10.1093/humupd/dmx031
7. Kamath MS, Antonisamy B, Selliah HY, La Marca A, Sunkara SK. Perinatal Outcomes Following IVF With Use of Donor Versus Partner Sperm. *Reprod BioMed Online* (2018) 36(6):705–10. doi: 10.1016/j.rbmo.2018.03.016
8. Gerkowicz SA, Crawford SB, Hipp HS, Boulet SL, Kissin DM, Kawwass JF. Assisted Reproductive Technology With Donor Sperm: National Trends and Perinatal Outcomes. *Am J Obstet Gynecol* (2018) 218:421.e1–21.e10. doi: 10.1016/j.ajog.2017.12.224
9. Yu B, Fritz R, Xie X, Negassa A, Jindal S, Vega M, et al. The Impact of Using Donor Sperm in Assisted Reproductive Technology Cycles on Perinatal Outcomes. *Fertil Steril* (2018) 110:1285–9. doi: 10.1016/j.fertnstert.2018.08.012
10. Da Luz CM, Caetano MA, Berteli TS, Vireque AA, Navarro PA. The Impact of Oocyte Vitrification on Offspring: A Systematic Review. *Reprod Sci* (2022). doi: 10.1007/s43032-022-00868-4
11. Guo Z, Xu X, Zhang L, Zhang L, Yan L, Ma J. Endometrial Thickness is Associated With Incidence of Small-for-Gestational-Age Infant S in Fresh *In Vitro* Fertilization-Intracytoplasmic Sperm Injection and Embryo Transfer Cycles. *Fertil Steril* (2020) 113:745–52. doi: 10.1016/j.fertnstert.2019.12.014
12. Shi Y, Sun Y, Hao C, Zhang H, Wei D, Zhang Y, et al. Transfer of Fresh Versus Frozen Embryos in Ovulatory Women. *N Engl J Med* (2018) 378:126–36. doi: 10.1056/NEJMoa1705334
13. Li J, Sun Q, Zhang M, Fu X, Zhang Y, Gao S, et al. Natural Cycles Achieve Better Pregnancy Outcomes Than Artificial Cycles in Non-PCOS Women Undergoing Vitrified Single-Blastocyst Transfer: A Retrospective Cohort Study of 6840 Cycles. *J Assist Reprod Genet* (2022). doi: 10.1007/s10815-022-02424-0
14. Liu X, Wang J, Fu X, Li J, Zhang M, Yan J, et al. Thin Endometrium Is Associated With the Risk of Hypertensive Disorders of Pregnancy in Fresh IVF/ICSI Embryo Transfer Cycles: A Retrospective Cohort Study of 9,266 Singleton Births. *Reprod Biol Endocrinol* (2021) 19:55. doi: 10.1186/s12958-021-00738-9
15. Liu Z, Zhang J, Zhao B, Xue X, Xu L, Wang F, et al. Population-Based Reference for Birth Weight for Gestational Age in Northern China. *Early Hum Dev* (2014) 90:177–87. doi: 10.1016/j.earlhumdev.2014.01.007
16. Roy TK, Bradley CK, Bowman MC, McArthur SJ. Single-Embryo Transfer of Vitrified-Warmed Blastocysts Yields Equivalent Live-Birth Rates and Improved Neonatal Outcomes Compared With Fresh Transfers. *Fertil Steril* (2014) 101:1294–301. doi: 10.1016/j.fertnstert.2014.01.046
17. Kato O, Kawasaki N, Bodri D, Kuroda T, Kawachiya S, Kato K, et al. Neonatal Outcome and Birth Defects in 6623 Singletons Born Following Minimal Ovarian Stimulation and Vitrified Versus Fresh Single Embryo Transfer. *Eur J Obstet Gynecol Reprod Biol* (2012) 161:46–50. doi: 10.1016/j.ejogrb.2011.12.005
18. Takahashi K, Mukaida T, Goto T, Oka C. Perinatal Outcome of Blastocyst Transfer With Vitrification Using Cryoloop: A 4-Year Follow-Up Study. *Fertil Steril* (2005) 84:88–92. doi: 10.1016/j.fertnstert.2004.12.051
19. Gosden R. Cryopreservation: A Cold Look at Technology for Fertility Preservation. *Fertil Steril* (2011) 96:264–8. doi: 10.1016/j.fertnstert.2011.06.029
20. Pereira N, Elias RT, Christos PJ, Petrini AC, Hancock K, Lekovich JP, et al. Supraphysiologic Estradiol Is an Independent Predictor of Low Birth Weight in Full-Term Singletons Born After Fresh Embryo Transfer. *Hum Reprod* (2017) 32:1410–7. doi: 10.1093/humrep/dex095
21. Bonagura TW, Pepe GJ, Enders AC, Albrecht ED. Suppression of Extravillous Trophoblast Vascular Endothelial Growth Factor Expression and Uterine Spiral Artery Invasion by Estrogen During Early Baboon Pregnancy. *Endocrinology* (2008) 149:5078–87. doi: 10.1210/en.2008-0116
22. Imudia AN, Awonuga AO, Doyle JO, Kaimal AJ, Wright DL, Toth TL, et al. Peak Serum Estradiol Level During Controlled Ovarian Hyperstimulation Is Associated With Increased Risk of Small for Gestational Age and PE in Singleton Pregnancies After *In Vitro* Fertilization. *Fertil Steril* (2012) 97:1374–9. doi: 10.1016/j.fertnstert.2012.03.028
23. Hiura H, Hattori H, Kobayashi N, Okae H, Chiba H, Miyauchi N, et al. Genome-Wide microRNA Expression Profiling in Placentae From Frozen-Thawed Blastocyst Transfer. *Clin Epigenet* (2017) 9:79. doi: 10.1186/s13148-017-0379-6
24. Wang Z, Xu L, He F. Embryo Vitrification Affects the Methylation of the H19/Igf2 Differentially Methylated Domain and the Expression of H19 and Igf2. *Fertil Steril* (2010) 93:272933. doi: 10.1016/j.fertnstert.2010.03.025
25. Makinen S, Soderstrom-Anttila V, Vainio J, Suikkari AM, Tuuri T. Does Long *In Vitro* Culture Promote Large for Gestational Age Babies? *Hum Reprod* (2013) 28:828–34. doi: 10.1093/humrep/des410
26. Ishihara O, Araki R, Kuwahara A, Itakura A, Saito H, Adamson GD. Impact of Frozen-Thawed Single-Blastocyst Transfer on Maternal and Neonatal Outcome:

An Analysis of 277,042 Single Embryo Transfer Cycles From 2008 to 2010 in Japan. *Fertil Steril* (2014) 101:128–33. doi: 10.1016/j.fertnstert.2013.09.025
27. Zhu J, Lin S, Li M, Chen L, Lian Y, Liu P, et al. Effect of *In Vitro* Culture Period on Birthweight of Singleton Newborn. *Hum Reprod* (2014) 29:448–54. doi: 10.1093/humrep/det460
28. Waterland RA, Garza C. Potential Mechanisms of Metabolic Imprinting That Lead to Chronic Disease. *Am J Clin Nutr* (1999) 69:179–97. doi: 10.1093/ajcn/69.2.179
29. Millership SJ, Van de Pette M, Withers DJ. Genomic Imprinting and its Effects on Postnatal Growth and Adult Metabolism. *Cell Mol Life Sci* (2019) 76:4009–21. doi: 10.1007/s00018-019-03197-z
30. Xu R, Li C, Liu X, Gao S. Insights Into Epigenetic Patterns in Mammalian Early Embryos. *Protein Cell* (2021) 12:7–28. doi: 10.1007/s13238-020-00757-z
31. Kumasako Y, Otsu E, Utsunomiya T, Araki Y. The Efficacy of the Transfer of Twice Frozen Thawed Embryos With the Vitrification Method. *Fertil Steril* (2009) 91:383–6. doi: 10.1016/j.fertnstert.2007.11.079
32. Zheng X, Chen Y, Yan J, Wu Y, Zhuang X, Lin S, et al. Effect of Repeated Cryopreservation on Human Embryo Developmental Potential. *Reprod BioMed Online* (2017) 35:627–32. doi: 10.1016/j.rbmo.2017.08.016
33. Bradley CK, Livingstone M, Traversa MV, McArthur SJ. Impact of Multiple Blastocyst Biopsy and Vitrification-Warming Procedures on Pregnancy Outcomes. *Fertil Steril* (2017) 108:999–1006. doi: 10.1016/j.fertnstert.2017.09.013

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Endocrinology

TYPE Original Research
PUBLISHED 06 March 2023
DOI 10.3389/fendo.2023.859204



OPEN ACCESS

EDITED BY
Yiping Shen,
Harvard Medical School, United States

REVIEWED BY
Dagan Mao,
Nanjing Agricultural University, China
Hui Chen,
Third Affiliated Hospital of Sun Yat-sen University, China

*CORRESPONDENCE
Jiayin Liu
jyliu_nj@126.com

SPECIALTY SECTION
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

RECEIVED 21 January 2022
ACCEPTED 21 February 2023
PUBLISHED 06 March 2023

CITATION
Xia X, Zhang Y, Cao M, Yu X, Gao L, Qin L, Wu W, Cui Y and Liu J (2023) Adverse effect of assisted reproductive technology-related hyperoestrogensim on the secretion and absorption of uterine fluid in superovulating mice during the peri-implantation period.
*Front. Endocrinol.* 14:859204.
doi: 10.3389/fendo.2023.859204



# Adverse effect of assisted reproductive technology-related hyperoestrogensim on the secretion and absorption of uterine fluid in superovulating mice during the peri-implantation period

Xinru Xia[1], Yuan Zhang[1], Meng Cao[1], Xiang Yu[2], Li Gao[1], Lianju Qin[1], Wei Wu[1], Yugui Cui[1] and Jiayin Liu[1]*

[1]State Key Laboratory of Reproductive Medicine, Center for Clinical Reproductive Medicine, First Affiliated Hospital, Nanjing Medical University, Nanjing, China, [2]Department of Pediatrics, First Affiliated Hospital, Nanjing Medical University, Nanjing, China

**Objectives:** This study aimed to investigate the potential mechanism of hyperoestrogensim elicited by ovulation induction affects endometrial receptivity and leads to embryo implantation abnormality or failure.

**Study design:** Establishment of ovulation induction mouse model. Changes in mouse body weight, ovarian weight, serum E2 level and oestrous cycle were observed. During the peri-implantation period, morphological changes in the mouse uterus and implantation sites and the localization and protein levels of oestrogen receptors ERα and ERβ, the tight junction factors CLDN3 and OCLN, the aquaporins AQP3, AQP4 and AQP8, and the sodium channel proteins SCNN1α, SCNN1β and SCNN1γ were observed. The expression and cellular localization of ERα, CLDN3, AQP8 and SCNN1 β in RL95-2 cell line were also detected by western blotting and immunofluorescence.

**Results:** Ovarian and body weights were significantly higher in the 5 IU and 10 IU groups than in the CON group. The E2 level was significantly higher in the 10 IU group than in the CON group. The mice in the 10 IU group had a disordered oestrous cycle and were in oestrus for a long time. At 5.5 dpc, significantly fewer implantation sites were observed in the 10 IU group than in the CON ($p<0.001$) and 5 IU ($p<0.05$) groups. The probability of abnormal implantation and abortion was higher in the 10 IU group than in the CON and 5 IU groups. CLDN3, OCLN, AQP8 and SCNN1β in the mouse endometrium were localized on the luminal epithelium and glandular epithelium and expression levels were lower in the 10 IU group than in the CON group. The protein expression level of ERα was increased by 50% in the 10 IU group compared to the CON group. The expressions of CLDN3, AQP8, SCNN1β in RL95-2 cell line were significantly depressed by the superphysiological E2, ERα agonist or ERβ agonist, which could be reversed by the oestrogen receptor antagonist.

**Conclusion:** ART-induced hyperoestrogenism reduces CLDN3, AQP8 and SCNN1β expression through ERα, thereby destroying tight junctions and water and sodium channels in the endometrial cavity epithelium, which may cause abnormal implantation due to abnormal uterine fluid secretion and absorption.

KEYWORDS

assisted reproductive technology, hyperoestrogenism, embryo implantation, uterine fluid, superovulation

# 1 Introduction

Although assisted reproductive technology (ART) has been significantly improved and overcomes many potential causes of infertility, the pregnancy success rate is still relatively low, mainly due to the failure of embryo implantation (1–6).

Embryo implantation, the most critical step in mammalian pregnancy (7), is an extremely complex physiological process regulated by a variety of factors, and its underlying mechanism has not yet been fully elucidated (7, 8). It requires an implantable blastocyst and a receptive endometrium, which communicate and interact with each other to achieve conception (8, 9). Oestrogen and progesterone are the main hormones that regulate this process (6, 10–12). During ART, a large number of eggs need to be collected and fertilized to increase the number of high-quality embryos available for transfer. The development of multiple follicles is induced by hormone stimulation. Although this method can be used to select high-quality embryos for transfer, ovarian stimulation can also lead to superphysiological levels of E2 (13, 14). It has been reported that hyperoestrogensim during the fresh embryo transfer cycle of ovulation induction leads to a decrease in the embryo implantation rate and clinical pregnancy rate (15–22).

The main function of the endometrium, the main target of oestrogen, is embryo implantation. Oestrogen mainly acts through two classic oestrogen receptor subtypes, ERα and ERβ, in the endoderm. Studies have revealed that ERα knockout mice are infertile, while embryo implantation does not seem to be disturbed in ERβ knockout mice (23). Elevated levels of sex steroids may impair endometrial receptivity (5, 21, 22, 24), leading to decreases in the embryo implantation and clinical pregnancy rates. However, the mechanism underlying the effect of superphysiological E2 levels on embryo implantation is still unclear.

Tight junctions (TJs) exist between the epithelial or endothelial cells of vertebrates that function to tightly join the plasma membranes of adjacent cells, and no gaps are present in cell junctions (25). TJs mainly function to close the gaps between adjacent cells, prevent molecules in solution from penetrating the body through gaps between cells, and maintain the relative stability of the bodily environment (26). TJs are composed mainly of members of two protein superfamilies, namely, the transmembrane protein family, which includes occludin (OCLN) and CLDN, and the perimembrane protein family, which includes zonula occludens (ZO) (27). Whether ovarian stimulation alters the structure and function of TJs among endometrial epithelial cells by changing the expression of TJ proteins, leading to abnormal embryo implantation, has not been reported.

Aquaporins in the mammalian reproductive system mainly function to regulate the amount of water in the uterus and fallopian tubes (28–32). The latest research shows that the expression of Aqp3, Aqp4, Aqp5 and Aqp8 is induced by E2 but not P4, while the expression of Aqp1 and Aqp11 is increased by P4. P4 inhibits the expression of Aqp3 and Aqp4 induced by E2, and E2 inhibits the expression of Aqp1 and Aqp11 induced by P4. Aqp9 expression is not significantly altered. Ovarian stimulation is known to alter the expression of D4 Aqp3, Aqp5 and Aqp8 (33). However, whether ovarian stimulation changes the amount of water in the uterine cavity by changing the expression of aquaporins and then disrupting embryo implantation has not been addressed.

Ion channels have proven to be essential for reproduction. An increasing number of studies have shown that ion channels in the endometrium play an important role in regulating endometrial receptivity and embryo implantation. Abnormal expression or function of endometrial ion channels may lead to impaired endometrial receptivity and/or implantation failure. The epithelial sodium ion channel (ENaC), which is encoded by the SCNN1 gene of the ENaC superfamily, is highly expressed in epithelial cells of the lung, kidney, brain, and reproductive tract. In the female reproductive tract, ENaC regulates the absorption of uterine fluid during the reproductive cycle. Thus far, the α, β, γ, and δ subunits of mammalian ENaC have been cloned. However, which ENaC subunits play important roles in ovarian stimulation has not been determined.

Therefore, this research focuses on the specific mechanism by which hyperoestrogensim regulates embryo implantation during ART-induced ovulation induction. It is technically and ethically difficult to study the process of human embryo implantation *in vivo*. Therefore, we established a hyperoestrogenic mouse model and observed whether it sufficiently simulates the clinical ovulation stimulation cycle of the embryo implantation process. We also used cell line RL95-2 to simulate the process of human embryo implantation *in vitro*. To investigate whether high oestrogen levels affect the mouse endometrium during the peri-implantation period and thus cause embryo implantation anomalies or failures and explored the possible mechanisms.

# 2 Materials and methods

## 2.1 Animals

All procedures involving the use of animals were approved by the Experimental Animal Ethics Committee of the University of Nanjing Medical University (Nanjing, China) with the ethics number IACUC-1702002.

Eight-week-old specific pathogen-free (SPF) ICR mice were bred under controlled environmental conditions (12 h light/dark cycle, relative humidity of 40–70%, and temperature of 20–25°C).

## 2.2 Treatment with gonadotropin to induce superovulation

The oestrous cycle phase was determined based on vaginal smears and staining with methylene blue (Shanghai Yuanye Biotechnology Co., Ltd., CAS: 7220-79-3). The oestrous cycle was divided into proestrus, oestrus, metestrus and dioestrus.

Female mice in proestrus or oestrus were injected intraperitoneally (ip) with 10 IU pregnant mare serum gonadotropin (PMSG, Sigma, USA) and then with 10 IU human chorionic gonadotropin (HCG, Sigma) 48 h later (the 10 IU group). Female mice in metestrus were injected ip with 5 IU PMSG and then with 5 IU HCG 48 h later (the 5 IU group).

Female mice in dioestrus were injected ip with physiological saline and then with physiological saline 48 h later (the CON group). Immediately after the HCG or physiological saline injection, female mice were mated with males at a ratio of 2:1. Mating was confirmed the next morning by the presence of a vaginal plug, and this day was considered 0.5 days postcoitum (dpc). If no vaginal plug was observed, vaginal smears were performed for one week.

We have 6 mice in each group. The mice were weighed at 8 a.m. At 3.5, 4.5 and 5.5 dpc, 0.7–0.8 ml of peripheral blood was taken from the inner canthal vein after anaesthetization with 0.2 ml/10 g tribromoethanol. Female mice were sacrificed after the injection of 0.1 ml/10 g trypan blue solution (Sigma, catalogue number: 93595) into their tail vein, and successful injection was confirmed by the mouths and ears of the mice turning blue. The uterus and ovaries were removed immediately and rinsed in precooled phosphate-buffered saline (PBS) (Beyotime, China, catalogue number: ST476). The uterus and ovaries were harvested and photographed immediately and then used for subsequent experiments. The number of implantation sites was determined by trypan blue staining. The ovaries were weighed; half of the uterus was fixed in 4% paraformaldehyde solution, and the other half was placed in a cryotube and stored at -80°C. The blood taken from the inner canthal vein was centrifuged at 3500 rpm for 5 min, and the supernatant was then stored at -80°C before the analysis of 17β-oestradiol levels. The next morning, the 4% paraformaldehyde was replaced with 75% ethanol, and 5 μm sections were used for haematoxylin and eosin (H&E) staining and immunohistochemistry (IHC).

The body weight, size and weight of the ovaries, serum E2 level, oestrous cycle, size and number of implantation sites in the uterus, and localization and expression of oestrogen receptors, TJ proteins, aquaporins and sodium channel proteins in endometrial epithelial cells were assessed.

## 2.3 Cells and cell culture techniques

RL95-2 cells (an endometrial adeno-carcinoma cell line with microvilli on the cell surface) were maintained in 25-$cm^2$ flasks using Dulbecco's minimal essential medium (DMEM)/F12 supplemented with 10% (vol/vol) fetal bovine serum (FBS). The cells were maintained at 37°C in a humidified atmosphere and 5% CO2. RL95-2 cells were initially passaged using a standard trypsinization protocol, plated in 24-well culture dishes, and grown to 70% confluence. The cells were then grown in serum-free, phenol red-free medium for 12 hours before the experimental treatments. The cells were then treated for another 24 hours with either $10^{-6}$M E2 (Sigma, USA, catalogue number: E8875), $10^{-6}$M ERα agonist (PPT) (Tocris, UK, catalogue number: 1026), $10^{-6}$M ERβ agonist (DPN) (Tocris, UK, catalogue number: 1494), or $10^{-6}$M ER antagonist (ICI 182,780) (Tocris, UK, catalogue number: 1047).

## 2.4 Analysis of serum 17β-oestradiol levels

A chemiluminescence detection kit (oestradiol determination kit, Beckman Coulter) and a chemiluminescence instrument (UniCel DxI 800, Beckman Coulter) were used for this analysis. Both the intra- and interassay variation were within the set range.

## 2.5 H&E staining

The morphological features of the implantation site were analysed by H&E staining. Uterine tissues isolated from mice were fixed with 4% paraformaldehyde overnight, embedded in paraffin, and cut into 5-μm-thick sections. The sections were deparaffinized and hydrated by brief incubations in xylene, ethanol, and water. Then, the sections were stained with haematoxylin, rinsed, and stained with eosin. The stained sections were dehydrated by brief incubations in water, alcohol and xylene. After mounting, the sections were observed with a bright-field microscope.

## 2.6 IHC

The expression of oestrogen receptors, TJ proteins, aquaporins and sodium channel proteins was assessed by IHC. The primary antibodies utilized are listed in Table 1. PBS rather than the primary antibody served as the negative control.

Tissue was fixed overnight in 4% paraformaldehyde, embedded in paraffin, and cut into 5-μm-thick sections. The sections were deparaffinized, hydrated, and incubated in a histochemistry box containing 10 mM citrate buffer (pH 6.0) at 95°C for 15 min for

TABLE 1 Primary antibodies used.

| Primary antibody | Host | Company | Catalogue number | Dilution (IHC) | Dilution (WB) |
|---|---|---|---|---|---|
| **ERα** | Rabbit | abcam | ab32063 | 1:200 | 1:1000 |
| **ERβ** | Mouse | santa | sc-390243 | 1:100 | 1:500 |
| **CLDN3** | Rabbit | abcam | ab52231 | 1:100 | 1:500 |
| **OCLN** | Mouse | santa | sc-133256 | 1:200 | 1:1000 |
| **AQP3** | Rabbit | abcam | ab125219 | 1:100 | 1:500 |
| **AQP4** | Rabbit | abcam | ab259318 | 1:100 | 1:500 |
| **AQP8** | Mouse | santa | sc-81870 | 1:100 | 1:500 |
| **SCNN1α** | Rabbit | sigma | SAB5200105 | 1:100 | 1:500 |
| **SCNN1β** | Rabbit | sigma | SAB5200106 | 1:100 | 1:500 |
| **SCNN1γ** | Rabbit | sigma | SAB5200107 | 1:100 | 1:500 |

IHC, immunohistochemistry; WB, Western blot

antigen retrieval. The sections were pretreated with 3% H2O2 in 0.1 M Tris-buffered saline (TBS, pH 7.4) for 10 min to block endogenous peroxidase activity. The sections were washed with PBS three times for 3 min each and then treated with protein blocking solution at 37°C for 10 min and with a primary antibody (diluted 1:100 in 5% BSA or IHC primary antibody dilution buffer) overnight at 4°C. The sections were washed with PBS three times for 3 min each time, covered with enzyme-labelled anti-mouse/rabbit polymer or Solution C and incubated for 10 min at room temperature. They were then covered with horseradish peroxidase (HRP)-labelled streptavidin or Solution D and incubated at room temperature for 10 min. The slides were washed thoroughly with PBS (pH=7.4) between incubations.

After treatment with 3,3'-diaminobenzidine (DAB) chromogen substrate solution, peroxidase bound to the antibody complex was observed. The DAB reaction was monitored under a microscope to determine the optimal incubation time, and the reaction was stopped by washing with 0.1 M TBS multiple times. The immunolabelled sections were dehydrated *via* a graded ethanol series, cleared in xylene, and fixed. The slides were counterstained with haematoxylin before mounting. Brown deposits indicated positive signals and were evaluated under a Nikon Eclipse Ti microscope (Nikon, Japan).

## 2.7 Immunofluorescence analysis

RL95-2 cells were fixed in 4% paraformaldehyde for 20min. Sections were washed three times with phosphate buffered saline (PBS) for 5 min. Treat with 0.4% Triton X-100 for 10min, and then washed three times with PBS for 5min. Non-specific binding was blocked with 5% bovine serum albumin (BSA) for 30min. Sections were incubated with the following primary antibodies diluted (1:100) in blocking solution (5% BSA) overnight at 4°C. ERα (Abcam ab32063), CLDN3 (Invitrogen, 34-1700), AQP8 (bioworld, BS71279) and SCNN1β (Sigma, SAB5200106). Sections were then washed three times with PBS for 5 min. For the fluorescent detection Alexa Fluor™ 488 goat anti-rabbit (dilution 1:500, Thermo Fisher Scientific) secondary antibody was used and nuclear counterstaining was performed with 4,6-diamidino-2-phenylindole (DAPI, Life Technologies, 10236276001). Evaluation of the sections was performed using confocal microscopy (Nikon, Eclipse Ti, Japan).

## 2.8 Western blot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed routinely. Frozen uterine samples were homogenized in tissue lysis buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% NP40) containing 1% protease and phosphatase inhibitors. The tissue homogenates were clarified by centrifugation at 12,000×g for 15 min at 4°C. A BCA protein concentration determination kit (Biyuntian, China, catalogue number: P0010) was used to determine the protein concentration, and the clarified supernatants were mixed with 6× SDS loading buffer (5:1) and transferred to a 70°C water bath for 10 min to denature the proteins. The samples were separated on NuPAGE™ 7% Tris-acetate protein gels (Invitrogen, catalogue number: EA0358BOX) and electrotransferred onto a PVDF Hybond nitrocellulose membrane (Millipore). The membrane was blocked in TBST (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% (w/v) Tween 20) containing 5% skim milk for 1 h at room temperature. Then, the membrane was incubated with a primary antibody (1:1000 dilution) at 4°C overnight, and a rabbit anti-mouse GAPDH polyclonal antibody (1:5000 dilution, Bioworld, catalogue number: AP0063) was used as a control. After washing with TBST, the membrane was incubated in 5% skim milk in TBST containing a secondary antibody (diluted 1:5000, HRP-conjugated goat anti-rabbit IgG, Invitrogen, catalogue number: Ab6721; HRP-conjugated goat anti-mouse IgG), Invitrogen, catalogue number: Ab6789) for one hour at room temperature. The membrane was washed 3 times with TBST, and an ECL kit (Thermo, catalogue number: 1863096; 1863097) was then used to visualize the bands. The membrane was scanned with a luminescence imaging analyser. The relative protein levels were evaluated with ImageJ analysis software (National Institutes of Health, Maryland, Baltimore, USA). The concentration in each sample is expressed as the grey value relative to that of GAPDH or ACTIN.

## 2.9 Statistical analysis

The data are presented as the means ± SDs of three or more independent experiments. For group comparisons, one-way ANOVA or Student's *t* test was performed using Prism software version 5.0 for statistical data analysis (GraphPad Software, Inc.). Differences were considered significant at *p < 0.05, **p< 0.01, and ***p< 0.001.

# 3 Results

## 3.1 Characteristics of female mice after PMSG/HCG injection

To investigate the effects of superovulation treatments on female mice, 5 IU or 10 IU PMSG/HCG was injected ip into the mice. We assessed their body weight, ovarian morphology, ovarian weight, serum oestradiol levels and oestrous cycle. There was no significant difference in the mouse weight, which was measured every other day, among the different groups (Supplemental Figure 1).

## 3.2 Ovarian morphology and ovarian weight of the CON, 5 IU and 10 IU groups from 3.5 to 5.5 dpc

During the peri-implantation period (from 3.5 to 5.5 dpc), the ovaries of the mice in the 5 IU and 10 IU groups were larger than those of the mice in the CON group (Figure 1A), and the ovarian weight showed a dose-dependent increase, as the ovarian weights of the 5 IU and 10 IU groups were increased compared with that of the CON group (Figure 1B). However, there was no significant increase in the mouse weight (Supplemental Figure 1). These changes were consistent with the clinical characteristics of ovaries after superovulation.

## 3.3 Differences in the serum oestrogen levels and oestrous cycles between the CON and 10 IU groups

We measured the peripheral serum oestradiol and progesterone concentrations during the peri-implantation period. The serum oestradiol level in the 10 IU group was 2-fold higher than that in the CON group (Figure 2A), while the serum progesterone level in the 10 IU group increased by 100% at 3.5dpc and increased by 50% at 4.5dpc compared to the CON group, but the difference was not statistically significant (Supplemental Figure 2). The mice in the CON group had a normal oestrus cycle, progressing from proestrus to oestrus, metestrus and dioestrus. In contrast, the oestrous cycle of the 10 IU group was disordered, and these mice remained in oestrus for a long time (Figure 2B). The above results indicate that our model sufficiently simulates the endocrine hormone alterations caused by clinical superovulation and a long-term hyperoestrogen state.

## 3.4 Effect of superovulation on embryo implantation during the peri-implantation period

To study the effect of superovulation treatment on embryo implantation, we compared the uterine morphologies, implantation

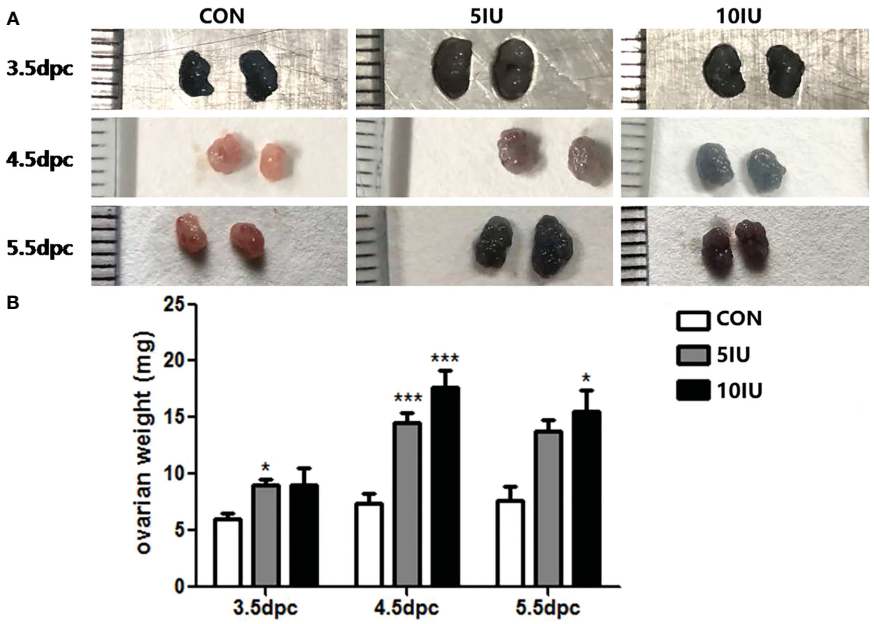



FIGURE 1
Ovarian morphologies and ovarian weights in the CON, 5 IU and 10 IU groups from 3.5 dpc to 5.5 dpc. **(A)** Changes in ovarian morphology from 3.5-5.5 dpc among the three groups. **(B)** Change in ovarian weight from 3.5-5.5 dpc among the three groups. The blue colour of the ovary in Figure A is due to the injection of trypan blue into the tail vein of the mouse. Compared with the control group, *p<0.05, ***p<0.001.

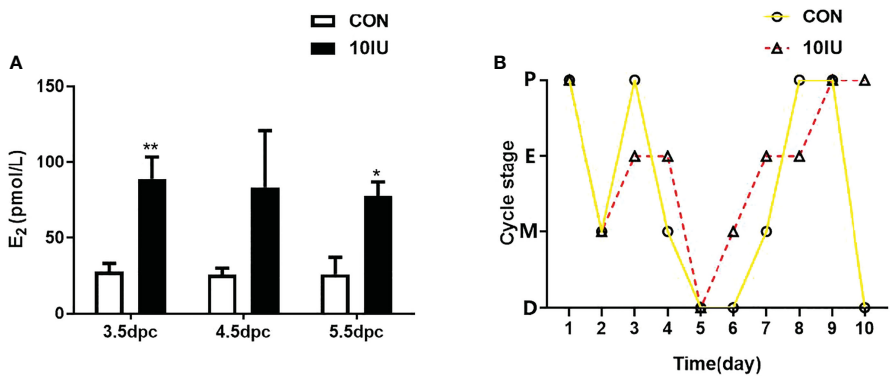



FIGURE 2
Serum oestrogen level and oestrus cycle changes between the CON and 10 IU groups. **(A)** Serum oestrogen levels of mice in the CON and 10 IU groups during the peri-implantation period. **(B)** Changes in the oestrous cycle of the mice in the CON and 10 IU groups. The abscissa depicts the number of days of detection, and the ordinate depicts the oestrus cycle. P: pro-oestrus, E: oestrus, M: meta-oestrus, D: dioestrus. Compared with the control group, *p<0.05, **p<0.01.

site numbers, and H&E staining of implantation sites among the three different groups.

## 3.5 Uterine morphologies and implantation site numbers in the CON, 5 IU and 10 IU groups during the peri-implantation period

At 3.5 dpc, no embryos were implanted in the uteri of mice in the CON group, but the uteri of mice in the 10 IU group showed obvious oedema and stubs. At 4.5 dpc, the uteri of mice in the CON group began to show implantation sites, but those of mice in the 5 IU group and 10 IU group did not yet show obvious implantation sites. At 5.5 dpc, the uteri of mice in the CON group showed complete implantation, while implantation in the uteri of mice in the 5 IU group and the 10 IU group occurred later than that in the CON group, and the spacing between the implantation sites was uneven (Figure 3A). There were significantly fewer implantation sites in the 10 IU group than in the CON group ($p<0.001$) and the 5 IU group ($p<0.05$) (Figure 3B).

## 3.6 Endometrial implantation sites in the CON, 5 IU and 10 IU groups at 5.5 dpc

To investigate the effect of hyperoestrogenism on implantation sites in mice at 5.5 dpc, we performed H&E staining. Morphological changes in the uteri were observed. Embryos in the CON group were evenly spaced and of the same size, and no gap existed between the embryo and the inner membrane. The implantation sites were mainly normal, the embryo spacing was even, and the gap between the embryo and the inner membrane was not obvious in the 5 IU group compared with the CON group; however, the embryo sizes differed between the two groups. In the 10 IU group, the gap between the embryo and the inner membrane was large, the embryo spacing was uneven, and the embryos were too small, resulting in abnormal implantation (Figure 3C). The probability of entry and miscarriage was higher in the 10 IU group than in the CON and 5 IU groups (Figure 3D).

## 3.7 Possible mechanism by which hyperoestrogenism affects embryo implantation

To study the possible mechanism by which hyperoestrogenism disrupts embryo implantation during ovulation induction, IHC and western blotting were performed to assess the localization and expression of the oestrogen receptors, tight junction factors, the aquaporins and the sodium channel proteins in the endometria of 5.5 dpc mice and RL95-2cell line.

## 3.8 Localization and western blot analyses of the ERα and ERβ proteins in the mouse uterus during the peri-implantation period

The results showed that ERα was localized in the luminal and glandular epithelia of the mouse endometrium (Figure 4A). The protein expression of ERα increased by 50% in the 10 IU group compared to the CON group (Figure 4B), and ERβ was not obviously expressed (Figure 4A). In RL95-2 cell line, ERα localized in cytoplasm. High oestrogen levels and oestrogen receptor agonists increased the expression of ERα, while oestrogen receptor inhibitors can inhibited the expression of ERα (Figure 5). This result suggeststhat hyperoestrogenism regulates embryo implantation through ERα.

## 3.9 Localization and western blot analyses of the CLDN3 and OCLN proteins in the mouse uterus during the peri-implantation period

At 5.5 dpc, CLDN3 and OCLN were localized in the epithelium of the mouse endometrial cavity (Figure 6A). At 4.5 and 5.5 dpc, the CLDN3 protein level in the 10 IU group was lower than that in the CON group ($p<0.05$ and $p<0.01$, respectively, Figure 6B). In RL95-2

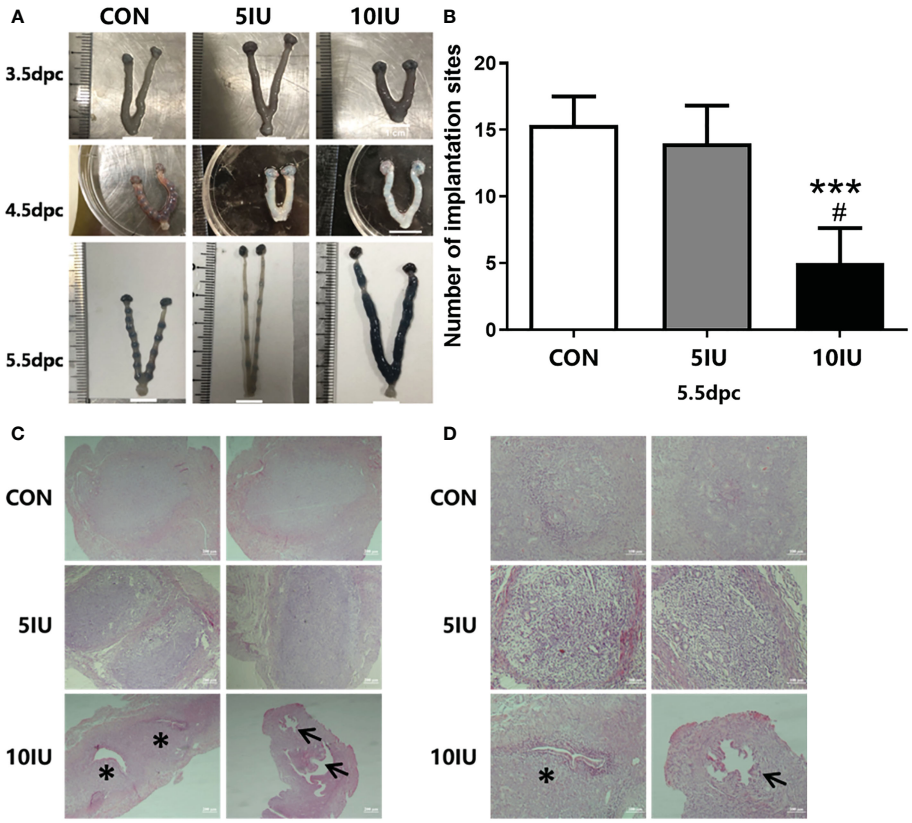



FIGURE 3
Uterine morphologies of mice during the peri-implantation period, implantation site numbers and implantation sites in the endometrium at 5.5 dpc among the CON, 5 IU and 10 IU groups. **(A)** Morphological changes in the mouse uteri from 3.5-5.5 dpc. A: Morphological changes in the uteri of mice from 3.5-5.5 dpc among the three groups. The implantation sites were stained with trypan blue. **(B)** Comparison of the implantation site numbers among the three groups of mice at of 5.5 dpc. Compared with the CON group, ***p<0.001. Compared with the 5IU group, $^{\#}$p<0.05. **(C)** Implantation sites in the endometrium at 5.5 dpc among the CON, 5 IU and 10 IU groups. Bar=200 μm. **(D)** Implantation site in the endometrium at 5.5 dpc among the CON, 5 IU and 10 IU groups. Bar=100 μm. ***p<0.001, *p<0.05. The arrows indicate abnormal sites of embryo implantation. The asterisk indicates the site of miscarriage.

cell line, CLDN3 was mainly localized in membrane. High oestrogen levels and oestrogen receptor agonists depressed the expression of CLDN3, while oestrogen receptor inhibitors can promote the expression of CLDN3 (Figures 5, 7). This result suggeststhat high oestrogen levels can reduce the expression of CLDN3 through ERα.

## 3.10 Localization and western blot analyses of the AQP3, AQP4 and AQP8 proteins in the mouse uterus during the peri-implantation period

At 5.5 dpc, AQP3, AQP4, and AQP8 were not obviously expressed in the murine endometrial cavity epithelium or glandular epithelium (Figure 8A). At 4.5 dpc, the expression of AQP8 in the 10 IU group was significantly lower than that in the CON group ($p<0.05$). At 5.5 dpc, the expression of AQP8 in the 10 IU group was lower than that in the CON group (Figure 8B). In RL95-2 cell line, AQP8 was mainly localized in cytoplasm. High oestrogen levels and oestrogen receptor agonists depressed the expression of AQP8, while oestrogen receptor inhibitors can promote the expression of AQP8 (Figures 5, 7). This result suggeststhat high oestrogen levels can also reduce the expression of aquaporin AQP8 through ERα.

## 3.11 Localization and western blot analyses of the SCNN1α, SCNN1β and SCNN1γ proteins in the mouse uterus during the peri-implantation period

At 5.5 dpc, SCNN1α and SCNN1γ were not obviously expressed. SCNN1β was localized in the murine endometrial cavity epithelium and glandular epithelium (Figure 9A).

At 4.5 dpc, the expression of SCNN1β in the 5 IU group was significantly lower than that in the CON group ($p<0.05$); its expression in the 10 IU group was reduced, but the difference was not significant. At 5.5 dpc, the expression of SCNN1β did not significantly differ among the three groups (Figure 9B). In RL95-2 cell line, SCNN1β was mainly localized in cytoplasm and showed polarity. High oestrogen levels and oestrogen receptor agonists depressed the expression of SCNN1β, while oestrogen receptor inhibitors can promote the expression of SCNN1β (Figures 5, 7).

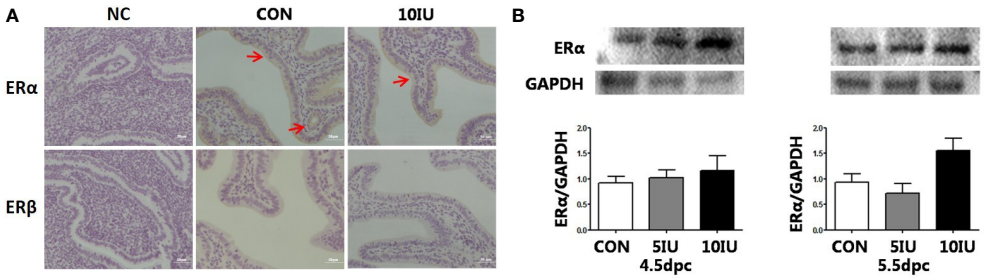



FIGURE 4
The localization and protein expression of the oestrogen receptors ERα and ERβ in the mouse endometrium at 4.5 dpc and 5.5 dpc in the CON, 5 IU and 10 IU groups. **(A)** Localization of ERα and ERβ in the endometria of mice at 5.5 dpc. Bar=50 μm. **(B)**: Protein expression of ERα in the endometria of mice in the CON, 5 IU and 10 IU groups at 4.5 dpc and 5.5 dpc. NC, negative control. The arrows indicate the localization of target protein.

# 4 Discussion

Exogenous gonadotropins are used to induce superovulation in humans and animals to increase the number of oocytes and embryos, thereby increasing the success rate of pregnancy (34). The PMSG/HCG regimen has been used in many laboratories to induce superovulation in mice for more than 60 years (35). However, superovulation in female mice increases the number of unhealthy follicles that are ovulated and leads to an increase in the number of low-quality oocytes (36–38). Superovulation treatment can induce changes in the maternal fallopian tubes that make them unsuitable for the transport of oocytes and cause alterations in the uterine environment, thereby impairing implantation and preventing subsequent pregnancy in female mice (39). Therefore, gonadotropin superovulation therapy seems to have an adverse effect on the maternal environment.

The doses of PMSG and hCG that are commonly used to construct mouse models of superovulation are 5 IU (40), 7.5 IU (41, 42), and 10 IU (43). The 10 IU dose was previously verified to seriously decrease the embryo implantation rate (43), but the specific mechanism has not been studied. This study revealed that the administration of PMSG and HCG to stimulate ovulation in mice simulated the increase in the ovarian volume caused by the clinical ovulation stimulation cycle, induced a continuous increase in serum oestrogen levels, and induced abnormal or failed embryo implantation in a dose-dependent manner.

E2 is reportedly a key determinant of the duration of the endometrial receptive implantation window (9) and mainly acts through nuclear oestrogen receptors (mainly ERα but not ERβ) (44, 45). Recently, Chai et al. compared the effects of high serum E2 levels on endometrial steroid receptor levels during the gonadotropin stimulation cycle and the natural cycle. Their results showed that oestrogen receptor expression was significantly reduced during the stimulation cycle (46). The results of this study showed that ERα expression but not ERβ expression was increased by 50% in the 10 IU group compared to the CON group at 5.5 dpc. However, the difference is not statistically significant. This finding confirms that at superphysiological concentrations, E2 exerts its effects *via* ERα.

Secreted uterine cavity fluid initially allows the transport of and provides support for sperm and untransferred embryos, while the absorption of uterine cavity fluid in early pregnancy leads to the

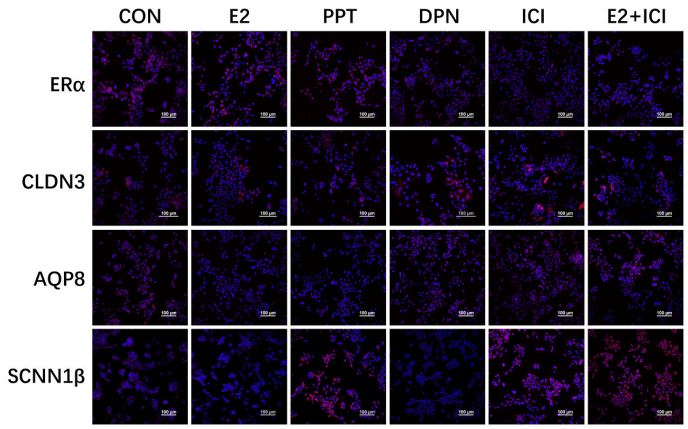



FIGURE 5
Localization of ERα, CLDN3, AQP8, and SCNN1β in RL95-2 cell line. CON: control group, E2: treat with $10^{-6}$M oestrogen, PPT: treat with $10^{-6}$M ERα agonist, DPN: treat with $10^{-6}$M ERβ agonist, ICI: treat with $10^{-6}$M oestrogen receptor antagonist, E2+ICI: treat with both $10^{-6}$M E2 and $10^{-6}$M ICI. The blue colour: nucleus stained by DAPI. The red colour: localization of target proteins.

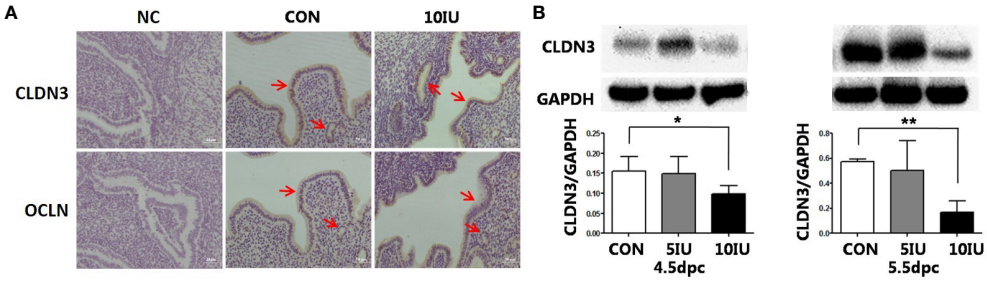



FIGURE 6
Localization and protein expression of the tight junction proteins CLDN3 and OCLN in the mouse endometrium at 4.5 dpc and 5.5 dpc among the CON, 5 IU and 10 IU groups. **(A)** Localization of CLDN3 and OCLN in the endometria of mice in the CON and 10 IU groups at 5.5 dpc. Bar=50 mm. **(B)** Protein expression of CLDN3 in the endometria of mice in the CON, 5 IU and 10 IU groups at 4.5 dpc and 5.5 dpc. NC: negative control Compared with the CON group, *p<0.05, **p<0.01. The arrows indicate the localization of target protein.

closure of the cavity and allows the blastocyst to establish close contact with the uterine epithelium. The content and volume of fluid in the uterine cavity are jointly regulated by TJs, water channels, and ion channels, among others (47).

The results of this study show that an imbalance in uterine cavity fluid during embryo implantation may cause embryo implantation failure. Embryo implantation was delayed after ovulation induction, the spacing between embryos was uneven, the number of embryos was reduced, and the incidence of abnormal implantation and miscarriage was high.

There is some evidence that TJs play a role in the uterus and implantation. Previous studies have shown that both CLDN3 and CLDN7 are expressed in the luminal and glandular epithelium in the mouse endometrium during the oestrous cycle and that CLDN10 is expressed in only the glandular epithelium. At 4.5 dpc, the time point at which embryo implantation occurs, the CLDN3 protein is localized at the top of the epithelium, while CLDN7 is not expressed in the epithelium at the implantation site. Moreover, CLDN3 and CLDN7 are not expressed in the matrix, but CLDN10 is strongly expressed in the primary decidual area (48). CLDN3 mRNA and protein are highly expressed in the luminal epithelia of mice on the 3rd and 4th days of pregnancy, but by the 5th day of pregnancy, when blastocysts are implanted, the expression of CLDN3 is downregulated. At this time, the downregulation of CLDN-3 expression may be beneficial for the loss of the luminal epithelial barrier, and changes in the expression and morphology of TJ proteins help blastocysts to invade the endometrium (49–52). At the same time, CLDN3 and CLDN10 are expressed in human endometrial epithelial cells (48). Thus, CLDN3 plays an important role in the process of embryo implantation. In this study, we evaluated the dynamic expression of CLDN3 and OCLN in mice subjected to PMSG+HCG-induced ovulation induction during the peri-implantation period. The results showed that CLDN3 and OCLN were localized in the

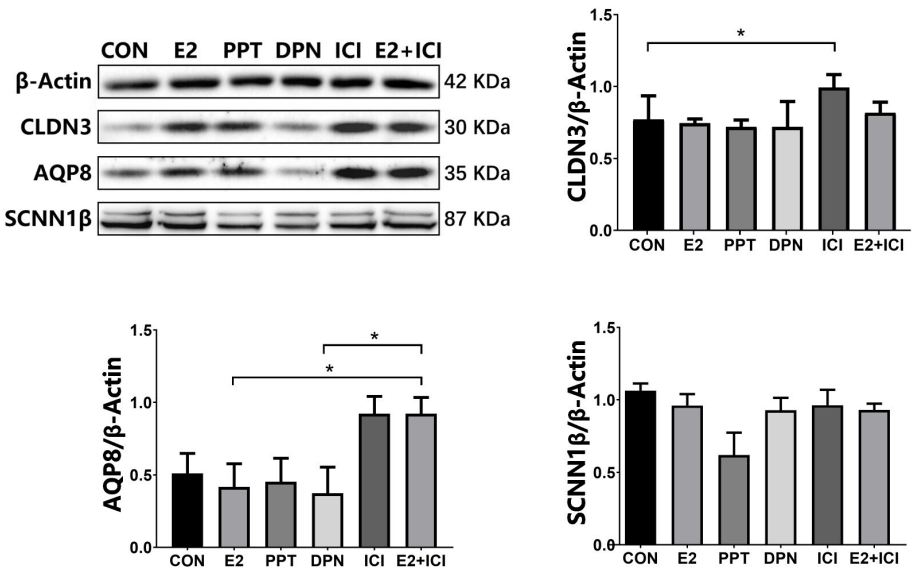



FIGURE 7
protein expression of CLDN3, AQP8, and SCNN1β in RL95-2 cell line. CON: control group, E2: treat with $10^{-6}$M oestrogen, PPT: treat with $10^{-6}$M ERα agonist, DPN: treat with $10^{-6}$M ERβ agonist, ICI: treat with $10^{-6}$M oestrogen receptor antagonist, E2+ICI: treat with both $10^{-6}$M E2 and $10^{-6}$M ICI. *p<0.05.

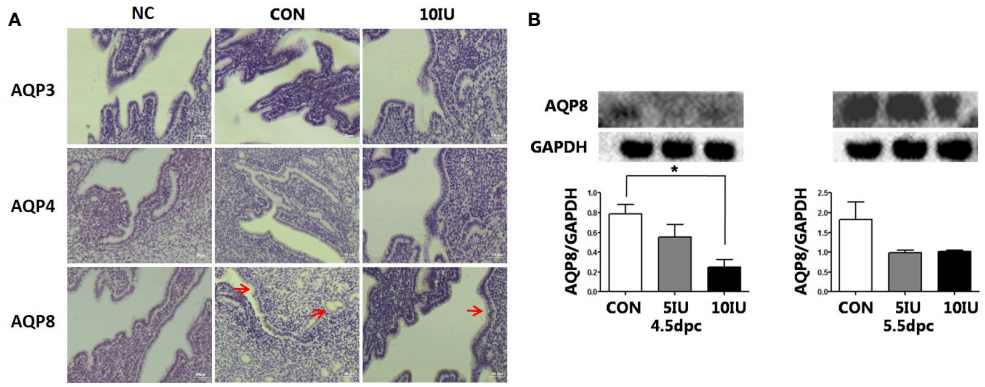



FIGURE 8
Localization and protein expression of the aquaporins AQP3, AQP4 and AQP8 in the mouse endometrium at 4.5 dpc and 5.5 dpc among the CON, 5 IU and 10 IU groups. **(A)** Localization of AQP3, AQP4 and AQP8 in the endometria of mice in the CON and 10 IU groups at 5.5 dpc. Bar=50 μm. **(B)** Protein expression of AQP8 in the endometria of mice in the CON, 5 IU and 10 IU groups at 4.5 dpc and 5.5 dpc. NC: negative control. Compared with the CON group, *p<0.05. The arrows indicate the localization of target protein.

luminal epithelium of the mouse endometrium at 5.5, 4.5 and 5.5 dpc and that the expression of CLDN3 in the 10 IU group was lower than that in the CON group. The decreased expression of CLDN3 is mainly mediated by ER α, which can be blocked by oestrogen receptor blocker ICI182780. These results suggest that the reduced CLDN3 expression in the present study suggest that the embryo implantation in the 10 IU group was affected by the highly female bodily environment.

The expression of AQP3 in the middle and late stages of human endometrial secretion is significantly higher than that in other stages. The protein expression of AQP5 in uterine cavity epithelial cells was shown to be increased in pregnant rats subjected to controlled ovarian hyperstimulation (COH) compared to normal pregnant rats. At the time of implantation, the distribution of AQP5 staining in rats subjected to COH was altered (53). There was no significant difference in the pregnancy rate between AQP8 knockout mice and wild-type mice. Compared with that in wild-type control mice, the number of embryos in pregnant AQP8 knockout mice was shown to be significantly increased (54).

Ovarian stimulation alters the expression of D4 Aqp3, Aqp5 and Aqp8 (33). Our results suggest that AQP8 plays a role in ovulation induction-induced hyperoestrogensim leading to abnormal or failed embryo implantation. Hyperoestrogenism decreased the expression of AQP8 through ERα. The decreased expression of AQP8 may affect the embryo implantation process by regulating the uterine fluid.

It was previously demonstrated that ENaC-α in the mouse endometrium is activated to the greatest extent at the time of implantation. ENaC deficiency or low expression of ENaC in the endometrium may lead to a low pregnancy success rate or abortion in patients undergoing *in vitro* fertilization (IVF) (55). In this study, high oestrogen levels during ART led to reduced expression levels of SCNN1β through ERα, which may account for the reduced endometrial receptivity, abnormal embryo implantation or implantation failure.

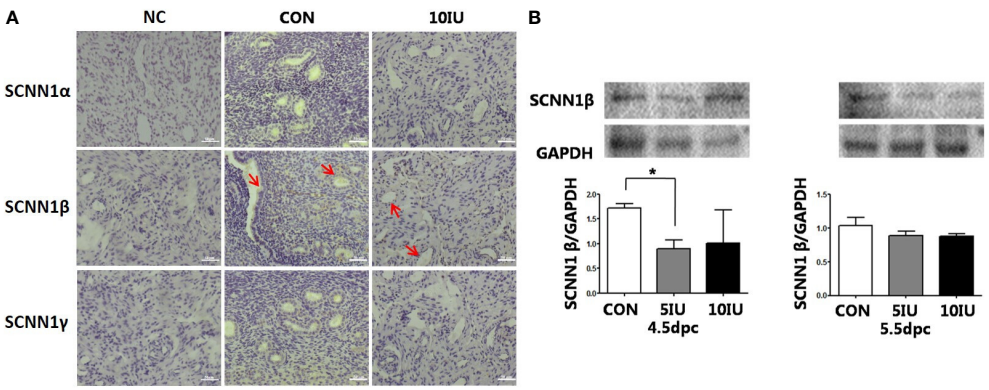



FIGURE 9
Localization and protein expression of the sodium channel proteins SCNN1α, SCNN1β and SCNN1γ in the mouse endometrium at 4.5 dpc and 5.5 dpc among the CON, 5 IU and 10 IU groups. **(A)** Localization of SCNN1α, SCNN1β and SCNN1γ in the endometria of mice in the CON and 10 IU groups at 5.5 dpc. Bar=50 μm. **(B)** Protein expression of SCNN1β in the endometria of mice in the CON, 5 IU and 10 IU groups at 4.5 dpc and 5.5 dpc. NC: negative control. Compared with the CON group, *p<0.05. The arrows indicate the localization of target protein.

At physiological concentrations, E2 and P regulate the volume of fluid in the uterine cavity during embryo implantation. In this study, we found that superphysiological concentrations of E2 significantly affected the expression of TJ, water channel, and sodium channel proteins in endometrial epithelial cells *in vivo*. This result provides a possible mechanism for low endometrial receptivity during the COH cycle. We found that superphysiological concentrations of E2 played a role in destroying TJs, water channels, and sodium channels. At superphysiological concentrations, E2 may impair embryo implantation by inducing fluid imbalance in the peri-implantation uterine cavity through ERα, thus hindering endometrial receptivity for implantation. We have shown that uterine fluid imbalance during COH is mediated by abnormal downregulation of CLDN3, AQP8 and SCNN1β expression. Our research lays the foundation for further research on the role of these factors in clinical phenomena such as embryo implantation failure and miscarriage.

## 5 Conclusions

These results suggest that in the 10 IU group, the implantation of mouse embryos was affected by the highly female bodily environment, implantation was delayed, the embryos were unevenly spaced, and the abortion rate was high, while the oestrogen environment in the 5 IU group had a smaller effect on receptivity. High oestrogen levels during ART alter the expression levels of TJ, aquaporin and sodium channels proteins through ERα, thereby destroying the TJs, water and sodium channels between the epithelium of the endometrial cavity and resulting in reduced endometrial receptivity, delayed implantation, and abnormal embryo implantation. These factors are associated with E2 and are the result of the co-regulation of multiple E2-related pathways.

## 6 Limitations

Further research is needed to clarify the downstream mechanism *via* which hyperoestrogensim related to ART mediates the regulation of TJ, aquaporin and sodium channel proteins.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The animal study was reviewed and approved by the Experimental Animal Ethics Committee of the University of Nanjing Medical University.

## Author contributions

XX wrote the draft of the manuscript. YZ performed the data analyses. MC, XY and LG conducted animal experiments and acquired the data. LQ, WW and YC reviewed and edited the manuscript. JL designed the study. All authors contributed to the article and approved the submitted version.

## Funding

This work was supported by grants from the National Nature and Science Foundation of China (81730041), the National Key Research and Development Program of China (2017YFC1001303, 2017YFC1001301), and a China Jiangsu Province Health Department programme (YXZXB2016001).

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.859204/full#supplementary-material

## References

1. Miller PB, Parnell BA, Bushnell G, Tallman N, Forstein DA, Higdon HL3rd, et al. Endometrial receptivity defects during IVF cycles with and without letrozole. *Hum Reprod* (2012) 27:881–8. doi: 10.1093/humrep/der452

2. Harton GL, Munne S, Surrey M, Grifo J, Kaplan B, McCulloh DH, et al. Diminished effect of maternal age on implantation after preimplantation genetic diagnosis with array comparative genomic hybridization. *Fertil Steril* (2013) 100:1695–703. doi: 10.1016/j.fertnstert.2013.07.2002

3. Norwitz ER, Schust DJ, Fisher SJ. Implantation and the survival of early pregnancy. *N Engl J Med* (2001) 345:1400–8. doi: 10.1056/NEJMra000763

4. Ng KYB, Mingels R, Morgan H, Macklon N, Cheong Y. *In vivo* oxygen, temperature and pH dynamics in the female reproductive tract and their importance in human conception: a systematic review. *Hum Reprod Update* (2018) 24:15–34. doi: 10.1093/humupd/dmx028

5. Li MQ, Jin LP. Ovarian stimulation for *in vitro* fertilization alters the protein profile expression in endometrial secretion. *Int J Clin Exp Pathol* (2013) 6:1964–71.

6. Karizbodagh MP, Rashidi B, Sahebkar A, Masoudifar A, Mirzaei H. Implantation window and angiogenesis. *J Cell Biochem* (2017) 118:4141–51. doi: 10.1002/jcb.26088

7. Mardon HJ, Bagchi MK, Bagchi IC, Peng C, Karpovich N, Wang Y. Hormonal and paracrine regulation of embryonic implantation: A workshop report. *Placenta* (2007) 28(Suppl A):S82–4. doi: 10.1016/j.placenta.2007.01.018

8. Wang H, Dey SK. Roadmap to embryo implantation: clues from mouse models. *Nat Rev Genet* (2006) 7:185–99. doi: 10.1038/nrg1808

9. Zhang S, Lin H, Kong S, Wang S, Wang H, Wang H, et al. Physiological and molecular determinants of embryo implantation. *Mol Aspects Med* (2013) 34:939–80. doi: 10.1016/j.mam.2012.12.011

10. Young SL. Oestrogen and progesterone action on endometrium: a translational approach to understanding endometrial receptivity. *Reprod BioMed Online* (2013) 27:497–505. doi: 10.1016/j.rbmo.2013.06.010

11. Wang X, Wu SP, DeMayo FJ. Hormone dependent uterine epithelial-stromal communication for pregnancy support. *Placenta* (2017) 60(Suppl 1):S20–6. doi: 10.1016/j.placenta.2017.07.003

12. Paulson RJ. Hormonal induction of endometrial receptivity. *Fertil Steril* (2011) 96:530–5. doi: 10.1016/j.fertnstert.2011.07.1097

13. Hu XL, Feng C, Lin XH, Zhong ZX, Zhu YM, Lv PP, et al. High maternal serum estradiol environment in the first trimester is associated with the increased risk of small-for-gestational-age birth. *J Clin Endocrinol Metab* (2014) 99:2217–24. doi: 10.1210/jc.2013-3362

14. Jarvela IY, Pelkonen S, Uimari O, Makikallio K, Puukka K, Ruokonen A, et al. Controlled ovarian hyperstimulation leads to high progesterone and estradiol levels during early pregnancy. *Hum Reprod* (2014) 29:2393–401. doi: 10.1093/humrep/deu223

15. Adeviye Ersahin A, Acet M, Ersahin SS, Dokuzeylul Gungor N. Frozen embryo transfer prevents the detrimental effect of high estrogen on endometrium receptivity. *J Turk Ger Gynecol Assoc* (2017) 18:38–42. doi: 10.4274/jtgga.2016.0186

16. Absalan F, Ghannadi A, Kazerooni M. Reproductive outcome following thawed embryo transfer in management of ovarian hyperstimulation syndrome. *J Reprod Infertil* (2013) 14:133–7.

17. Joo BS, Park SH, An BM, Kim KS, Moon SE, Moon HS. Serum estradiol levels during controlled ovarian hyperstimulation influence the pregnancy outcome of *in vitro* fertilization in a concentration-dependent manner. *Fertil Steril* (2010) 93:442–6. doi: 10.1016/j.fertnstert.2009.02.066

18. Kyrou D, Popovic-Todorovic B, Fatemi HM, Bourgain C, Haentjens P, Van Landuyt L, et al. Does the estradiol level on the day of human chorionic gonadotrophin administration have an impact on pregnancy rates in patients treated with rec-FSH/GnRH antagonist? *Hum Reprod* (2009) 24:2902–9. doi: 10.1093/humrep/dep290

19. Pellicer A, Valbuena D, Cano F, Remohi J, Simon C. Lower implantation rates in high responders: Evidence for an altered endocrine milieu during the preimplantation period. *Fertil Steril* (1996) 65:1190–5. doi: 10.1016/S0015-0282(16)58337-X

20. Arslan M, Bocca S, Arslan EO, Duran HE, Stadtmauer L, Oehninger S. Cumulative exposure to high estradiol levels during the follicular phase of IVF cycles negatively affects implantation. *J Assist Reprod Genet* (2007) 24:111–7. doi: 10.1007/s10815-006-9101-x

21. Simon C, Cano F, Valbuena D, Remohi J, Pellicer A. Clinical evidence for a detrimental effect on uterine receptivity of high serum oestradiol concentrations in high and normal responder patients. *Hum Reprod* (1995) 10:2432–7. doi: 10.1093/oxfordjournals.humrep.a136313

22. Ullah K, Rahman TU, Pan HT, Guo MX, Dong XY, Liu J, et al. Serum estradiol levels in controlled ovarian stimulation directly affect the endometrium. *J Mol Endocrinol* (2017) 59:105–19. doi: 10.1530/JME-17-0036

23. Hapangama DK, Kamal AM, Bulmer JN. Estrogen receptor beta: the guardian of the endometrium. *Hum Reprod Update* (2015) 21:174–93. doi: 10.1093/humupd/dmu053

24. Dey SK, Lim H, Das SK, Reese J, Paria BC, Daikoku T, et al. Molecular cues to implantation. *Endocr Rev* (2004) 25:341–73. doi: 10.1210/er.2003-0020

25. Murphy CR, Rogers PA, Hosie MJ, Leeton J, Beaton L. Tight junctions of human uterine epithelial cells change during the menstrual cycle: a morphometric study. *Acta Anat (Basel)* (1992) 144:36–8. doi: 10.1159/000147282

26. Zihni C, Mills C, Matter K, Balda MS. Tight junctions: from simple barriers to multifunctional molecular gates. *Nat Rev Mol Cell Biol* (2016) 17:564–80. doi: 10.1038/nrm.2016.80

27. Otani T, Furuse M. Tight junction structure and function revisited. *Trends Cell Biol* (2020) 30:805–17. doi: 10.1016/j.tcb.2020.08.004

28. Aralla M, Borromeo V, Groppetti D, Secchi C, Cremonesi F, Arrighi S. A collaboration of aquaporins handles water transport in relation to the estrous cycle in the bitch uterus. *Theriogenology* (2009) 72:310–21. doi: 10.1016/j.theriogenology.2009.01.023

29. Skowronski MT, Skowronska A, Nielsen S. Fluctuation of aquaporin 1, 5, and 9 expression in the pig oviduct during the estrous cycle and early pregnancy. *J Histochem Cytochem* (2011) 59:419–27. doi: 10.1369/0022155411400874

30. Huang HF, He RH, Sun CC, Zhang Y, Meng QX, Ma YY. Function of aquaporins in female and male reproductive systems. *Hum Reprod Update* (2006) 12:785–95. doi: 10.1093/humupd/dml035

31. Zhu C, Jiang Z, Bazer FW, Johnson GA, Burghardt RC, Wu G. Aquaporins in the female reproductive system of mammals. *Front Biosci (Landmark Ed)* (2015) 20:838–71. doi: 10.2741/4341

32. Day RE, Kitchen P, Owen DS, Bland C, Marshall L, Conner AC, et al. Human aquaporins: regulators of transcellular water flow. *Biochim Biophys Acta* (2014) 1840:1492–506. doi: 10.1016/j.bbagen.2013.09.033

33. de Oliveira V, Schaefer J, Abu-Rafea B, Vilos GA, Vilos AG, Bhattacharya M, et al. Uterine aquaporin expression is dynamically regulated by estradiol and progesterone and ovarian stimulation disrupts embryo implantation without affecting luminal closure. *Mol Hum Reprod* (2020) 26:154–66. doi: 10.1093/molehr/gaaa007

34. B.A. Practice Committee of American Society for Reproductive Medicine. Gonadotropin preparations: Past, present, and future perspectives. *Fertil Steril* (2008) 90:S13–20. doi: 10.1016/j.fertnstert.2008.08.031

35. Fowler RE, Edwards RG. Induction of superovulation and pregnancy in mature mice by gonadotrophins. *J Endocrinol* (1957) 15:374–84. doi: 10.1677/joe.0.0150374

36. Ertzeid G, Storeng R. Adverse effects of gonadotrophin treatment on pre- and postimplantation development in mice. *J Reprod Fertil* (1992) 96:649–55. doi: 10.1530/jrf.0.0960649

37. Walton EA, Evans G, Armstrong DT. Ovulation response and fertilization failure in immature rats induced to superovulate. *J Reprod Fertil* (1983) 67:91–6. doi: 10.1530/jrf.0.0670091

38. Van der Auwera I, D'Hooghe T. Superovulation of female mice delays embryonic and fetal development. *Hum Reprod* (2001) 16:1237–43. doi: 10.1093/humrep/16.6.1237

39. Van der Auwera I, Pijnenborg R, Koninckx PR. The influence of *in-vitro* culture versus stimulated and untreated oviductal environment on mouse embryo development and implantation. *Hum Reprod* (1999) 14:2570–4. doi: 10.1093/humrep/14.10.2570

40. Karaer O, Vatansever HS, Oruc S, Ozbilgin K, Cilaker S, Koyuncu MF. The aromatase inhibitor anastrozole is associated with favorable embryo development and implantation markers in mice ovarian stimulation cycles. *Fertil Steril* (2005) 83:1797–806. doi: 10.1016/j.fertnstert.2005.01.100

41. Ma WW, Xiao J, Song YF, Ding JH, Tan XJ, Song KK, et al. Effect and underlying mechanism of bu-Shen-An-Tai recipe on ovarian apoptosis in mice with controlled ovarian hyperstimulation implantation dysfunction. *J Huazhong Univ Sci Technolog Med Sci* (2017) 37:401–6. doi: 10.1007/s11596-017-1747-3

42. Fayazi M, Beigi Boroujeni M, Salehnia M, Khansarinejad B. Ovarian stimulation by exogenous gonadotropin decreases the implantation rate and expression of mouse blastocysts integrins. *Iran BioMed J* (2014) 18:8–15. doi: 10.6091/ibj.1236.2013

43. Fossum GT, Davidson A, Paulson RJ. Ovarian hyperstimulation inhibits embryo implantation in the mouse. *J In Vitro Fert Embryo Transf* (1989) 6:7–10. doi: 10.1007/BF01134574

44. Paria BC, Reese J, Das SK, Dey SK. Deciphering the cross-talk of implantation: advances and challenges. *Science* (2002) 296:2185–8. doi: 10.1126/science.1071601

45. Pampfer S, Donnay I. Apoptosis at the time of embryo implantation in mouse and rat. *Cell Death Differ* (1999) 6:533–45. doi: 10.1038/sj.cdd.4400516

46. Chai J, Lee KF, Ng EH, Yeung WS, Ho PC. Ovarian stimulation modulates steroid receptor expression and spheroid attachment in peri-implantation endometria: studies on natural and stimulated cycles. *Fertil Steril* (2011) 96:764–8. doi: 10.1016/j.fertnstert.2011.06.015

47. Ruan YC, Chen H, Chan HC. Ion channels in the endometrium: regulation of endometrial receptivity and embryo implantation. *Hum Reprod Update* (2014) 20:517–29. doi: 10.1093/humupd/dmu006

48. Schumann S, Buck VU, Classen-Linke I, Wennemuth G, Grummer R. Claudin-3, claudin-7, and claudin-10 show different distribution patterns during decidualization and trophoblast invasion in mouse and human. *Histochem Cell Biol* (2015) 144:571–85. doi: 10.1007/s00418-015-1361-z

49. Liang X, Zhang XH, Han BC, Lei W, Qi QR, Wang TS, et al. Progesterone and heparin-binding epidermal growth factor-like growth factor regulate the expression of tight junction protein claudin-3 during early pregnancy. *Fertil Steril* (2013) 100:1410–8. doi: 10.1016/j.fertnstert.2013.07.001

50. Wang X, Matsumoto H, Zhao X, Das SK, Paria BC. Embryonic signals direct the formation of tight junctional permeability barrier in the decidualizing stroma during embryo implantation. *J Cell Sci* (2004) 117:53–62. doi: 10.1242/jcs.00826

51. Whitby S, Zhou W, Dimitriadis E. Alterations in epithelial cell polarity during endometrial receptivity: A systematic review. *Front Endocrinol (Lausanne)* (2020) 11:596324. doi: 10.3389/fendo.2020.596324

52. Satterfield MC, Dunlap KA, Hayashi K, Burghardt RC, Spencer TE, Bazer FW. Tight and adherens junctions in the ovine uterus: Differential regulation by pregnancy and progesterone. *Endocrinology* (2007) 148:3922–31. doi: 10.1210/en.2007-0321

53. Lindsay LA, Murphy CR. Ovarian hyperstimulation affects fluid transporters in the uterus: A potential mechanism in uterine receptivity. *Reprod Fertil Dev* (2014) 26:982–90. doi: 10.1071/RD12396

54. Sha XY, Xiong ZF, Liu HS, Zheng Z, Ma TH. Pregnant phenotype in aquaporin 8-deficient mice. *Acta Pharmacol Sin* (2011) 32:840–4. doi: 10.1038/aps.2011.45

55. Ruan YC, Guo JH, Liu X, Zhang R, Tsang LL, Dong JD, et al. Activation of the epithelial na+ channel triggers prostaglandin E(2) release and production required for embryo implantation. *Nat Med* (2012) 18:1112–7. doi: 10.1038/nm.2771

# Frontiers in Endocrinology

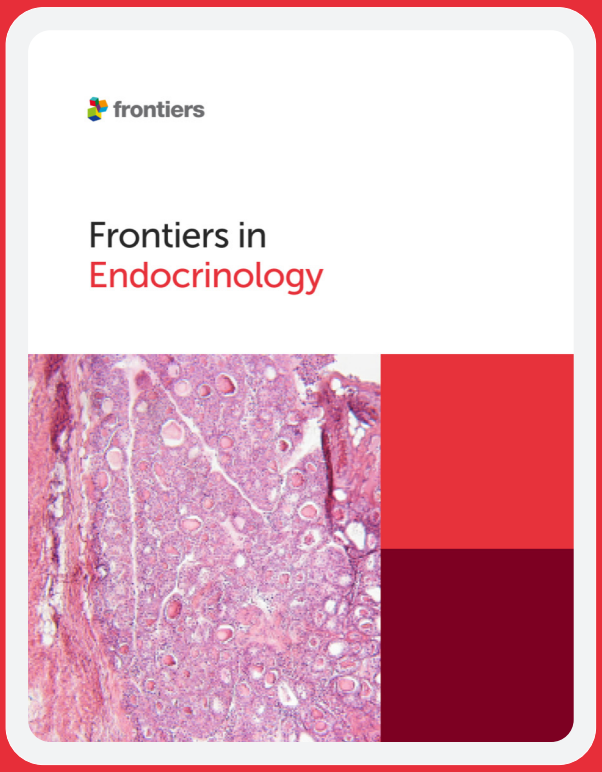
**Explores the endocrine system to find new therapies for key health issues**

The second most-cited endocrinology and metabolism journal, which advances our understanding of the endocrine system. It uncovers new therapies for prevalent health issues such as obesity, diabetes, reproduction, and aging.

---

## Discover the latest Research Topics

See more →




**Frontiers**

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

**Contact us**

+41 (0)21 510 17 00
frontiersin.org/about/contact