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Advances in the study of the developmental process and gene state of gametes and embryos

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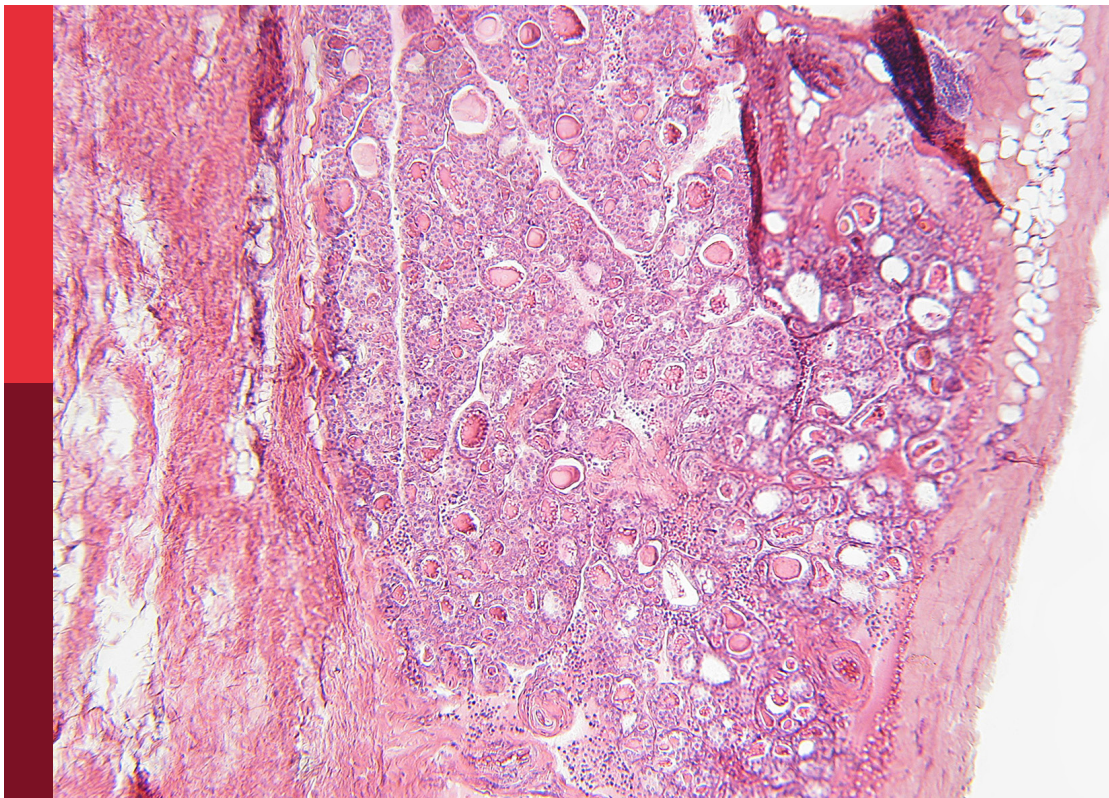

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Advances in the study of the developmental process and gene state of gametes and embryos

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Can time-lapse culture combined with artificial intelligence improve ongoing pregnancy rates in fresh transfer cycles of single cleavage stage embryos?

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Purpose: With the rapid advancement of time-lapse culture and artificial intelligence (AI) technologies for embryo screening, pregnancy rates in assisted reproductive technology (ART) have significantly improved. However, clinical pregnancy rates in fresh cycles remain dependent on the number and type of embryos transferred. The selection of embryos with the highest implantation potential is critical for embryologists and influences transfer strategies in fertility centers. The superiority of AI over traditional morphological scoring for ranking cleavage-stage embryos based on their implantation potential remains controversial.

Methods: This retrospective study analyzed 105 fresh embryo transfer cycles at the Centre for Reproductive Medicine from August 2023 to March 2024, following IVF/ICSI treatment at the cleavage stage. All embryos were cultured using time-lapse technology and scored using an automated AI model (iDAScore V2.0). Embryos were categorized into three groups based on the iDAScore V2.0: Group A (8 cells, iDA: 1.0-5.7); Group B (8 cells, iDA: 5.8-8.0); and Group C (>8 cells, iDA: 5.8-8.0). Clinical treatment outcomes, embryonic development, and pregnancy outcomes were analyzed and compared across the groups.

Results: Baseline characteristics such as patient age, AMH levels, AFC, and basal sex hormones showed no significant differences among the three groups ($p > 0.05$). The iDAscores were significantly higher in Group C (7.3 ± 0.5) compared to Group B (6.7 ± 0.5) and the iDAscores were significantly higher in Group B (6.7 ± 0.5) compared to Group A (4.8 ± 1.0) ($p < 0.001$).The mean number of high-quality embryos was highest in Group C (4.7 ± 3.0), followed by Group B (3.6 ± 1.7) and Group A (2.1 ± 1.2) ($p < 0.001$). There was no statistical difference ($p = 0.392$) in the ongoing pregnancy rate for single cleavage-stage transfers

between Group B (54.5%, 30/55) and Group A (38.1%, 8/21), although there was a tendency for Group B to be higher.

Conclusion: Combining time-lapse culture with AI scoring may enhance ongoing pregnancy rates in single cleavage-stage fresh transfer cycles.

KEYWORDS

time-lapse culture, artificial intelligence, single cleavage stage embryo transfer, iDAscores, fresh cycle

Introduction

Assisted Reproductive Technology (ART) has evolved significantly over the past 46 years and its effectiveness is widely recognized. To date, more than 10 million babies have been born through ART, effectively addressing the fertility issues of many infertile families (1). However, the live birth rate remains at 30-40%, indicating substantial room for improvement. Key factors for successful pregnancies include the *in vitro* culture of high-quality embryos, selection of embryos with the highest implantation potential, and synchronization with the optimal uterine implantation window. The rapid advancements in industrialization and artificial intelligence (AI) have introduced time-lapse culture, facilitating the *in vitro* culture and selection of embryos with superior implantation potential. While several studies have demonstrated that time-lapse culture can improve pregnancy rates, others have reported no significant impact on live birth rates (2–4).Specifically, using a time-lapse selection model to choose blastocysts for fresh single embryo transfer on Day 5 has not shown improvement in ongoing pregnancy rates compared to traditional morphology-based selection (5).

Despite the ongoing debate regarding the effectiveness of time-lapse culture, our study demonstrates that time-lapse culture combined with AI improves pregnancy rates in fresh cycles. Furthermore, single cleavage-stage transfers effectively reduce multiple birth rates and the risk of canceling fresh cycle blastocyst transfers. In developing countries, due to economic conditions and health insurance systems, physicians and patients may opt to transfer multiple embryos to achieve higher pregnancy rates. However, this practice increases the risk of multiple pregnancies, which in turn elevates the rates of miscarriages and perinatal complications for both mothers and infants, including preterm births and low-birth-weight babies (6, 7). Selective single embryo transfers are internationally recommended, primarily for blastocysts due to their high developmental potential. However, in fresh cycles, blastocyst transfer poses risks such as increased cancellation rates, reduced pregnancy rates due to the closure of the endometrial implantation window caused by certain ovulation regimens (e.g., antagonists), and an imbalance in the sex ratio (8). Single cleavage-stage transfers can effectively mitigate these issues. The current challenge is that traditional morphological assessment has limited predictive value for the developmental potential of cleavage-stage embryos, with implantation rates remaining around 20-40%. Ranking cleavage-stage embryos by their developmental potential is a significant challenge for embryologists. When only a single cleavage-stage embryo is transferred, the pressure on embryologists to make accurate selections increases, particularly when many high-quality embryos are available. Traditional morphological scoring can be subjective and inconsistent, leading to arbitrary selections (9). Current time-lapse culture technology, combined with AI-based selection systems, can rank embryos more effectively based on developmental dynamics, potentially outperforming traditional methods. This approach can shorten the time to first pregnancy, especially in fresh transfer cycles (10). This study is the first to analyze pregnancy outcomes based on different iDAScore groupings under the same morphological scoring of cleavage-stage embryos. This comparison allows for an evaluation of the validity of traditional morphological scoring versus AI-enhanced time-lapse culture scoring, providing data to support the broader application of time-lapse culture.

Materials and methods

Patients and study design

This retrospective analysis included patients who underwent IVF/ICSI-assisted conception at the Reproductive Center of the Affiliated Hospital of Guangdong Medical University between August 1, 2023, and March 30, 2024. All procedures involving human participants adhered to the ethical standards of the institutional and national research committee, as well as the 1964 Helsinki Declaration and its subsequent amendments. Written informed consent was obtained from all patients, and they were informed that they could withdraw from the study at any time. Inclusion criteria for patients were: (i) age ≤38 years; (ii) long agonist protocols or antagonist protocols; (iii) fresh transfer on day 3 of a single cleavage-stage embryo cycle; (iv) embryos cultured in a time-lapse system (EmbryoScope+). Exclusion criteria included:

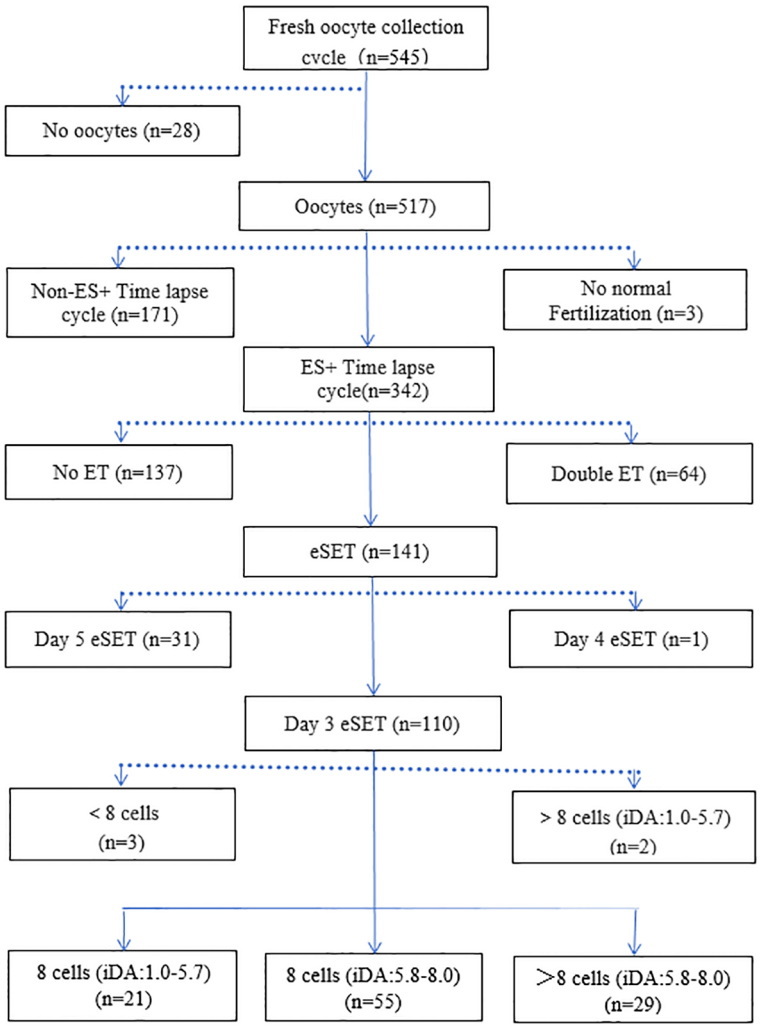

FIGURE 1
Flow chart of the study. All 545 fresh Oocytes retrieval cycles were left after inclusion exclusion criteria with 105 fresh cycles of single cleavage stage transplantation cycles.

(i) reproductive system abnormalities and chromosomal anomalies; (ii) a history of uterine surgery; (iii) missing data. The iDAScore values of the transferred embryos were evaluated using iDAScore Version 2.0. After applying the inclusion and exclusion criteria, 105 fresh cycles of single cleavage-stage embryo transfers were included from the initial 545 fresh oocyte retrieval cycles (Figure 1). The iDAScore is an automated AI model, and day 3 embryo morphological assessment was conducted based on the Istanbul consensus report, which serves as a conventional morphological grading method. Embryos were divided into three groups according to the iDAScore V2.0 score of 8 cells and >8 cells: Group A: 8 cells (iDA: 1.0-5.7); Group B: 8 cells (iDA: 5.8-8.0); Group C: >8 cells (iDA: 5.8-8.0).

Controlled ovarian stimulation

Patients underwent controlled ovarian stimulation using either a long gonadotropin-releasing hormone (GnRH) agonist (afolin, Huiling, Germany) protocol or a GnRH antagonist (Cetrotide, Merck Serono, Germany) protocol, based on their ovarian response and medical history of ART treatment. Human chorionic gonadotropin (HCG, Zhuhai Lizhu, China; Ovidrel, Merck Serono, Germany) was administered when the diameter of at least one follicle reached 19 mm, two follicles reached 18 mm, or three follicles reached 17 mm. Additionally, the blood E2 level had to reach 250-300 pg/mL for each dominant follicle (≥ 16 mm), or more than 60% of the follicles greater than 16 mm. The injection of HCG was 5000-10,000 IU on the same night.

Fertilization, embryo culture and transfer

IVF or intracytoplasmic sperm injection (ICSI) insemination was performed based on the patient's condition. IVF was performed 38-40 hours after HCG administration at a concentration of approximately 100,000 spermatozoa/700 μL microdrop. Oocytes were denuded of cumulus cells 4-6 hours after IVF insemination to evaluate the

extrusion of the second polar body. Oocytes showing a second polar body were transferred to the EmbryoScope+ for culture. Alternatively, oocyte denudation was performed 38-40 hours after HCG administration, and ICSI was conducted 2 hours later. After microinjection, all oocytes were individually cultured in the EmbryoScope+ time-lapse incubator under 6% CO_2 and 5% O_2 at 37°C. Embryo culture was carried out using continuous medium GTL culture medium (Vitrolife, Gothenburg, Sweden) until 66-68 hours after fertilization.Embryo grading refer to the Istanbul Consensus (11).

The iDAScore AI model, developed using deep learning and a neural network, was trained to analyze sequences of time-lapse images. The only inputs to the model were images from the time-lapse sequences, and the outputs were numerical scores from 1.0 to 8.0 (Day 3 Models) that correlated with the likelihood of an FHB. Therefore, iDAScore did not use any human-annotated data for training. The selection of embryos for transfer was based primarily on the iDAScore 2.0. Selected single embryos were incubated in G2 culture medium until the time of transfer.

A serum hCG level > 5 U/L was considered positive. Twenty-eight days after transplantation, a guided B ultrasound examination was performed to confirm the presence of an intrauterine pregnancy, and early cardiac motion indicated a clinical pregnancy.

Statistical analysis

Data analysis was performed using SPSS 26.0 statistical software. The adoption rate (%) of enumeration data was used to indicate the comparison of rates between groups, using the χ^2 test. Normally distributed data are expressed as mean ± SD, and the independent sample t-test was used for comparisons. The LSD t-test was used for pairwise multiple comparisons. A p-value < 0.05 was considered statistically significant.

Results

Participant characteristics

After applying the inclusion and exclusion criteria, a total of 105 fresh cycles of single cleavage-stage embryo transplantation were included in the analysis. Embryos with fewer than 8 cells rarely achieved the highest iDAScore, with only 3 fresh transfers in this category. Similarly, embryos with iDAScores of >8 cells in the 1.0-5.7 range had only two cases, which were excluded from the study due to the small sample size (Figure 1).

General data comparison focused on patients with different iDA scores of 8 cells and >8 cells. No statistically significant differences were found in baseline characteristics such as age, AMH, AFC, and basal sex hormones among the two groups of patients (p<0.05) (Table 1).

Stimulation cycle characteristics

The stimulation cycle was predominantly dominated by long agonist protocols (64.8%), while the antagonist protocol accounted for 35.2%. The difference in the percentage of protocols for ovulation promotion was not statistically significant in group A compared to group B, and group B compared to group C (Table 2).

Embryo laboratory data

The iDAScores were significantly higher in Group C (7.3 ± 0.5) compared to Group B (6.7 ± 0.5), and significantly higher in Group B compared to Group A (4.8 ± 1.0) (p < 0.001). However, there were no statistically significant differences between the groups in terms of fertilization program, number of oocytes, fertilization rate, oocyte cleavage rate, 4-cell rate, and 8-cell rate on DAY 2 and DAY 3.

The mean number of high-quality embryos was higher in Group B (3.6 ± 1.7) compared to Group A (2.1 ± 1.2) (p < 0.001). The number of blastocysts formed was higher in Group B (4.1 ± 2.2) than in Group A (2.5 ± 2.4) (p = 0.009). Additionally, the rate of blastocyst formation was higher in Group B (56.4 ± 28.4%) compared to Group A (37.9 ± 25.0%) (p = 0.008) (Table 3).

Clinical outcomes

There was no statistically significant difference (p = 0.392) in the ongoing pregnancy rate for single cleavage-stage transfers between Group B (54.5%, 30/55) and Group A (38.1%, 8/21), although there was a tendency for the rate to be higher in Group B (Table 4).

IDAscores predict the developmental potential of 8-cell cleavage stage embryos

Figure 2 illustrates embryos assessed by embryologists at the 8-cell level with varying developmental potentials. IDAscores proved to be reliable predictors of the probability of embryo development into blastocysts. For instance, embryos with iDAscores above 5.7, such as Well 10 (iDA=6.8), Well 5 (iDA=5.7), and Well 13 (iDA=5.7), developed into good-quality blastocysts. In contrast, embryos with iDAscores below 5.7, such as Well 2, Well 6, and Well 9, did not form usable blastocysts by Day 5. Additionally, Supplementary Video 1 showcases the embryo developmental kinetics from 6.8 hours to 117.2 hours post-fertilization, highlighting variations in blastocyst formation despite similar Day 3 embryo quality assessments.

Discussion

ART has evolved significantly over the years, leading to the emergence of various new technologies and techniques, such as advanced culture fluids (12, 13), heavy oils for assisted reproduction cultures (14), non-invasive preimplantation genetic testing (niPGT) (15), Raman spectroscopy applications (16, 17), time-lapse incubators (3, 18), and artificial intelligence applications (19, 20). Clinicians, embryologists, and scientists are continually striving to improve pregnancy rates, which are now approaching a plateau.

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TABLE 1 Patients' characteristics.

Characteristics	Group(A)	Group(B)	Group(C)	p-value
iDAScore v2.0 grouping	8 cells (iDA:1.0-5.7)	8 cells (iDA:5.8-8.0)	>8 cells (iDA:5.8-8.0)	
No. of cycles	21	55	29	/
Maternal age, (years)	31.4 ± 3.1	31.16 ± 3.5	32.8 ± 3.5	p=0.096
Body mass index (kg/m^2)	22.1 ± 3.9	22.3 ± 2.7	23.0 ± 2.3	p=0.459
Infertility duration (years)	3.4 ± 2.7	3.0 ± 2.1	4.1 ± 3.0	p=0.174
Anti-mullerian hormone (AMH),(ng/mL)	3.1 ± 1.6	3.7 ± 2.5	4.4 ± 3.1	p=0.193
AFC, (num)	9.2 ± 4.6	11.1 ± 5.3	12.9 ± 7.5	p=0.099
Basic hormone				
Basal FSH(mIU/ml)	7.8 ± 2.8	6.8 ± 1.7	6.1 ± 1.6	p=0.056
Basal LH(mIU/ml)	6.8 ± 2.1	6.5 ± 3.8	7.3 ± 4.2	p=0.616
Basal PRL(ng/ml)	27.6 ± 20.3	30.5 ± 41.9	25.2 ± 23.6	p=0.811
Basal E_2(pg/ml)	53.9 ± 34.7	45.6 ± 22.7	53.8 ± 54.8	p=0.515
Basal T(ng/ml)	0.3 ± 0.2	0.3 ± 0.1	0.3 ± 0.2	p=0.394
Basal P(ng/ml)	0.4 ± 0.2	0.4 ± 0.3	0.6 ± 1.0	p=0.356
Type of infertility				
Primary infertility(%),n/N	47.6(10/21)	41.8(23/55)	37.9(11/29)	p=0.791
Secondary infertility(%),n/N	52.4(11/21)	58.2(32/55)	62.19(18/29)	
Cause of infertility				
Tubal factor(%)	47.6(10/21)	38.2(21/55)	41.4(12/29)	p=0.535
Male factor(%)	33.3(7/21)	25.5(14/55)	10.3(3/29)	
Ovulation disorders(%)	9.5(2/21)	18.2(10/55)	17.2(5/29)	
Combination(%)	9.5(2/21)	7.3(4/55)	20.7(6/29)	
Endometriosis(%)	0(0/21)	5.5(3/55)	3.4(1/29)	
Unknown(%)	0(0/21)	3.6(2/55)	6.9(2/29)	
Ovarian hypoplasia(%)	0(0/21)	1.8(1/55)	00/29)	

Data are means ± SD or n.

TABLE 2 Stimulation cycle characteristics.

Characteristics	Group(A)	Group(B)	Group(C)	p-value
iDAScore v2.0 grouping	8 cells (iDA:1-5.7)	8 cells (iDA:5.8-8)	>8 cells (iDA:5.8-8)	
No. of cycles	21	55	29	
Ovulation programme				
Long agonist protocols(%)	76.2 (16/21)	63.6 (35/55)	58.6 (17/29)	p=0.425
Antagonist protocol(%)	23.8 (5/21)	36.4 (20/55)	41.4 (12/29)	
Initial Gn dose (IU)	160.7 ± 61.0	158.4 ± 53.8	146.6 ± 41.7	p=0.544
Length of Gn stimulation (days)	11.1 ± 1.6	10.7 ± 1.8	11.1 ± 2.1	p=0.615
Total dose of Gn (IU)	2172.0 ± 615.7	2024.6 ± 603.4	2052.1 ± 2701.2	p=0.661

(Continued)

TABLE 2 Continued

Characteristics	Group(A)	Group(B)	Group(C)	p-value
iDAScore v2.0 grouping	8 cells (iDA:1-5.7)	8 cells (iDA:5.8-8)	>8 cells (iDA:5.8-8)	
Ovulation programme				
E_2 levels at the trigger day (pg/ml)	2633.2 ± 1359.5	2796.8 ± 1330.7	2465.37 ± 1088.9	p=0.538
LH levels at the trigger day (mIU/ml)	2.3 ± 0.9	2.7 ± 1.3	2.5 ± 1.5	p=0.422
P levels at the trigger day (mIU/ml)	0.6 ± 0.3	0.7 ± 0.3	0.7 ± 0.3	p=0.142
Endometrial thickness at the trigger day (mm)	12.5 ± 2.5	11.7 ± 2.2	11.3 ± 2.2	p=0.180

Data are means ± SD or n.

The ultimate goal for every reproductive medicine practitioner is to select embryos non-invasively, economically, objectively, and conveniently, and implant them into the patient's uterus to achieve pregnancy and successfully deliver a healthy baby (21). Literature has shown that both fresh cycle transfers and freeze-thaw cycle transfers result in good pregnancy rates when ovarian hyperstimulation syndrome (OHSS) is well controlled, but fresh cycle transfers effectively shorten the patient's waiting time for implantation (22). Time-lapse culture combined with artificial intelligence to assess embryo kinetic parameters may serve as a non-invasive, effective, and concise method to enhance clinical pregnancy rates in fresh cycles. Ma et al. reported a correlation

TABLE 3 Comparison of various fertilization programmes and embryo development data.

Characteristics	Group(A)	Group(B)	Group(C)	*p*-value
iDAScore v2.0 grouping	8 cells (iDA:1.0-5.7)	8 cells (iDA:5.8-8.0)	>8 cells (iDA:5.8-8.0)	
Cycle, n	21	55	29	
iDAScore v2.0	4.8 ± 1.0[a]	6.7 ± 0.5[b]	7.3 ± 0.5	*p*<0.001
Fertilization program				
IVF(%)	81.0(17/21)	69.1(38/55)	89.7(26/29)	*p*=0.104
ICSI(%)	19.0(4/21)	30.9(17/55)	10.3(3/29)	
Number of oocytes (n)	11.1 ± 5.2	12.31 ± 4.1	13.7 ± 6.0	*p*=0.183
MII (n)	9.4 ± 4.0	10.1 ± 3.5	12.0 ± 5.4	*p*=0.068
Number of normal fertilization (n)	6.8 ± 3.6	7.7 ± 2.5	8.7 ± 4.9	*p*=0.174
Normal Fertilization Rate (%)	62.0 ± 19.4	64.4 ± 18.3	65.1 ± 22.0	*p*=0.850
Number of cleavage (n)	8.0 ± 3.7	8.8 ± 2.8	9.6 ± 4.8	*p*=0.282
Oocyte cleavage rate (%)	97.8 ± 5.5	97.5 ± 8.7	98.2 ± 3.8	*p*=0.897
4-cell counts for Day2(n)	3.1 ± 1.3	4.3 ± 2.0	4.7 ± 3.5	*p*=0.065
4-cell rate for Day2(%)	52.9 ± 23.1	57.13 ± 20.6	53.64 ± 25.0	*p*=0.682
8-cell counts for Day3(n)	2.8 ± 1.5	3.5 ± 1.6	3.2 ± 2.6	*p*=0.331
8-cell rate for Day3(%)	49.3 ± 29.0	48.3 ± 19.0	37.8 ± 24.0	*p*=0.087
Number of good quality embryos on Day 3(n)	2.1 ± 1.2[a]	3.6 ± 1.7[b]	4.7 ± 3.0	*p*<0.001
Rate of good quality embryos on Day 3(%)	39.2 ± 25.8	50.0 ± 21.0	58.0 ± 27.0	*p*=0.025
Number of blastocysts formed(n)	2.5 ± 2.4[a]	4.1 ± 2.2	5.1 ± 4.0	*p*=0.009
Rate of blastocyst formation (%)	37.9 ± 25.0[a]	56.4 ± 28.4	63.2 ± 30.5	*p*=0.008

Data are means ± SD or n.
[a]compared with group B (p<0.05); [b]compared with group C (p<0.05).

TABLE 4 Comparison of clinical pregnancy, early abortion and ongoing pregnancy between various.

Characteristics	Group(A)	Group(B)	Group(C)	*p*-value
iDAScore v2.0 grouping	8 cells (iDA:1.0-5.7)	8 cells (iDA:5.8-8.0)	>8 cells (iDA:5.8-8.0)	
No. of cycles	21	55	29	
Clinical pregnancy (%)	47.6(10/21)	60.0(33/55)	69.0(20/29)	*p*=0.315
Early abortion (%)	20.0(2/10)	10.0(3/33)	20.0(4/20)	*p*=0.466
Ongoing pregnancy(%)	38.1(8/21)	54.5(30/55)	55.2(16/29)	*p*=0.392

between 3,448 blastocyst biopsies and iDAscores, finding that time-lapse culture combined with iDAscores predicted embryonic viability, with a higher probability of viable blastocysts associated with higher iDAscores (23).

The choice between Day 3 cleavage stage embryo transfer and Day 5 blastocyst stage transfer in fresh cycles, and whether to transfer one or two embryos, are critical questions for clinicians and embryologists. Fresh transfer of blastocysts increases the implantation rate due to the selection of viable embryos through extended *in vitro* culture but also raises the rate of preterm birth (24). Some ovulation protocols, such as antagonist protocols, may miss the implantation window due to early closure of endometrial receptivity (25, 26), reducing the pregnancy rate of blastocyst transfer. The potential long-term effects of blastocyst culture, such as shortened telomeres and senescent phenotypes in offspring, should also be consideredt (27). Fresh blastocyst transfers also risk cancellation due to failure to develop or lack of transferable blastocysts on Day 5. Additionally, sex bias due to blastocyst transfer is a significant factor, with stricter regulations in some countries to avoid severe sex ratio imbalances (8).

The current 2024 updated ESHRE guideline on the number of embryos to transfer during IVF/ICSI recommends that selective single embryo transfers be performed up to the age of 38 years (moderate quality evidence), and also for women aged 38 years and above (very low quality evidence) (28). Transferring two or more cleavage stage embryos in fresh cycles has been the dominant strategy in developing countries due to the limited predictive value of traditional morphological assessments of implantation potential. This approach aims to guarantee pregnancy rates in a single transfer, despite the increased risk of multiple pregnancies

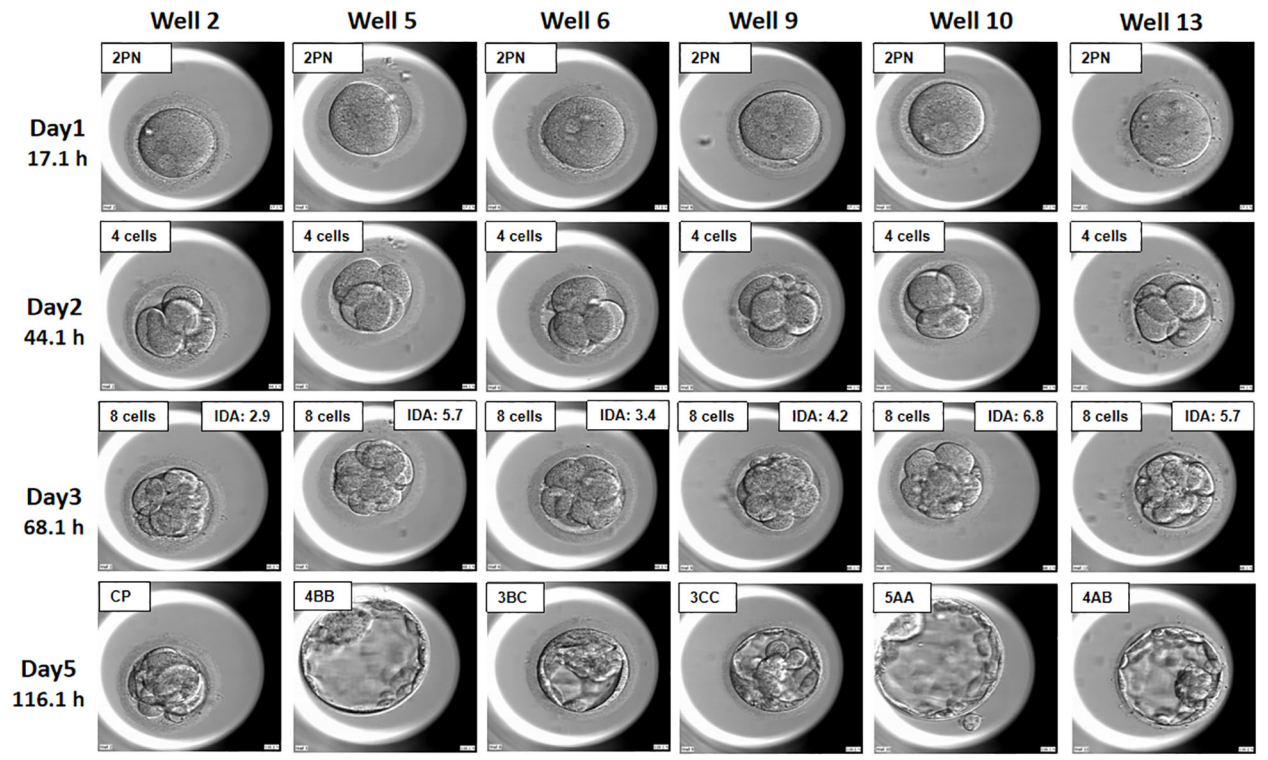

FIGURE 2
IDAscores predict the developmental potential of 8-cell cleavage stage embryos. Well 2, Well 5, Well 6, Well 9, Well 10and Well 13 were all rated as (8 cells) high-quality embryos by conventional morphology scores at 68.1 hours after fertilization (DAY3), but at 116.1 hours after fertilization (DAY5), only embryos from Well 5, Well 13 with an IDA score of 5.7, and Well 10, with an IDAscore of 6.8, developed into high-quality blastocysts in Day 5.

and associated maternal and perinatal complications, such as gestational diabetes, pre-eclampsia, preterm labor, and low birth weight (29, 30).

Currently, traditional morphological evaluation can effectively distinguish between good quality and poor-quality embryos. However, for patients with a high number of good quality embryos, there is confusion in embryo sorting, making it difficult to select the best embryos. Different embryologists may select different embryos, which introduces subjectivity and arbitrariness, thus there is no guarantee that the transferred embryos are the most promising for single cleavage stage embryo transfer (20). Time-lapse culture combined with artificial intelligence screening can score and rank cleavage stage embryos based on their kinetic parameters, accurately predicting their developmental potential. Transferring Day 3 highly scored single cleavage stage embryos facilitates embryo and endometrium interactions, effectively reduces *in vitro* culture time, and ensures the pregnancy rate while minimizing the risks associated with blastocyst transfer. In this study, we found that although all embryos were morphologically assessed as good quality 8-cell embryos, their developmental potential and ongoing pregnancy rates varied according to their iDAscores (Figure 2). Embryos with higher iDAscores had higher ongoing pregnancy rates, though the difference was not statistically significant, likely due to the small sample size. Another key finding was that Day 3 embryos with both high cell counts and high iDAscores exhibited higher ongoing pregnancy rates. While the previous Istanbul consensus indicated that neither too slow nor too fast embryo development is optimal (11), our study found that Day 3 embryos with higher cell counts and iDAscores had the highest implantation rates. More data is needed to validate this phenomenon.

Time-lapse culture combined with iDAscores scoring also predicts the developmental potential of each embryo (31), allowing embryologists to accurately decide on strategies for transferring, freezing, and continuing to culture embryos based on the patient's overall embryo score. If the patient has embryos with high iDAscores, a single cleavage stage embryo transfer is chosen. If the patient has low iDAscores, the number of embryos to be transferred can be determined by considering the patient's individual situation, height, and uterine condition.

This personalized approach can simultaneously ensure the pregnancy rate and effectively reduce the number of embryos transferred, thereby reducing the rate of multiple births. This is important for stabilizing the pregnancy rate of a reproductive center and for maintaining the center's reputation. Currently, due to the high cost of time-lapse incubators and special petri dishes, full time-lapse incubation may not be feasible in many fertility centers in developing countries.The limited number of time-lapse incubators can be prioritized for patients considering fresh cycle transfers to ensure pregnancy rates. For other patients unable to undergo a fresh transfer, the pregnancy rate can be maintained by transferring blastocysts in a freeze-thaw cycle, as the blastocyst culture effectively eliminates embryos with low developmental potential, thus improving the implantation rate.

The potential benefits of time-lapse culture with artificial intelligence have been added.The time-lapse culture generate a large amount of embryo image data that records a large number of kinetic parameters of embryo development. If these large amounts of data need to be labeled by embryologists, then this seriously affects the efficiency of embryologists in evaluating embryos, and also prevents the evaluation of effective parameter weights. And the time-lapse culture combined with artificial intelligence can effectively solve this problem. It can reduce the workload of embryologists and assist embryologists in selecting embryos with the highest developmental potential.

In summary, the strategy of time-lapse culture combined with AI for fresh cycle single cleavage stage embryo transfer is a non-invasive, rapid method that helps resolve transfer sequencing confusion for embryologists evaluating patients with multiple embryos. Combining time-lapse culture with AI scoring may enhance ongoing pregnancy rates in single cleavage-stage fresh transfer cycles.

Data availability statement

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

Ethics statement

The studies involving humans were approved by Ethics Committee of Affiliated and Clinical Research of Guangdong Medical University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

XW: Data curation, Resources, Writing – original draft. QW: Data curation, Formal Analysis, Investigation, Writing – original draft. WH: Conceptualization, Data curation, Formal Analysis, Writing – original draft. LY: Data curation, Formal Analysis, Methodology, Software, Writing – original draft. TM: Conceptualization, Formal Analysis, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

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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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1449035/full#supplementary-material

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Feasibility of preimplantation genetic testing for aneuploidy on frozen-thawed embryos following conventional IVF insemination

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Objective: Intracytoplasmic sperm injection (ICSI) is commonly employed in preimplantation genetic testing (PGT) to minimize the risk of foreign sperm DNA contamination. Cryopreserved embryos from patients with recurrent miscarriage or repeated implantation failure, who have undergone conventional *in vitro* fertilization (IVF), can be thawed and biopsied for PGT. Therefore, we aimed to assess the accuracy and effectiveness of preimplantation genetic testing for aneuploidy (PGT-A) on frozen embryos using conventional IVF (c-IVF) insemination methods.

Methods: From January 2021 to November 2023, our center conducted 107 thawed cryopreserved embryo biopsy cycles to screen for PGT-A. Among them, 58 cycles used c-IVF insemination, and 49 used ICSI insemination. Basic patient clinical information, laboratory data, PGT test results, and clinical outcome data were collected. To minimize the confounding effects of patient characteristics and embryo quality on PGT-A outcomes, clinical outcomes, and contamination assessment, these variables were included in the analysis. We then evaluated the blastocyst euploidy rate, clinical outcomes, and accuracy of PGT-A results between the two groups and analyzed potential contamination in the c-IVF insemination group.

Results: A total of 320 blastocysts underwent PGT-A testing, with 179 blastocysts from c-IVF insemination and 141 from ICSI insemination. Considering participants' baseline characteristics and embryological outcomes, no significant differences were found between the two groups regarding infertility type, average age, body mass index, percentage of PGT-A indications, or quality of embryonic development. Regarding PGT-A results, all 320 biopsy samples were successfully analyzed, showing no statistical variance in chromosomal euploidy, abnormality, or mosaicism rates between the two insemination methods. No parental contamination was detected in the c-IVF insemination group. When assessing clinical outcomes, parameters such as biochemical pregnancy, clinical pregnancy, and miscarriage rates did not exhibit significant

discrepancies between the two groups, and no misdiagnoses were reported during the study period.

Conclusion: Embryo transfer and PGT-A results are not affected by potential parental contamination in frozen-thawed embryos conceived via c-IVF. PGT-A guided embryo transfer in thawed embryos conceived by c-IVF is a viable and clinically effective approach.

KEYWORDS

PGT-A, conventional IVF insemination, ICSI, frozen-thawed embryos, biopsy, parental contamination

1 Introduction

Preimplantation genetic testing (PGT) has rapidly advanced in terms of technology and application. Initially utilized to prevent the vertical transmission of monogenic diseases, it is now predominantly employed for selecting chromosomally normal embryos with higher implantation potential, thereby reducing the risk of transferring genetically abnormal embryos (1, 2).

ICSI has been widely used in PGT. Including the Preimplantation Genetic Diagnosis International Society (PGDIS), the American Society for Reproductive Medicine (ASRM), and the European Society of Human Reproduction and Embryology (ESHRE) PGT Consortium, specialized societies recommend the use of ICSI for PGT (3–5). It was well known that spermatozoa and cumulus cells could adhere to the zona pellucida (ZP), which was major contamination factors during PGT procedures. Wilton et al. summarized the causes of misdiagnosis in PGD, in which ICSI must be performed for all PCR diagnosis to avoid paternal contamination and cumulus cells must be removed prior to biopsy to avoid maternal contamination (2). ICSI was recommended for all PCR cases to reduce the chance of paternal contamination from extraneous sperm attached to the zona pellucida or non-decondensed sperm within blastomeres (6, 7). Lynch, C et al. investigated the necessity of using ICSI in preimplantation genetic testing (PGT), particularly concerning the risk of paternal cell contamination, concluding that using c-IVF as a fertilization method in PGT-A presents a low risk of paternal cell contamination (8). However, the extensive use of ICSI in ART for non-male factor infertility has been controversial. The current available studies that compare ICSI and c-IVF in couples with males presenting with normal total sperm count and motility, show neither method was superior to the other, in achieving live birth, adverse events (multiple pregnancy, ectopic pregnancy, pre-eclampsia and prematurity), also alongside secondary outcomes, clinical pregnancy, viable intrauterine pregnancy or miscarriage (9). Paffoni et al. found that ICSI was associated with lower live birth rates compared to c-IVF when ART was indicated solely due to female factors. Additionally, the use of ICSI in unexplained infertility cycles did not demonstrate a significant improvement (10). Wang et al. found that ICSI did not improve the live birth rate after the first transplant compared with c-IVF in patients with non-severe male factor infertility, although the mean ovum fertilization rate was significantly higher in the ICSI group than in the c-IVF group (75.0% vs. 66.7%). In addition, the study found no significant differences between the two groups in secondary outcome measures, such as fertilization failure, miscarriage, preterm birth, and low birth weight (11). The ESHRE Add-ons working group recommends that the application of ICSI in clinical practice should be carefully tailored to specific circumstances and patient needs, rather than being used as an additional option in terms of cost and indication (12). The analysis data indicates that the average cost of an ICSI cycle is £226 higher than that of an c-IVF cycle (13). In practical IVF procedures, compared with c-IVF, ICSI involves a more complex operational process, placing higher demands on personnel and equipment.

Currently, PGT utilizes amplification techniques, specifically whole-genome amplification (WGA) of trophectoderm (TE) biopsy cells. Recent research findings indicate that during the WGA of TE biopsy cells in fresh PGT cycles using c-IVF, sperm DNA cannot be amplified. The levels of paternal and maternal contamination are extremely low and nearly negligible, suggesting that the likelihood of misdiagnosis is minimal (14–16). Huang et al. and Dong et al. demonstrated the feasibility of PGT-A under c-IVF by establishing a method for analyzing parental contamination (17, 18). Zhang et al. retrospectively analyzed pregnancy outcomes in frozen PGT cycles of patients who primarily experienced abortion with chromosomal aberration or recurrent implantation failure after c-IVF or ICSI treatment and observed that the rates of clinical pregnancy, early miscarriage, and ongoing pregnancy were similar between the two groups of insemination methods (19). In cases of repeated implantation failures or miscarriages attributed to chromosomal abnormalities in c-IVF cycles, performing PGT-A on frozen embryos is considered necessary. It is noteworthy that frozen embryos not initially intended for PGT may contain sperm and cumulus cells attached to the zona pellucida. Presently, there is a paucity of studies addressing the amplification of sperm or cumulus cells post-freezing in liquid nitrogen and its potential implications for PGT-A outcomes. However, more research evidences are needed on

the feasibility of PGT of frozen embryos fertilized with c-IVF to prove safety and effectiveness. The Individual of pregnant woman and quality of transplanted embryo was not controlled in previous studies. This study aims to evaluate the impact of PGT-A testing using c-IVF frozen embryos, examining the influence of c-IVF sperm or cumulus cells on PGT-A results and exploring the potential utility of this approach in PGT-A cycles.

2 Materials and methods

2.1 Ethics approval

This study was approved by the Ethics Committee of Reproductive Medicine of Zhongshan Boai Hospital. A total of 107 couples signed consent forms that were approved by the local ethics committee. Of these, 58 couples underwent c-IVF insemination and 49 underwent ICSI insemination. The frozen embryos were subsequently thawed and biopsied for PGT-A testing.

2.2 Study design and patients

In this study, we reviewed 107 cycles of PGT-A testing of frozen embryo biopsies performed between January 2021 and November 2023. The study included patients with common indications for PGT-A, such as advanced age (≥38 years old), repeated implantation failure (three or more times), and recurrent miscarriage (two or more times). The study was divided into two main groups: one involved frozen embryos inseminated using c-IVF, with peripheral blood samples from both partners extracted for linked single-nucleotide polymorphism (SNP) analysis to detect embryo contamination; the other involved frozen embryos inseminated using ICSI, a recommended PGT-A insemination method owing to minimal contamination from parental sources, where embryos were only thawed and biopsied for testing. The objectives were to assess euploidy, chromosomal abnormality, mosaicism, biomedical pregnancy, clinical pregnancy, and early miscarriage rates between c-IVF and ICSI frozen blastocysts, and the contamination and detection accuracy of paternal and maternal sources in frozen embryos from c-IVF.

2.3 Thawing, biopsy and freezing of embryos

The embryo was vitrified and thawed using RapidWarm™ Cleave reagent (Vitrolife Sweden AB). Thawing solution 1, 2, 3, 4was placed in a 37°C incubator for 30 min for equilibration, whereas thawing solutions 2, 3, and 4 were equilibrated at room temperature for the same duration. After resuscitating the embryos, the front section of the cryotop carrier rod was quickly immersed in pre-warmed thawing solution 1 for 30 seconds, thawing solution 2 for 1 min, thawing solution 3 for 2 min, and thawing solution 4 for 5 min, and finally placed in pre-warmed G2 solution. Following blastocyst recovery, the embryos were cultured for 2–4 h before biopsy. Preheated G-PGD or G-MOPS Plus (Vitrolife, Goteborg, Sweden) was used as biopsy fluid. Under an inverted microscope, the inner cell mass of the blastocyst was identified and fixed at 9–12 o'clock, and the hatched TE cells were aspirated into the biopsy pipette. Three laser pulses of 2.0 ms (ZYLOS-tk®, Hamilton Thorne, MA, USA) were used to loosen cell junctions before a mechanical 'flick' method was applied to remove 4–10 TE cells. The cells were then washed and placed in a 0.2 mL polymerase chain reaction tube pre-added with 1–2.5 μL phosphate buffered saline for PGT detection. In cases where the blastocyst had not hatched, the laser perforated the zona pellucida for 2.0 ms to induce collapse before the biopsy. After biopsy, the blastocysts were vitrified and frozen within 1 h at room temperature using Vitrification Refrigerant Set (Kitazato Corporation). The vitrification cryogenic solution and the equilibrium solution were equilibrated for 30 min at room temperature. The embryos were aspirated and immersed in an equilibrium solution for 10 min and then the embryos were moved between different drops of vitrification cryogenic solution and rinsed 2-3 times within 60s. After that, the embryos were transferred to a freezing carrier rod and promptly placed in liquid nitrogen for storage.

2.4 Whole-genome amplification

WGA was performed as previously described (20) using a ChromSwift kit (Yikon, China) with multiple annealing and looping-based amplification cycles (MALBAC) in accordance with the manufacturer's protocol.

2.5 Determination of blastocyst ploidy status using next generation sequencing

To analyze the ploidy status of the blastocysts, the DNA amplified across WGA-MALBAC was sequenced using a MiSeq sequencer with a single-ended read length of 55 bp. Approximately two million raw reads were generated for each TE biopsy sample. Raw data were analyzed as described previously (20). The original reads were aligned and compared with the UCSC hg19 human reference genome to filter out low-quality and duplicate sequences for copy number variation (CNV) analysis. The R language program was used to visualize the CNVs of the 24 chromosomes. Embryos were diagnosed as euploid when the extent of mosaicism was < 30%. When the CNV was > 4 Mb and the extent of mosaicism was > 70%, the embryo was diagnosed as aneuploid. When the CNV size was > 4 Mb and the extent of mosaicism was between 30% and 70%, the embryo was diagnosed with chromosomal mosaicism.

2.6 Determination of parental contamination for c-IVF inseminated embryos

Parental contamination was identified using the quantitative parental contamination test (qPCT), as described by Dong et al. (18). Briefly, the gene SNP method utilizes the Infinium Asian Screening Array bead chip technology (Illumina, Inc., San Diego, CA, USA) to analyze the genotypes of both blastocysts and parents.

Following the extraction of peripheral blood DNA from the parents, the WGA DNA of each blastocyst was linearly amplified, fragmented, precipitated, and hybridized according to the manufacturer's instructions. Subsequently, an iScan system (Illumina, Inc., San Diego, CA, USA) was used to scan the chip signal, enabling analysis of the B allele frequency (BAF) for each sample. The expected BAFs for genotypes AA, AB, and BB were approximately 0, 0.5, and 1, respectively. Hence, if the father's SNP was BB or AA and the mother's SNP was AA or BB, the embryo genotype was anticipated to be AB with a BAF of 0.5. Deviations in BAF could indicate parental contamination or biases in alleles that often stem from allelic amplification bias induced by WGA technology. During the data analysis, biased data should initially be disregarded. Ultimately, a BAF exceeding 0.6 or falling below 0.4 suggests parental bias and potential contamination.

2.7 Embryo transfer and follow-up

Endometrial preparation was performed using hormone replacement therapy. Blastocysts with normal chromosome copy numbers were thawed and transferred into the uterus five days after progesterone-initiated ovulation. Clinical pregnancy was confirmed if an intrauterine gestational sac was detected by ultrasound along with a fetal heartbeat 30–40 days after frozen embryo transfer (FET). To validate the accuracy of PGT-A results, noninvasive prenatal testing (NIPT) can be performed at 12–18 weeks of gestation or amniocentesis at 16–24 weeks of gestation.

2.8 Statistical analyses

Continuous variables are presented as the mean ± standard deviation. Categorical variables are presented as the number of observations and percentages. Differences in variables between the different insemination groups were analyzed using a 2-tailed Student's *t*-test (female age, body mass index [BMI], infertility type, and blastocyst development on day 5/6/7). The chi-square test was used to analyze the biomedical pregnancy, clinical pregnancy, miscarriage, euploid, abnormal, and mosaic rates and other data (percentage of infertility type, indication for PGT-A, days of blastocyst biopsy, and blastocyst development on day 5/6/7). Statistical significance was set at $P < 0.05$.

3 Results

3.1 Baseline characteristics and embryological outcomes of the study participants

PGT cycles with at least one successfully resuscitated embryo that could be used for embryo biopsy between January 2021 and November 2022 at our center were reviewed for eligibility. Finally, 107 PGT cycles with embryo biopsies performed after thawing were included in this study. Of these, 58 belonged to the c-IVF insemination group and 49 to the ICSI insemination group. The clinical data of both groups and the embryological data are summarized in Table 1.

3.2 The results of PGT

A total of 320 blastocysts underwent chromosomal analysis with a 100% success rate for PGT. The molecular karyotype findings were categorized into chromosomal euploidy, chromosomal abnormalities, and chromosomal mosaicism (Table 2). There were no statistically significant differences in the rates of chromosomal euploidy (48.60% vs. 50.35%), abnormalities (35.20% vs. 36.88%), or mosaicism (16.20% vs. 12.77%) between the two groups ($P = 0.69$). Figure 1 illustrates the distribution of chromosomal abnormalities. Karyotyping results for chromosomal abnormalities were similar between the c-IVF and ICSI insemination groups. Consistent with the findings of previous studies, the most common chromosomal abnormalities were observed on chromosomes 15, 16, 22, and 21. Furthermore, in the c-IVF insemination group, no samples showed parental contamination after combined analysis with parental alleles (contamination rate, 0.00%). Therefore, contamination with paternal DNA-containing sperm and cumulus cells was negligible in thawed embryos inseminated via c-IVF.

3.3 Clinical outcomes

According to the PGT results, embryos with the highest morphological score and without chromosomal abnormalities were prioritized for transfer into the uterus. A total of 158 euploid embryos were available for transplantation, with only one embryo transferred in all transfer cycles. The numbers of FET cycles in the c-IVF and ICSI groups were 45 and 39, respectively. Table 3 presents the primary clinical outcomes of the 84 FET cycles in these groups. Some of the cycles were ongoing pregnancies; therefore, we did not compare the live birth rates. The biochemical pregnancy rate (68.89% vs. 64.10%, $P = 0.64$), clinical pregnancy rate (57.78% vs. 64.10%, $P = 0.55$), and miscarriage rate (11.54% vs. 16.00%, $P = 0.70$) were not significantly different between the c-IVF and ICSI groups. In the c-IVF group, three cases of miscarriage occurred, with one patient undergoing abortion product chromosome testing showing euploidy, whereas the remaining two patients did not undergo testing. The ICSI group had four cases of miscarriage, none of which underwent chromosomal testing for abortion products, and one case was an ectopic pregnancy. No misdiagnoses were observed during the study period.

4 Discussion

Currently, research on PGT in embryos subjected to c-IVF insemination is limited and its clinical application remains controversial. One primary concern is the potential contamination of biopsy samples with paternal and maternal DNA. Consequently, in

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TABLE 1 Clinical information of the FET cycles and the laboratory data of embryological for each study group.

Characteristics	Study groups		P value (c-IVF vs ICSI)
	c-IVF group	ICSI group	
No. of cycles, n	58	49	–
Infertility type			
Primary infertility, n (%)	9 (15.52%)	10 (20.41%)	0.51
Secondary infertility, n (%)	49 (84.48%)	39 (79.59%)	
Female			
age (years, average ± SD)	35.74 ± 4.33	35.92 ± 5.03	0.25
BMI (kg/m2, average ± SD)	22.31 ± 3.76	22.51 ± 3.17	0.85
The indication for PGT-A			
recurrent abortion, n (%)	23 (39.66%)	19 (38.78%)	
Advanced maternal age, n (%)	24 (41.38%)	20 (40.82%)	0.98
repeated implantation failure, n (%)	11 (18.97%)	10 (20.41%)	
Blastocyst biopsy			
No. of blastocyst, n	179	141	
Blastocyst biopsy on Day5, n (%)	55 (30.73%)	43 (30.50%)	
Blastocyst biopsy on Day6, n (%)	121 (67.60%)	92 (65.25%)	0.41
Blastocyst biopsy on Day7, n (%)	3 (1.68%)	6 (4.26%)	
Blastocyst development on Day5/6/7			
EQ1 (average ± SD)	1.94 ± 1.53	1.87 ± 1.52	0.66
EQ2 (average ± SD)	1.09 ± 1.34	0.92 ± 1.10	0.44
EQ3 (average ± SD)	0.07 ± 0.26	0.08 ± 0.28	0.62
Total EQ1, n (%)	116 (64.80%)	92 (65.25%)	
Total EQ2, n (%)	59 (32.96%)	45 (31.91%)	0.95
Total EQ3, n (%)	4 (2.23%)	4 (2.84%)	

Blastocyst development on Day5/6/7, EQ1 means the embryo score is AA AB BA, EQ2 means the embryo score is BB AC, EQ3 means the embryo score is CA BC CB.

TABLE 2 The results of PGT-A in the two study groups.

Item	Study groups		P value (c-IVF vs ICSI)
	c-IVF group	ICSI group	
PCR failure, n (%)	0 (0.00%)	0 (0.00%)	–
Tested embryos			
Euploid, n (%)	87 (48.60%)	71 (50.35%)	0.69
Abnormal, n(%)	63 (35.20%)	52 (36.88%)	
Mosaic, n(%)	29 (16.20%)	18 (12.77%)	
parental contamination, n(%)	0 (0.00%)	0 (0.00%)	–

PCR failure—no embryonal DNA is available for PCR; euploid indicates that the embryo is chromosomally normal and is available for transfer; abnormal indicates that the embryo has CNVs > 4M and mosaicism > 70%; mosaic indicates that the embryo has CNVs > 4M and mosaicism is between 30% and 70%.

routine PGT treatment cycles, the main guidelines and expert consensus of ESHRE, ASRM and PGTIS advocate the use of ICSI as the preferred method of insemination to minimize the risk of contamination from spermatozoa and cumulus cells attached to the embryonic zona pellucida. Considering the off-indication use, potential impact on embryo development and advantages compared with c-IVF, the necessity of ICSI for PGT needs further exploration.

PGT relies on single-cell WGA for detection. The main purpose of WGA technology is to amplify DNA from a small number of biopsy cells, typically 4–10, to generate sufficient DNA templates for subsequent chromosome or gene analysis. Historically, paternal contamination has been a concern in PGT. Various widely used WGA kits are available on the market, including SurePlex, multiple displacement amplification (MDA), MALBAC, and RepSeq. Because of the unique packaging of DNA in sperm, which involves complex structural components, such as the nuclear matrix and small nuclear circles, the chromatin in sperm nuclei is highly condensed. Prior to WGA, lysis with specific reagents, such as proteases or dithiothreitol, is necessary to decondense the sperm chromosomes (21, 22). Some studies suggest that sperm cell amplification may require modified SurePlex or MDA methods before PGT technology can be used for genetic testing of individual sperm. Essentially, when sperm undergo traditional WGA methods, their DNA content may be insufficient for PGT (8). This opens the possibility of using c-IVF insemination methods during PGT cycles.

In a retrospective analysis, Feldman et al. compared the accuracy of c-IVF and ICSI insemination in PGT (14). This study observed comparable PGT results and contamination rates between the two groups, with negligible paternal sperm DNA contamination in the c-IVF insemination group. Additionally, De Munck et al. assessed the effectiveness of c-IVF and ICSI methods in PGT-A cycles involving 30 couples with non-male factor infertility in a prospective study (16). No significant differences were observed between the two groups in terms of fertilization rate, embryo development ability, blastocyst euploidy rate, contamination rate, aneuploid parent source, or uniparental diploid parent source in the PGT cycle. In Zhang et al. study, only one of 150 trophectoderm biopsy samples testing showed maternal contamination level of 10%, and the PGT-A results was validated with 100% consistent by inner cell mass and trophectoderm (19). This further supports the conclusion that c-IVF insemination may be not a risk factor for PGT-A cycle, suggesting that ICSI should be preferred for men with infertility.

The aforementioned studies were preliminary explorations of the use of c-IVF insemination methods in fresh PGT-A cycles. Patients who have previously undergone c-IVF insemination during embryo transfer cycles, often because of factors such as advanced maternal age, recurrent miscarriage, or repeated implantation failure, are now being considered for PGT-A. This test necessitates the thawing and biopsy of frozen embryos. There are

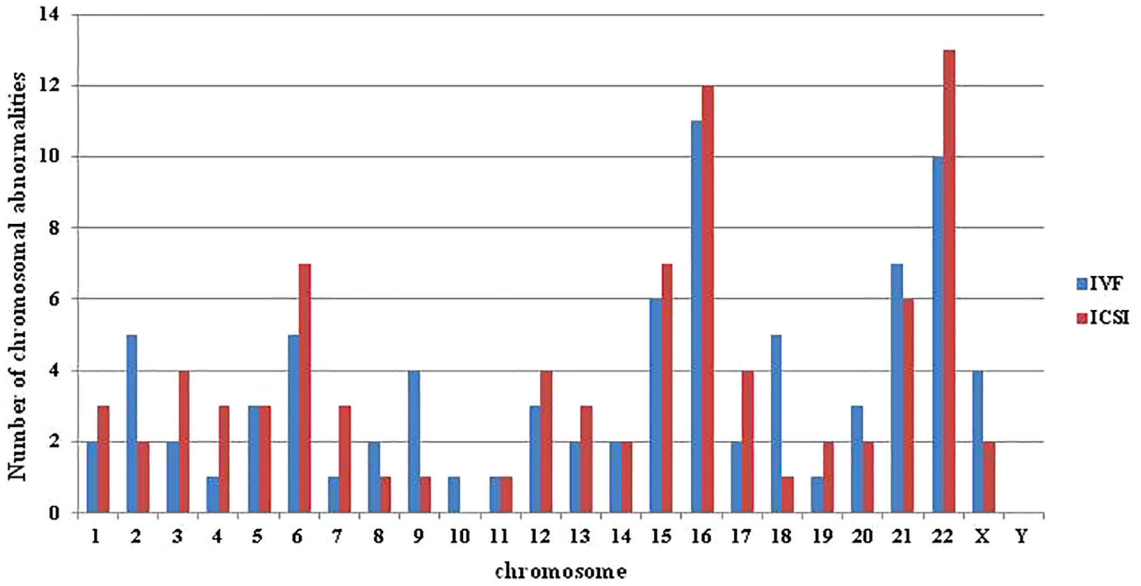

FIGURE 1
The distribution of abnormalities in each chromosome in the c-IVF and ICSI groups. Abnormalities included chromosome monomer, multibody, and fragment abnormalities, but did not include mosaicism.

two main issues when considering thaw biopsy of frozen embryos from c-IVF insemination. First is that some frozen blastocysts from c-IVF insemination may contain a zona pellucida containing both sperm and cumulus cells. Second is whether sperm and cumulus cells can be easily amplified after freezing in liquid nitrogen. To prevent contamination of biopsy samples with maternal cumulus cells, embryologists meticulously remove cumulus cells from the zona pellucida of the embryo. Additionally, our study utilized the qPCT method described previously (23–25) to assess the susceptibility of frozen embryos inseminated via c-IVF to parental contamination. Recently, researchers conducted a prospective analysis of the risk of parental contamination during PGT-A cycles involving cryopreserved embryos inseminated via c-IVF (18). The contamination rate of 0.83% (1/120) is very close to the 0.00% (0/179) in this study, and no sperm contamination is found. The findings indicated that frozen embryos fertilized with c-IVF undergo PGT-A with minimal risk of contamination from the parents and that MALBAC technology can be effectively utilized for testing frozen embryos resulting from c-IVF in PGT-A cycles.

TABLE 3 Clinical outcomes of the two groups.

Item	Study groups		P value (c-IVF vs ICSI)
	c-IVF group	ICSI group	
FET cycles,n	45	39	–
Biomedical pregnancy rate, n (%)	31 (68.89%)	25 (64.10%)	0.64
Clinical pregnancy rate, n (%)	26 (57.78%)	25 (64.10%)	0.55
Miscarriage rate, n (%)	3 (11.54%)	4 (16.00%)	0.70
The accuracy of PGT-A results, n (%)	23 (100.00%)	21 (100.00%)	–

Biochemical pregnancy is confirmed by elevated levels of human chorionic gonadotropin but does not result in the formation of a pregnancy sac.
Clinical pregnancy refers to B-ultrasound that can find the pregnancy sac.
Miscarriage includes spontaneous abortion and induced abortion.
The accuracy of the PGT-A results indicates the consistency of PGT-A and NIPT or amniocentesis results.

In the study, the individual and embryonic development characteristics of the patients enrolled in the c-IVF and ICSI groups were analyzed, including female age, BMI, indication, biopsy embryo age and embryo morphology. There was no significant difference between the two groups. Subsequent comparative analysis of PGT-A results and transplantation clinical outcomes between the two groups showed no difference. The research results of Sahin showed that the chromosomal abnormalities rates in embryos fertilized by c-IVF and ICSI were 65% and 69.9% (26), respectively, which were higher than the results of this study. In the Palmerola et al. study, there was no significant difference in the test failure rate (4.4% vs 6.3%), euploidy rate (27.9% vs 30%), aneuploidy rate (45.4% vs 43.1%) and the test failure rate (4.4% vs 6.3%) between the c-IVF and the ICSI group. Though not significant, they identified a trend toward higher rate of mosaicism in IVF (25.9%) versus ICSI (20.9%) (27), which is consistent with the results of this study. De Munck study results showed no significant difference on euploidy rate (49.8% vs 44.1%) between the c-IVF and ICSI PGT-A (16). The findings of Zhang et al. suggested that the choice between c-IVF and ICSI with PGT-A may not significantly impact clinical pregnancy outcomes, as both groups demonstrated comparable pregnancy rates of 59.4% and 55.6%, respectively (19). Interestingly, when comparing the distribution of different chromosomal abnormalities between c-IVF and ICSI with PGT-A in our study, we found a higher

prevalence of abnormalities in chromosomes 2, 9, and 18 in the c-IVF group, although the sample size was limited. The discrepancies in PGT-A results and clinical outcomes across different studies can be attributed to various factors, including patient selection criteria, testing methods, and study design.

Although our retrospective study offers support for the use of c-IVF in PGT-A cycles, it has some limitations. This study was limited to 58 c-IVF insemination cycles and a limited number of patients. It is crucial to recognize that PGT-M and PGT-SR involve distinct treatment options and present varying risk profiles. Therefore, our findings should not be extrapolated to patients undergoing PGT-M (monogenic disorders) or PGT-SR (structural chromosomal rearrangements). Moreover, because of the standard treatment process for PGT-A, embryos in the ICSI group were not tested for parental contamination using qPCT, leading to an inability to assess parental contamination in this group. Finally, a key drawback of this clinical application strategy is the requirement for invasive embryo biopsy. The repeated thawing and freezing of embryos may raise safety concerns.

In summary, c-IVF as an insemination method for PGT-A is associated with a lower risk of parental contamination and misdiagnosis. This study demonstrates the feasibility of performing PGT-A on frozen embryos resulting from c-IVF insemination. Clinicians recommend that patients with cryopreserved embryos obtained from c-IVF should consider this option.

5 Conclusion

This study verified the efficacy of PGT-A detection in frozen embryos resulting from c-IVF insemination by analyzing the outcomes of PGT on frozen embryos from both c-IVF insemination and ICSI insemination. The findings of this research could potentially be extrapolated to the implementation of PGT-SR or PGT-M in future studies.

Data availability statement

The data that support the findings of this study have been deposited into CNGB Sequence Archive (CNSA) of China National GeneBank DataBase (CNGBdb) with accession number CNP0006266.

Ethics statement

The studies involving humans were approved by Reproductive Medicine of Zhongshan Boai Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements. 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

XW: Writing – original draft, Writing – review & editing. ZL: Writing – original draft, Writing – review & editing. LC: Conceptualization, Data curation, Writing – review & editing. JH: Methodology, Writing – review & editing. WY: Conceptualization, Data curation, Writing – review & editing. ZO: Visualization, Writing – review & editing. NL: Resources, Visualization, Writing – review & editing. JL: Visualization, Writing – review & editing. XF: Conceptualization, Investigation, Project administration, Writing – review & editing. XL: Conceptualization, Investigation, Project administration, Writing – review & editing.

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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.

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The association between pregnancy outcomes and frozen-thawed embryo transfer cycles based on D3 cell count in high-quality blastocysts

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Objective: To investigate the number of cells in D3-stage embryos of high-quality blastocysts as a contributing factor, to evaluate the clinical pregnancy outcomes in frozen-thawed embryo transfer cycles, and to determine the impact of D3-stage cell count on pregnancy outcomes.

Methods: Patients under 38 years old who underwent frozen-thawed single high-quality blastocyst transfer at our center were selected. Based on the cell count of D3 cleavage-stage embryos forming blastocysts, patients were divided into three groups: ≤6 cells, 7-9 cells, and ≥10 cells. A multivariate regression analysis was used to establish the prediction model, analyzing the impact of different D3 cleavage-stage cell counts on clinical pregnancy outcomes to guide clinical laboratories in selecting blastocysts with the best pregnancy outcomes for transfer.

Results: This study identified a significant association between D3 cell count, blastocyst development stage, and embryo age. Embryos with a higher D3 cell count (≥10) were more likely to reach advanced blastocyst stages and form blastocysts by D5, whereas embryos with fewer D3 cells (≤6) were more likely to form blastocysts on D6. While D3 cell count significantly influenced blastocyst stage and timing of embryo development, no significant differences were observed between groups regarding clinical pregnancy, implantation, or live birth rates. Notably, embryos with fewer D3 cells exhibited a significantly lower miscarriage rate than other groups. Multivariate regression analysis showed a significant correlation between blastocyst stage, embryo age, and D3 cell count, particularly in D5 embryos and more advanced blastocysts. The increased miscarriage rate may be related to lower D3 cell count, and inadequate endometrial preparation was associated with poorer pregnancy outcomes. The type of infertility was also linked to D3 cell count, with secondary infertility patients showing more significant influencing factors.

Conclusion: D3 cell count and related factors play a critical role in pregnancy outcomes during frozen-thawed high-quality blastocyst transfer cycles. Optimizing embryo age, selecting blastocysts at different stages, and refining endometrial preparation protocols are likely to enhance clinical pregnancy and live birth rates.

KEYWORDS

frozen-thawed embryo transfer, high-quality blastocyst, D3 cell count day, pregnancy outcome, single blastocyst transplantation

Introduction

In assisted reproductive technology (ART), the transfer of a single blastocyst is widely accepted as it is crucial for achieving a successful pregnancy and is also important for the health of both the mother and the baby (1). Recently, novel strategies for embryo selection have emerged, such as delayed imaging (2), preimplantation genetic testing for aneuploidy (PGT-A) (3), and the analysis of gene expression in cumulus cells and metabolite levels in culture media (4, 5). Despite these advances, morphological scoring remains the predominant assessment method for evaluating embryo quality.

Blastocyst formation is a complex and continuous process involving key events such as blastomere proliferation during cleavage, compaction, and the development of the blastocyst cavity (6, 7). Embryonic genome activation, occurring during the 4-cell to 8-cell stage of cleavage, marks a crucial transition in blastocyst development (8). It is widely recognized that the ideal embryo for transfer is an 8-cell stage embryo with uniformly sized blastomeres on Day 3, as deviations in blastomere number can lower implantation rates (9). Blastomere count is thus a critical metric for evaluating blastocyst development, directly influencing its quality and potential for implantation.

The impact of blastomere number on the pregnancy outcomes of blastocyst transfer is debated. Some researchers argue that blastocysts derived from Day 3 embryos with a higher number of cells exhibit greater developmental potential, leading to increased rates of implantation and live births (10, 11). Conversely, other studies suggest that blastocysts derived from fewer blastomeres exhibit comparable implantation and live birth rates, though embryos with faster cleavage rates may have an increased risk of chromosomal abnormalities (12, 13). Therefore, definitive conclusions from research are still lacking.

Research on how the number of cells in D3 embryos affects pregnancy outcomes in frozen-thawed high-quality blastocyst transfer cycles is currently limited. This study aims to reveal the potential impact of high-quality blastocysts from different blastomere counts during frozen-thawed transfer cycles through in-depth analysis. The goal is to establish a feasible predictive model, providing clinicians with more accurate criteria for embryo selection.

Materials and methods

Data collection

A retrospective analysis was conducted on infertile couples undergoing frozen-thawed embryo transfer cycles at the Maternal and Child Health Care Center of Yulin from January 2018 to November 2023. The study was approved by the Ethics Committee of the Maternal and Child Health Hospital of Yulin. Before participation, all subjects were informed about the study details and provided written consent.

Data collection included variables such as age, body mass index (BMI), duration of infertility, type and causes of infertility, fertilization method, endometrial preparation protocol, endometrial thickness, implantation rate, clinical pregnancy rate, miscarriage rate, multiple pregnancy rate, and live birth rate.

Inclusion and exclusion criteria

Inclusion criteria

Female age <38 years. Transfer of high-quality single blastocysts post-thawing, defined as blastocysts with a Gardner score of ≥3BB (14). This includes blastocysts with a well-developed blastocyst cavity, a clear inner cell mass, and a well-defined trophoblast layer. Normal endometrial thickness and morphology at the time of transfer. "Normal" endometrial thickness is defined as ≥7 mm, and the endometrial morphology should be trilaminar as observed via ultrasound, indicating a receptive uterine environment for embryo implantation. Normal blastocyst expansion, defined as the blastocyst cavity volume having returned to at least half of its pre-freeze volume, indicating that the blastocyst is in an appropriate state for implantation. Transfer of high-quality single blastocysts that are at the 2PN (two pronucleus) stage, assessed approximately 16-18 hours post-insemination on Day 1.

Exclusion criteria

Patients diagnosed with uterine abnormalities including hydrosalpinx, endometriosis, or uterine fibroids. Patients where either partner has a documented chromosomal abnormality. Patients with blastocysts that did not fully expand or collapsed after thawing. Patients undergoing embryo transfer during the cleavage stage. Patients undergoing fresh transfer cycles. High-quality blastocysts developed from Day 3 embryos graded as third level.

Patient group

A total of 574 FET cycles meeting the above criteria were performed, corresponding to 574 individual treatment cycles. Some patients underwent more than one cycle, but to control for potential patient bias, only the first treatment cycle per patient was included in the primary analysis. Patients were divided into three groups based on the number of cells in the D3 embryos that formed the transferred blastocysts: Group A (≤6 cells, n = 66 cycles), Group B (7-9 cells, n = 381 cycles), and Group C (≥10 cells, n = 127 cycles). The order of transfer was prioritized based on the blastocyst grading for each patient.

Methods for grading cleavage-stage embryos and blastocysts

According to the "Istanbul Consensus" (9), the grading system for cleavage-stage embryos considers cell number, fragmentation amount, cell size, texture, color, and the uniformity and arrangement of pronuclei. Cleavage-stage embryos are classified into high-quality, moderate-quality, and poor-quality embryos. High-quality embryos have 8-9 cells, less than 10% fragmentation, cell sizes appropriate for the developmental stage, and no multinucleation. Moderate-quality embryos have more than 6 cells, 10%-25% fragmentation, most cells of appropriate size for the developmental stage, and no multinucleation. Poor-quality embryos are characterized by fewer than 6 cells, more than 25% fragmentation, irregular cell sizes, and multinucleation.

The survival rate of frozen-thawed blastocysts is evaluated based on the expansion of the blastocyst cavity within 2-4 hours post-recovery. The quality of thawed blastocysts is assessed using the Gardner grading system (14). Blastocysts with inner cell mass or trophectoderm cells graded as B are deemed usable. High-quality blastocysts are defined as those with inner cell mass and trophectoderm cells both graded ≥B and reaching stage 3 or higher. The high-quality blastocyst rate is the proportion of high-quality blastocysts to the total number of blastocysts at stage 2 or above. The integrity rate represents the proportion of fully recovered embryos to the total number of thawed embryos.

Embryo vitrification and thawing protocols

In our embryo cryopreservation protocol, we use vitrification to preserve blastocysts on days 5-6. This process involves Cryotop (Kitazato, Japan) and commercial vitrification reagents (VT601, Kitazato). The warming process utilizes the warming kit (VT602) from Kitazato (Tokyo, Japan). We adhere to the manufacturer's vitrification and warming protocols, as described in previous studies Vitrified blastocysts are thawed and cultured *in vitro* for 2-4 hours before transfer. If blastocysts collapse during warming, the FET cycle is typically canceled or a re-thawing is performed. Outcomes of each individual blastocyst transfer are recorded.

Preparation of endometrium and embryo transfer

Our reproductive center employs three protocols for uterine preparation before embryo transfer: hormone replacement cycle, natural cycle, and ovulation induction cycle. In the natural cycle, monitoring of endometrial and follicular development starts from day 10 of the menstrual cycle using ultrasound. Luteal phase support therapy begins when the leading follicle reaches 18-24 mm in diameter and the endometrial thickness is ≥8 mm (defined as day 0), with embryo transfers scheduled on days 0, 3, or 5 accordingly. For the hormone replacement cycle, oral estradiol valerate (Tonicare, Bayer, Germany) is initiated on days 2-3 of menstruation until the endometrium reaches ≥8 mm, followed by progesterone injection (20 mg/IU, Zhejiang Xianju) for luteal support, with transfers scheduled on days 0, 3, or 5. In the ovulation induction cycle, letrozole is orally administered for 5 days starting from days 3-5 after menstruation, coupled with human menopausal gonadotropin (HMG) for ovulation induction. Progesterone is administered once the dominant follicle reaches ≥17 mm and the endometrial thickness is ≥8 mm, preparing the endometrium for embryo transfer.

In all cycles, we consistently use COOK catheters manufactured by COOK Medical, headquartered in Indiana, USA, for transferring selected embryos into the uterine cavity under ultrasound guidance. This standardized catheter use ensures methodological consistency and minimizes potential variations that could occur with different catheters.

Clinical outcomes

In assessing pregnancy outcomes, we analyze several indicators: clinical pregnancy, clinical pregnancy rate, implantation rate, multiple pregnancy rate, miscarriage rate, and live birth rate. The clinical pregnancy rate is calculated as the number of cycles with the presence of a gestational sac and fetal heartbeat at 4 weeks post-embryo transfer, divided by the total number of embryo transfer cycles. β-hCG levels are tested on the 11th day after D5/D6 frozen-thawed blastocyst transfer, with levels above 50 mIU/mL considered hCG-positive. Clinical pregnancy is confirmed if both a gestational sac and fetal heartbeat are detected by the 4th week after embryo transfer. In cases where multiple live births occur following single embryo transfer (SET), this is likely due to the natural splitting of a single blastocyst into monozygotic twins, a phenomenon that, although rare, occurs occasionally in assisted reproductive technology (ART). To avoid errors, our embryo transfer process involves strict double-checking by two individuals. Implantation rate is calculated by determining the

number of gestational sacs per transferred embryo at the 6th week of pregnancy. Miscarriage rate is computed as the number of miscarriages divided by the number of clinical pregnancies. The rate of multiple births is derived from dividing the number of multiple pregnancies by the number of clinical pregnancies. Live birth rate signifies the proportion of live births per clinical pregnancy.

Statistical analysis

This study determined the required sample size using G*Power 3.1.9.7 and applied a Chi-square test to assess differences in pregnancy rates across three groups. The statistical parameters were: an effect size (w) of 0.3 (medium), a significance level (α) of 0.05, statistical power (1-β) of 0.80, and degrees of freedom (df) of 2. Based on these inputs, G*Power calculated a total sample size of 108, with approximately 36 frozen-thawed blastocyst transfer cycles per group. This sample size provides 80% power to detect significant differences among the groups, satisfying the statistical requirements. Statistical analyses were performed using SPSS 25.0 (Chicago, SPSS). Categorical variables were described as frequencies (percentages) and assessed using the chi-square test. Continuous variables were presented as mean ± SD; t-tests and one-way ANOVA evaluated group differences, with *post hoc* tests for multiple comparisons. A *P*-value < 0.05 was considered statistically significant. Logistic regression analysis identified differences among influencing factors.

Results

Comparison of baseline data

The baseline comparison across the three groups revealed no significant differences in age ($\chi2 = 4.215$, $P=0.122$), body mass index (BMI) ($\chi2 = 2.254$, $P=0.324$), or infertility duration ($\chi2 = 1.256$, $P=0.534$). However, the unexplained infertility rate was significantly higher in Group A than in Groups B and C ($\chi2 = 7.335$, $P=0.026$). Regarding fertilization methods, there were no significant differences between the groups for *in vitro* fertilization (IVF) ($\chi2 = 3.553$, $P=0.171$), intracytoplasmic sperm injection (ICSI) ($\chi2 = 5.707$, $P=0.095$), or R-ICSI ($\chi2 = 0.616$, $P=0.736$). For endometrial preparation methods, the natural cycle usage rate in Group C was significantly lower than in Groups A and B ($\chi2 = 6.733$, $P=0.035$), while the ovulation induction cycle usage rate in Group C was significantly higher than in Groups A and B ($\chi2 = 10.511$, $P=0.001$). No significant differences were observed in the use of hormone replacement therapy ($\chi2 = 1.923$, $P=0.382$) or in endometrial thickness among the groups ($\chi^2 = 0.754$, $P=0.684$) (see Table 1).

Comparison of pregnancy outcomes

The comparison of pregnancy outcomes among the three groups revealed that the proportion of stage 5 or higher blastocysts in Group C (≥10 cells) was significantly greater than in Groups A and B ($\chi2 = 9.099$, $P=0.011$). No significant differences were observed in the proportions of stage 3 and stage 4 blastocysts among the groups ($\chi2 = 4.413$, $P=0.110$; $\chi2 = 0.554$, $P=0.758$). Group C also had a significantly higher proportion of D5 blastocysts compared to Groups A and B ($\chi2 = 59.615$, $P=0.000$), while Group A had a significantly higher proportion of D6 blastocysts than Groups B and C ($\chi2 = 59.615$, $P=0.000$). Clinical pregnancy rates did not differ significantly among the groups ($\chi2 = 1.344$, $P=0.511$). Similarly, no significant differences were found in multiple pregnancy rates ($\chi2 = 0.196$, P=0.906) or implantation rates ($\chi2 = 1.344$, $P=0.511$). The miscarriage rate in Group B was significantly higher than in Groups A and C ($\chi2 = 7.464$, $P=0.024$). Finally, live birth rates were not significantly different across the groups ($\chi2 = 3.299$, $P=0.194$) (see Table 2).

Multivariate regression analysis

Multivariate logistic regression analysis of group A

A multivariate logistic regression model was developed with Group C as the reference category. This model incorporated variables such as blastocyst stage, embryo age, endometrial preparation protocol, infertility type, and miscarriage rate. The analysis revealed that blastocyst grading significantly influenced the outcome (RR = 0.063, 95% CI: 0.016–0.246, $P < 0.001$). Likewise, embryo age had a substantial effect on the outcome (RR = 4.309E+31, 95% CI: 3.366E+30–5.516E+32, $P < 0.001$). Conversely, the endometrial preparation protocol did not significantly impact the outcome (RR = 0.916, 95% CI: 0.265–3.157, $P = 0.889$), nor did infertility type (RR = 0.727, 95% CI: 0.165–3.213, $P = 0.674$). In contrast, miscarriage rate had a significant effect on the outcome (RR = 5.938, 95% CI: 2.189–16.105, $P < 0.001$) (see Table 3).

Multivariate logistic regression analysis for group B

A multivariate logistic regression model was constructed, with Group C serving as the reference, incorporating factors such as blastocyst stage, embryonic age, endometrial preparation protocol, infertility type, and miscarriage rate. The results for Group B showed that the blastocyst stage significantly impacted the outcome (RR = 0.162, 95% CI: 0.079–0.330, $P < 0.001$). Embryonic age also significantly influenced the outcome (RR = 21.935, 95% CI: 2.485–193.951, $P = 0.005$). Endometrial preparation protocol was significantly associated with the outcome (RR = 0.281, 95% CI: 0.149–0.529, $P < 0.001$). Furthermore, infertility type had a significant impact on the outcome (RR = 8.250, 95% CI: 3.783–17.991, $P < 0.001$), and miscarriage rate was likewise significantly associated with the outcome (RR = 2.864, 95% CI: 1.477–5.553, $P = 0.002$) (see Table 4).

Discussion

In assisted reproductive technology (ART), selecting the optimal embryo for transfer is critical to achieving favorable pregnancy outcomes. Identifying embryos with the highest

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TABLE 1 Comparison of basic data of high quality blastocysts from different sources of D3 blastomeres number during frozen-thawed embryo transfer cycle.

Index	≤6 cells Group A	7~9 cells Group B	≥10 cells Group C	*P*
No. of cycles (n)	66	381	127	
Age (y)	32.67 ± 3.9	31.94 ± 3.6	31.53 ± 3.7	0.122
Body mass index (kg/m^2)	22.27 ± 3.2	22.08 ± 3.0	22.55 ± 3.0	0.324
Infertility duration (a)	3.96 ± 2.68	4.37 ± 3.0	4.19 ± 2.8	0.534
Types of infertility (%)				0.031
Primary infertility	40.9% (27/66)	32.5% (124/381)[a]	44.9% (57/127)	
Secondary infertility	59.1% (39/66)	67.5% (257381)[a]	55.1% (70/127)	
Infertility factor (%)				
Female factor	68.2% (45/66)	70% (282/381)	75.6% (96/127)	0.525
Male factor	9% (6/66)	6.0% (23/381)	11.8% (15/127)	0.095
Combined factors	7.6% (5/66)	11.4% (43/381)	4.7% (6/127)	0.078
Unexplained factors	7.6% (5/66)[A]	1.3% (5/381)	1.6% (2/127)	0.026
Multiple factors	7.6% (5/66)	7.3% (28/381)	6.3% (8/127)	0.912
Fertilization method (%)				
IVF	68.2% (45/66)	75.1% (286/381)	80.3% (102/127)	0.171
ICSI	31.8% (21/66)	22.3% (85/381)	18.1% (23/127)	0.095
RICSI	0	2.6% (10/381)	1.6% (2/127)	0.736
Inner membrane Scheme				
The natural cycle	27.3% (18/66)	24.4% (93/381)	14.2% (18/127)[a]	0.035
The Inducing ovulatory cycle	15.1% (10/66)	26% (99/381)	38.6% (49/127)[A]	0.001
Hormone replacement therapy	57.6% (38/66)	49.6% (189/381)	47.2% (60/127)	0.382
Endometrial thickness in ET (mm)	9.49 ± 1.74	9.62 ± 1.8	9.53 ± 1.6	0.684

P<0.01 indicates a highly significant difference and is represented by uppercase letters; P<0.05 indicates a significant difference and is represented by lowercase letters.

implantation potential continues to pose a significant challenge in managing patients undergoing *in vitro* fertilization and embryo transfer (IVF-ET). The findings of this study demonstrate that the number of cells in D3 embryos significantly influences the developmental stage and embryonic age of blastocysts. D3 embryos with the higher number of cells tend to develop into high-quality blastocysts earlier, specifically on day 5, and achieve a better blastocyst grade. By optimizing the selection of embryos based on embryonic age and different stages of blastocyst development, along with endometrial preparation protocols, it is possible to improve or achieve equally high clinical pregnancy rates and live birth rates.

Existing evidence suggests that the morphological grading of D3 embryos is correlated with the pregnancy outcomes of cleavage-stage embryos, as higher-quality embryos are more likely to result in successful implantation and pregnancy. However, the impact of D3 Cell Count on clinical pregnancy outcomes in frozen-thawed embryo transfer cycles involving high-quality blastocysts remains underexplored. Wang et al. (15) identified the number and growth of viable blastomeres as key indicators for predicting embryo developmental potential and clinical outcomes. High-quality embryos show that minimal damage during thawing does not impair their developmental potential or implantation rates. Sayme et al. (16) evaluated 195 oocytes from 40 patients undergoing antagonist Intracytoplasmic Sperm Injection (ICSI) treatment cycles. The cleavage-stage embryo arrangement was found to influence blastocyst formation rates. Specifically, embryos arranged in a tetrahedral configuration developed into a greater number of high-quality blastocysts compared to those with non-tetrahedral arrangements. The shorter time between ICSI and blastocyst formation in high-quality blastocysts suggests a strong correlation between embryo structural arrangement and the timing and quality of blastocyst formation. Liu et al. (17) noted that although C-grade embryos (>10 cells, symmetric blastomeres, and/or <20% fragmentation) possess relatively good developmental potential, A-grade embryos (6-10 cells, symmetric blastomeres, and/or <20% fragmentation) yield better clinical pregnancy outcomes. Xia et al.

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TABLE 2 Comparison of pregnancy outcomes in high-quality blastocysts from different sources of D3 blastomeres number during frozen-thawed embryo transfer cycles.

Index	≤6 cells Group A	7~9 cells Group B	≥10 cells Group C	*P*-value
No. of cycles(n)	66	381	127	
Blastocyst Stage				
Stage 3	22.7%(15/66)	18.6%(71/381)	11.8%(15/127)	0.110
Stage 4	68.2%(45/66)	71.4%(272/381)	68.5%(87/127)	0.758
Stage 5 and above	9.1%(6/66)	10%(38/381)	19.7%(25/127)[a]	0.011
Embryonic Age				0.000
D5	68.2%(45/66)	93.4%(356/381)[a]	99.2%(126/127)[A]	
D6	31.8%(21/66)	6.6%(25/381)	0.8%(1/127)	
Clinical pregnancy rate(%)	53%(35/66)	57.2%(218/381)	61.4%(78/127)	0.511
Multiple pregnancy rate(%)	0	5%(11/218)	3.8%(3/78)	0.906
implantation rate(%)	53%(35/66)	57.2%(218/381)	61.4%(78/127)	0.511
Miscarriage rate(%)	5.7%(2/35)	17.9%(39/218)[a]	9.4% (12/127)	0.024
Live birth rate(%)	94.3%(33/35)	82.1%(179/218)	83.3%(65/78)	0.194

P<0.01 indicates a highly significant difference and is represented by uppercase letters; P<0.05 indicates a significant difference and is represented by lowercase letters.

(18) recommended prioritizing high-quality blastocysts for selective frozen-thawed single embryo transfer. Despite the limited effect of blastocyst cleavage stage grading on pregnancy outcomes, it is preferable to choose embryos available on Day 2. If Day 3 embryos are not available, embryos with more than six cells on Day 2 should be prioritized for transfer. Similarly, Mi et al. (19) suggested that the number of cells on Day 2 influences blastocyst formation and clinical pregnancy rates. Consistent with our findings, the number of cells in D3 embryos significantly impacts the staging and embryonic age of blastocysts.

Wu et al. (20) demonstrated that in women under 35, live birth rates (LBR) significantly varied according to the number of cells on Day 3. Their findings indicate that as the number of Day 3 cells increases, LBR also rises following blastocyst transfer in younger women. Zhang et al. (21) identified a significant correlation between Day 3 morphological quality and the clinical pregnancy rate (CPR) and LBR in low-quality blastocysts. They suggested that when only low-quality euploid blastocysts are available for transfer, priority should be given to those derived from high-quality Day 3 embryos. Coticchio et al. (22) presented different results, showing that the formation rate of grade A blastocysts, based on inner cell mass (ICM) and trophectoderm (TE) morphology, was negatively correlated with the time to reach the blastocyst stage—indicating that higher-quality blastocysts formed in a shorter amount of time. Consistent with previous research, our study found that the number of cells in D3 embryos significantly influences blastocyst staging and embryonic age, with embryos containing more blastomeres often reaching high-quality blastocyst status earlier (on Day 5) and at a more advanced stage. High-quality blastocysts are associated with higher pregnancy and live birth rates. However, unlike previous studies, which included low-quality blastocyst transfers and observed significant differences in pregnancy outcomes between groups, our study focused exclusively on high-quality blastocysts, revealing no significant differences in pregnancy outcomes across groups.

Hu et al. (23) found no significant differences in pregnancy and birth outcomes between high-quality expanded blastocysts formed

TABLE 3 The Results of multivariate Logistic Regression Screening of Group A.

Group	Variables	Regression Coefficient	Standard Error	*Wald chi-square value*	*P*	*RR*	95%*CI*
A	Intercept	-54.654	1.249	1913.603	0.000		
	Blastocyst Stage	-2.764	0.694	15.871	0.000	0.063	0.016-0.246
	Embryonic Age	72.841	1.301	3135.854	0.000	4.309E+31	(3.366E+30)-(5.516E+32)
	Endometrial preparation protocol	-0.088	0.632	0.020	0.889	0.916	0.265-3.157
	Types of infertility	-0.319	0.758	0.177	0.674	0.727	0.165-3.213
	Miscarriage rate	1.781	0.509	12.245	0.000	5.938	2.189-16.105

TABLE 4 The Results of multivariate Logistic Regression Screening of Group B.

Group	Variables	Regression Coefficient	Standard Error	*Wald chi-square value*	*P*	*RR*	95%*CI*
B	Intercept	-1.543	1.235	1.560	0.212		
	Blastocyst Stage	-1.821	0.364	25.020	0.000	0.162	0.079-0.330
	Embryonic Age	3.088	1.111	7.725	0.005	21.935	2.485-193.591
	Endometrial preparation protocol	-1.270	0.323	15.424	0.000	0.281	0.149-0.529
	Types of infertility	2.110	0.398	28.143	0.000	8.250	3.783-17.991
	Miscarriage rate	1.052	0.338	9.700	0.002	2.864	1.477-5.553

on Day 6 and those formed on Day 5. However, for low-quality blastocysts, the clinical pregnancy rate was higher on Day 5 compared to Day 6. In patients with inner cell mass (ICM) graded B or higher, the live birth rate was higher for blastocysts on Day 5 compared to those on Day 6. However, for patients with ICM graded C, there was no significant difference in live birth rates between blastocysts developed on Day 5 and Day 6. Ozgur et al. (24) found that fully expanded blastocysts (grade ≥3) with AA and BA scores had similar implantation rates, while those with AB scores had relatively lower rates. Logistic regression analysis indicated that female age, blastocyst age, blastocyst expansion score, trophectoderm score, and the number of frozen blastocysts were important predictors of clinical implantation. These findings are consistent with ours, as our study also found no significant differences in pregnancy outcomes between frozen-thawed cycles involving high-quality blastocysts formed on Day 5 and Day 6. In contrast, Coticchio et al. (22) reported that as blastocyst development time increased, implantation rates, sustained pregnancy rates, and live birth rates progressively decreased, even when adjusted for maternal age. Compared to Day 5 blastocysts, Day 6 blastocysts had significantly lower probabilities of implantation, clinical pregnancy, sustained pregnancy, and live birth (25). The divergence between these results and ours may be due to the fact that, although vitrification technology has advanced, it still cannot entirely prevent cell damage during freezing. We hypothesize that high-quality blastocysts (with more inner cell mass and trophectoderm cells) may better compensate for freezing-induced damage following thawed embryo transfer, but this assumption has not been directly proven by specific data or research in our study. Therefore, pregnancy outcomes between Day 5 and Day 6 high-quality blastocysts in frozen-thawed cycles show minimal differences. Additionally, embryo cell division requires time, and embryos with more blastomeres on Day 3 can more quickly increase the number of ICM and trophectoderm cells, allowing them to develop into high-quality blastocysts in a shorter time frame. This hypothesis aligns with our findings, as our study demonstrated that the number of cells in D3 embryos significantly impacts blastocyst staging and embryonic age.

The number of cells in D3 embryos is clearly associated with the formation of high-quality blastocysts. However, it remains uncertain whether this relationship is also influenced by factors such as fertilization methods, endometrial preparation protocols, or types of infertility. Martins et al. (26) proposed that differentially expressed proteins in semen might serve as biomarkers for diagnosing primary and secondary infertility. Their research further indicated that sperm maturation failure and immune responses are significant causes of both primary and secondary male infertility, affecting the development of high-quality embryos. In contrast, Abebe MS et al. (27) reported that the proportions of primary and secondary infertility in Africa are approximately equal, suggesting that embryo quality should be similar; however, this is not always observed. Similar to our study, this research found that the type of infertility is somewhat associated with the number of cells in D3 embryos, with patients suffering from secondary infertility showing higher pregnancy success rates. We believe this may be due to the fact that patients with secondary infertility have already experienced pregnancy and childbirth, demonstrating the potential for successful fertilization, embryo development, and pregnancy. However, patients with primary infertility may have other factors hindering pregnancy, which still require further investigation and elimination.

Jiang et al. (28) observed that fertilization rates, high-quality embryo rates, embryo implantation rates, clinical pregnancy rates, and live birth rates were comparable between early rescue ICSI and half-ICSI. Similarly, Ten et al. (29) noted that embryos obtained through conventional IVF had superior quality, as indicated by a higher number of grade A embryos, compared to those obtained via ICSI. Contrary to these findings, our study showed that the fertilization method did not affect the formation of high-quality blastocysts. Additionally, Madani et al. (30) reported no significant differences in embryo implantation rates, ongoing pregnancy rates, miscarriage rates, or live birth rates among natural cycles, ovulation induction cycles, and hormone replacement cycles, which contrasts with our results. Our study found that inappropriate endometrial preparation protocols may lead to poorer pregnancy outcomes, and optimizing endometrial protocols may improve pregnancy outcomes.

This study underscores the critical role of selecting suitable embryos for transfer in assisted reproductive technology, as it has a significant impact on pregnancy outcomes. Multivariate regression analysis identified several key factors influencing the number and quality of embryo cells. There is a significant correlation between blastocyst stage, embryonic age, and the number of cells in D3 embryos, particularly in Day 5 embryos and more advanced-stage blastocysts. The increase in miscarriage rates may be related to the

number of cells in D3 embryos, and insufficient endometrial preparation partially affects clinical pregnancy outcomes. The type of infertility is also somewhat associated with the number of cells in D3 embryos, with patients suffering from secondary infertility showing higher pregnancy success rates.

A key limitation of this study is that the sample was obtained from a single reproductive center. Consequently, despite the large sample size and the rigorous study design, the results may not fully generalize to other regions or institutions. Variations in patient demographics, treatment protocols, and laboratory conditions across different reproductive centers could influence embryo transfer success rates and pregnancy outcomes. As a result, the external validity of the findings may be limited. To strengthen the generalizability of these results, future studies should be conducted across multiple centers to verify the findings in diverse clinical settings.

In summary, the results of this study demonstrate that D3 cell count and related factors play a critical role in pregnancy outcomes during frozen-thawed high-quality blastocyst transfer cycles. Optimizing embryo development time, selecting blastocysts at different stages, and refining endometrial preparation protocols may help improve clinical pregnancy and live birth rates.

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 reviewed and approved by the Ethics Committee of Maternal and Child Health Care Hospital of Yulin, Yulin, Guangxi 537000, China (Approval No.: YLSFYLLKY2024-03-04-11). Written informed consent was obtained from all study participants.

Author contributions

XL: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. YZ: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. ZY: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. LZ: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. YL: Conceptualization, Writing – original draft, Writing – review & editing. JJ: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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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.

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Effects and safety of propofol intravenous anesthesia in transvaginal oocyte retrieval on outcomes of *in vitro* fertilization and embryo transplantation

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Purpose: Propofol, a widely utilized anesthetic, is employed to alleviate pain and anxiety in outpatient oocyte retrieval procedures. However, its potential impact and safety profile in the context of *in vitro* fertilization and embryo transfer (IVF-ET) remain unclear.

Methods: This retrospective study enrolled 1187 patients undergoing IVF-ET, and divided into two groups depending on whether they received propofol (propofol group, n=140) or not (control group, n=1047) for anesthesia during oocyte retrieval.

Results: The baseline characteristics were comparable between the groups. Compared with control group, the number of oocytes retrieved in propofol group was more (p=0.012), while both the estradiol (E2) level on the trigger day and the pre-ovulatory follicle count were higher in propofol group ((p<0.01). Additionally, the rate of preterm delivery was significantly higher in the propofol group (p<0.001). To further analyze the effect of propofol on the oocyte retrieval rate, patients were divided into three subgroups depending on the pre-ovulatory follicle count (≤10, 11–20, and >20) to eliminate the influence of inconsistency in the estimation of the pre-ovulatory follicle count between the two groups. Analysis revealed that the use of propofol during oocyte retrieval was particularly advantageous in the subgroup with a pre-ovulatory follicle count of 11–20, yielding a higher oocyte retrieval rate (p<0.001).

Conclusion: The use of propofol in oocyte retrieval did not adversely affect fertilization, embryo quality, or clinical outcomes. Moreover, it was found to increase the oocyte retrieval rate among patients with an estimated pre-ovulatory follicle count of 11–20. These findings offer valuable evidence supporting the clinical application of propofol in oocyte retrieval procedures.

KEYWORDS

oocyte retrieval, *in vitro* fertilization, pregnancy rate, embryo quality, propofol

Introduction

Infertility is the inability to conceive within 1 year of unprotected intercourse and has been identified as a public health priority (1). In recent years, the incidence of infertility has increased annually, and the prevalence of infertility is about 25% among couples of reproductive age in China (2). As one of the leading treatments for infertility, *in vitro* fertilization and embryo transfer (IVF-ET) is a major assisted reproductive technique with a high success rate (3). During the process of IVF-ET, the most fearful part for patients is oocyte retrieval. To reduce the pain and fear of patients, anesthesia is applied gradually for oocyte retrieval. However, the safety of anesthesia on IVF-ET outcomes is yet unclear.

Transvaginal ultrasound-guided oocyte retrieval is a painful assisted reproductive technology procedure. A small number of patients are in severe pain due to anatomic changes in the pelvis, such as adenomyosis and chronic pelvic inflammation (4). Hitherto date, both general and regional anesthetics, including paracervicals, spinals, and epidurals have been used, and various methods of conscious sedation and analgesia have been attempted to reduce the pain of patients (5). Whenever favorable analgesia with sedation and rapid recovery is desired, propofol and remifentanil are administered in ambulatory settings due to their pharmacokinetic profile (6, 7). Propofol (2,6-diisopropylphenol, Diprivan, ICI Pharmaceuticals, Manchester, UK) is widely used either as an adjunct in general anesthesia or as a sole anesthetic agent by continuous intravenous administration and intermittent bolus injections for minor surgical interventions. For several years, this anesthetic was used for painless oocyte retrieval in IVF (8). Some studies have shown that propofol does not affect the postoperative levels of female sex hormones (serum estradiol and follicle-stimulating hormone [FSH]) after gynecologic surgery (9). Nonetheless, the potential impact of propofol used in oocyte retrieval on oocyte fertilization, embryo quality, and clinical pregnancy also need to be explored further. The results of the studies on the concentrations of propofol in follicular fluid during oocyte retrieval in women showed that the anesthetic accumulated in the follicular fluid in a dose- and time-dependent manner, which might have potential adverse effects on the follicular quality (10). A prolonged duration of anesthesia (>30 min) might seem to decrease implantation and clinical pregnancy rates (7, 11). Some studies showed that propofol has no effect on polar body extrusion in oocyte maturation, pronucleus formation, and embryo development of mice (12). However, a recent study showed that the embryo number and quality, and pup count of rats decreased with an increasing time of propofol administration (13). Another study showed that propofol does not affect the fertilization rate compared to an anesthetic ketamine (11); however, the data of patients without anesthesia were not included and compared to the propofol data in the study. Therefore, the effect of propofol on fertilization, embryo quality, and offspring is still inconclusive and needs to be studied further.

Therefore, the present study was undertaken to explore the effects and safety of propofol on the oocyte retrieval rate, embryo quality, and pregnancy outcomes.

Methods

Reasons for selecting retrospective analysis in this study

The use of a retrospective design in the study investigating the effects of propofol on *in vitro* fertilization and embryo transfer (IVF-ET) has several justifications, particularly when compared to prospective or randomized approaches. Here are the key reasons:

Ethical Considerations: Retrospective studies are often chosen when there are ethical concerns that prevent the use of a traditional experimental design. In the context of IVF-ET, it may not be ethical to withhold anesthesia from a control group, making a retrospective design more suitable.

Efficiency in Time and Budget: Retrospective studies are more efficient in terms of time and budget. They require fewer subjects and utilize pre-existing secondary research data, which is cost-effective and less time-consuming compared to the extensive planning and execution required for prospective or randomized studies.

Studying Rare or Unusual Exposures: Retrospective cohort studies are particularly useful when studying rare or unusual exposures, as well as diseases with a long latency or incubation period. This is relevant in IVF-ET where the use of propofol may be less common or have long-term effects that are not yet fully understood.

Relatively Inexpensive and Quick: The use of previously collected and stored records in a database means that retrospective cohort studies are relatively inexpensive and quick to perform. This is an advantage over prospective studies, which require significant resources for data collection and follow-up.

Sequence of Risk and Outcome Factors: Both retrospective and prospective cohort studies allow for the recording of exposure to risk factors before the outcome occurs, which is crucial for evaluating the sequence of risk and outcome factors.

However, it is important to acknowledge the limitations of retrospective studies, such as the risk of research biases, including recall bias and observer bias due to reliance on memory and self-reported data. Additionally, retrospective studies cannot establish causality, leading to lower internal and external validity. Despite these limitations, retrospective studies can provide valuable preliminary data that can inform the design of larger, more rigorous prospective or randomized trials.

Subjects

This retrospective study consecutively enrolled 1187 patients who were treated with IVF-ET cycles between June 2016 and 2017 at the Reproductive Center of Second Affiliated Hospital of Wenzhou Medical University as the study subjects. Patient-related data were retrieved from the hospital's electronic database system.

The inclusion criteria were as follows: (1) all candidates were aged 21–46-years-old and met the IVF-ET indications; (2) all

candidates undergoing IVF-ET for the first time; (3) all candidates were subjected to the standardized long agonist protocol; (4) the number of oocytes collected was >4.

The exclusion criteria were as follows: (1) endometrial adhesion, submucosal myoma, or uterine diameters >65 mm: these conditions can significantly affect the uterine environment and the success of IVF-ET procedures. Including patients with these conditions could introduce confounding variables that might skew the results, making it difficult to attribute outcomes to propofol anesthesia alone; (2) obvious infection after oocyte retrieval: infections can have serious implications for patient health and can also affect the success of IVF-ET; (3) history of cardiopulmonary disease, hypertension, opioids, and benzodiazepines, which can have systemic effects on the body, potentially influencing the outcomes of IVF-ET (For example, cardiopulmonary disease can affect oxygenation and blood flow, which are critical for embryo development); (4) complications with malignant tumors or other systemic diseases, such as an active stage of systemic lupus erythematosus, that were not suitable for pregnancy.

Study design

Oocyte retrieval with an anesthetic is not a routine operation during IVF-ET in China. The pain caused by oocyte retrieval is tolerable by most patients. However, patients who could not stand the pain and met the criteria of anesthetic surgery could select anesthetics during oocyte retrieval. Therefore, the patients who were administered propofol as an anesthetic during oocyte retrieval were consecutively enrolled in the current study as the propofol group (n=140). The other patients enrolled during the same period who met the inclusion criteria comprised the control group (n=1047). All oocyte retrieval procedures were performed by the same gynecologist. Oocyte retrieval rate, metaphase II (MII) oocyte rate, two pronuclear (2PN) rate, cleavage rate, high-quality embryo rate, and frozen embryo rate were analyzed. Among the 1187 patients, 1043 underwent fresh embryo transfer (propofol group n=112, control group n=931); then, the biochemical pregnancy, clinical pregnancy rate, early spontaneous abortion rate, ectopic pregnancy rate, multiplets pregnancy rate, preterm delivery rate, neonate weight, and sex ratio were analyzed between the two groups (Figure 1).

In order to further analyze the correlation between the oocyte retrieval rate and the oocyte retrieval operation with or without anesthetics, the patients were divided into three subgroups depending on the pre-ovulatory follicle count (≤10, 11–20, and >20) to eliminate the influence of inconsistent estimated pre-ovulatory follicle count between the propofol and control groups (Figure 1).

Superovulation program and embryo culture

All patients were subjected to a long-term protocol of pituitary downregulation with gonadotrophin-releasing hormone (GnRH) agonist for controlled ovarian hyperstimulation (COH) as described previously (14).

Patients who underwent oocyte retrieval with anesthesia were deprived of food and water for >10 h. Then, intravenous access was established and transferred into the operation room after bladder voiding. Propofol was administered as the anesthetic agent for the oocyte retrieval procedure. Dosing was tailored to each patient's weight, age, and overall health status, with a standard dosage ranging from 2 to 3 milligrams per kilogram of body weight. The operation was efficiently performed and completed within a 20-minute timeframe. The vital signs were closely monitored during the operation, including the blood pressure (BP), mean arterial pressure (MAP), heart rate (HR), and oxygen saturation (SpO2), and oxygen was administered at 2-3 L/min. Postoperative monitoring included electrocardiogram

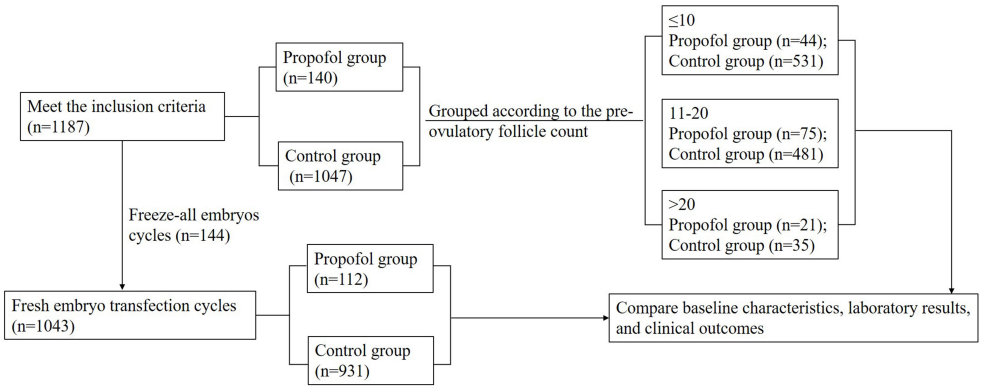

FIGURE 1
Flowchart. A total of 1187 cycles were consecutively enrolled in our single reproductive center during June 2016–2017. Among these, 1043 cycles underwent fresh embryo transfection. Patients who employed propofol as an anesthetic for oocyte retrieval were consecutively enrolled in the propofol group (n=140), and the others comprised the control group (n=1047). Then, each group was divided into three subgroups according to the pre-ovulatory follicle count (≤10, 11–20, and >20). The baseline information, laboratory data, and clinical outcomes were analyzed between each subgroup.

(ECG), non-invasive blood pressure (NIBP), and SpO2 every 15 min, along with pain (VAS scores) and nausea/vomiting (PONV) evaluation.

The embryos were cultured as described previously, and the embryos were scored according to the 2011 Istanbul Consensus (15, 16). Briefly, fertilization was observed 20 h after insemination based on the appearance of 2PN. The embryos were cultured in G-1 plus and G-2 plus medium (Vitrolife Co., Ltd, Australia) at 37°C in a humidified atmosphere with 6% CO_2. A D3 embryo with 7–9 blastomere cells of an A or B grade was considered a high-quality D3 embryo (15).

ET and luteal phase support

ET was performed under the guidance of transabdominal ultrasound. The starting time of luteal phase support depended on the serum P4 level on the day of hCG trigger, as described previously (17). Briefly, when P was <1.2 ng/mL, luteal support was initiated 1 day after oocyte retrieval. When P was ≥1.2 ng/mL, luteal support was initiated 2 days after ovulation. If P was ≥1.5 ng/mL, ET was canceled. Crinone gel and dydrogesterone tablets were used for luteal phase support until days 13-14 after ET. In the case of pregnancy, luteal support was continued until weeks 10–12 of pregnancy.

Outcome assessment

The present study aimed to analyze the laboratory data, including the oocyte retrieval rate, MII oocyte rate, 2PN rate, cleavage rate, high-quality embryo rate, frozen embryo rate, and the clinical outcomes, which included biochemical pregnancy, clinical pregnancy rate, early spontaneous abortion rate, ectopic pregnancy rate, multiplets pregnancy rate, preterm delivery rate, and neonate weight.

We also defined the laboratory and pregnancy outcomes in this study according to "CSRM consensus on the key indicators for quality control in IVF laboratory" (18).

1. Oocyte retrieval rate: number of oocytes retrieved/pre-ovulatory follicle count (follicle diameter ≥12 mm was counted on the hCG injection day)×100%.
2. MII oocyte rate: number of MII oocytes/number of oocytes retrieved×100%.
3. 2PN rate: number of 2PN fertilized oocytes/number of MII oocytes×100%.
4. Cleavage rate: number of cleaved embryos/number of 2PN fertilized oocytes×100%.
5. High-quality embryo rate: number of high-quality D3 cleaved–embryos/number of cleavage embryos×100%. Note, a D3 embryo with 7–9 blastomere cells of an A or B grade was considered a high-quality D3 embryo.
6. Frozen embryo rate: number of frozen embryos/(total number of embryos cultured–total number of embryos transferred)×100%.
7. Biochemical pregnancy rate: biochemical pregnancy cycle number/fresh transfer cycle number×100%. The biochemical pregnancy was diagnosed when serum β-hCG level was >25 IU/L on the 14^{th} day after embryo transfer.
8. Clinical pregnancy rate: clinical pregnancy cycle number/fresh transfer cycle number×100%. Clinical pregnancy was diagnosed when ≥1 pregnancy sacs were observed on ultrasound. The rate included normal intrauterine pregnancy, ectopic pregnancy, and heterotopic pregnancy.
9. Early spontaneous abortion rate: number of spontaneous abortion cycles within 12 weeks of pregnancy/number of clinical pregnancy cycles×100%.
10. Ectopic pregnancy rate: number of ectopic pregnancy cycles/number of clinical pregnancy cycles×100%.
11. Multiplets pregnancy rate: multiple pregnancy cycles/number of clinical pregnancy cycles×100%.
12. Live birth rate: number of live births/transfer cycles×100%.
13. Preterm delivery rate: number of live births before 37 weeks/number of live births×100%.

Statistical analysis

The data were analyzed using the SPSS software (Version 17.0; SPSS, Chicago, IL, USA). Student's *t*-test was used to analyze the continuous variables, and the chi-square test was used for non-continuous variables. Before data analysis, a normality test was conducted. For percentage data that deviates from a normal distribution, such as multiple pregnancy rates and preterm birth rates, an arcsine transformation was performed. After the transformation, the data conformed to a normal distribution, and the t-test was continued for analysis. All data are presented as mean ± standard deviation (SD), and $p<0.05$ was considered statistically significant.

Results

General characteristics

In this study, we analyzed 1187 IVF-ET cycles in patients who, for the first time, underwent the assisted reproductive technology (ART) treatment (Figure 1). We compared the patients' clinical outcomes, including the baseline information, the clinical parameters, the laboratory data, and the clinical pregnancy outcomes. The baseline information, including age, infertility duration, body mass index (BMI), basal FSH, LH, E2, P, and the antral follicle count (AFC) were similar between the two groups (Table 1).

Clinical parameters

All patients received a long-term protocol, and the clinical parameters are shown in Table 2. The level of E2 (2403.7 ±

TABLE 1 General characteristics of patients at baseline.

	Propofol group (n=140)	Control group (n=1047)	*p*-value
Maternal age (years)	30.6 (4.4)	32.1 (4.6)	0.439
Infertility duration (years)	3 (2.4)	3.1 (1.9)	0.12
Maternal BMI (kg/m^2)	20.9 (2.9)	21.6 (2.6)	0.067
Basal FSH (mIU/mL)	6.7 (2.6)	6.7 (2.2)	0.065
Basal LH (mIU/mL)	4.2 (1.8)	4.3 (1.9)	0.263
Basic E2 level (pg/mL)	50.6 (18.4)	51.9 (16.6)	0.124
Progesterone (ng/mL)	0.51 (0.27)	0.51 (0.26)	0.339
AFC	13.3 (5.8)	11.3 (5.8)	0.841

Data are presented as mean (SD). No statistically significant differences were observed between the two groups. BMI: body mass index; FSH: follicle-stimulating hormone; LH: luteinizing hormone; E2: estradiol; AFC: antral follicle count.

1276.6 pg/mL *vs.* 2092.0 ± 1015.9 pg/mL, p=0.001) and the pre-ovulatory follicle count (14.2 ± 6.8 *vs.* 11.1 ± 4.7, p<0.001) was significantly higher in the propofol group than in control group. On the other hand, the total dose of Gn and days of Gn were similar between the two groups.

In vitro fertilization outcome parameters

The laboratory results are summarized in Table 3. The number of oocytes retrieved, number of MII oocytes, number of 2PN oocytes, number of D3 high-quality embryos, and the number of frozen embryos of the propofol group were significantly higher in the propofol group than in the control group (p<0.05). Conversely, no significant difference was detected in the MII oocyte rate, 2PN rate, number of cleavage oocytes, cleavage rate, D3 high-quality embryo rate, and frozen embryo rate between the two groups. However, the oocyte retrieval rate was significantly higher in the control group (109 ± 36.4%) than in the propofol group (107 ± 41.1%, p=0.007).

TABLE 2 Clinical parameters.

	Propofol group (n=140)	Control group (n=1047)	*p*-value
Total does of Gn (U)	2163.8 (794.0)	2490.6 (927.0)	0.154
Gn duration (days)	11.6 (2.2)	11.6 (2.6)	0.135
E2 on the trigger day (pg/mL)	2403.7 (1276.6)	2092.0 (1015.9)	0.001
Pre-ovulatory follicle count	14.2 (6.8)	11.1 (4.7)	0

Data are presented as mean (SD). Gn: gonadotrophin; E2: estradiol.

TABLE 3 *In vitro* fertilization laboratory data.

	Propofol group (n=140)	Control group (n=1047)	*p*-value
No. of oocytes retrieved	13.6 (5.2)	11.4 (4.3)	0.012
Oocyte retrieval rate (%)	107 (41.1)	109 (36.4)	0.007
No. of MII oocytes	11.9 (4.9)	9.7 (4.0)	0.007
MII oocyte rate (%)	87.7 (13.4)	85.4 (15)	0.081
No. of 2PN oocytes	9.5 (4.1)	7.8 (3.5)	0.038
2PN rate (%)	80.2 (15.4)	80.4 (16.5)	0.297
No. of cleavage embryos	9.2 (4.0)	7.6 (3.5)	0.139
Cleavage rate (%)	97.5 (6.1)	97.8 (6.6)	0.612
No. of D3 high-quality embryos	3.7 (2.6)	2.7 (2.3)	0.004
High-quality embryo rate (%)	41.2 (25.6)	34.2 (24.7)	0.283
No. of frozen embryos	4.6 (3.3)	3.2 (2.8)	0.004
Frozen embryo rate (%)	60.4 (33.2)	51.7 (34.8)	0.299

Data are presented as mean (SD). No.: number; MII: metaphase II; 2PN: two pronucleus.

Pregnancy outcome parameters

A total of 1043 patients underwent fresh embryo transfer (propofol group n=112, control group n=931). As shown in Table 4, the propofol and control groups had similar biochemical pregnancy rates, clinical pregnancy rates, early spontaneous abortion rates, ectopic pregnancy rates, multiplets pregnancy rates, live birth rates, neonate weight, and male to female ratio. However, a statistically significant difference was detected in the preterm delivery rates between the propofol group [9/65 (13.8%)] and the control group [12/591 (2%), $p<0.001$], and twin pregnancy was the main cause of preterm delivery [7/65 (10.8%) *vs.* 6/591 (1%), $p<0.001$].

Oocyte retrieval under painless operation is valuable to obtain oocytes

To investigate the relationship between the oocyte retrieval rate and the use of anesthesia during the procedure, patients were stratified into three distinct subgroups based on the number of pre-ovulatory follicles. This stratification aimed to mitigate the impact of potential disparities in the estimation of pre-ovulatory follicle counts between the propofol and control groups. The three

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TABLE 4 *In vitro* fertilization-embryo transfer pregnancy outcomes.

	Propofol group (n=112)	Control group (n=931)	*p*-value
Biochemical pregnancy rate	8/112 (7.1%)	55/931 (5.9%)	0.604
Clinical pregnancy rate	60/112 (53.6%)	534/931 (57.4%)	0.445
Early spontaneous abortion rate	9/60 (15%)	61/534 (11.4%)	0.415
Ectopic pregnancy rate	0/60 (0%)	5/931(0.5%)	0.569
Multiplets pregnancy rate	18/60 (30%)	133/534 (24.9%)	0.39
Live birth rate	65/112 (58.0%)	591/931 (63.5%)	0.26
Preterm delivery rate	9/65 (13.8%)	12/591 (2.0%)	0
Preterm delivery rate of twin pregnancy	7/65 (10.8%)	6/591 (1.0%)	0
Neonate weight (g)	2826.0 (673.0)	2922.0 (657.0)	0.281
Male to female ratio	36/29 (1.34:1)	289/302 (0.96:1)	0.321

Data are presented as mean (SD) or n (%).

subgroups were as follows: pre-ovulatory follicle count ≤10 group (Group 1; painless group, n=44; control group, n=531), pre-ovulatory follicle count 10–20 group (Group 2; painless group, n=75; control group, n=481), and pre-ovulatory follicle count >20 group (Group 3; painless group, n=21; control group, n=35). Pre-ovulatory follicle is a non-invasive diagnostic tool that assists clinicians in estimating the quantity of oocytes that can be harvested and the likelihood of success in an IVF cycle. The thresholds established signify varying degrees of ovarian reserve.

The baseline information, clinical parameters, and laboratory data were compared among the subgroups. The baseline information and clinical parameters are summarized in supplementary Supplementary Tables S1, S2. Furthermore, no significant differences were observed in age, infertility duration, basal FSH, LH, E2, and P, AFC, days of Gn, total dose of Gn, E2 on the trigger day, and pre-ovulatory follicle count between Groups 1 and 2, whereas the pre-ovulatory follicle count was higher in the propofol group than in the control group in Group 3 (26.1 ± 5.8 *vs.* 23.1± 2.2, *P*=0.002).

The IVF outcome parameters are listed in Table 5; the results in the propofol group were similar to those of the control group in Group 1, while in Group 2, the number of oocytes retrieved (14.8 ±

TABLE 5 *In vitro* fertilization laboratory data.

	Group 1 (≤10)			Group 2 (11–20)			Group 3 (>20)		
	Propofol group (n=44)	Control group (n=531)	*p*-value	Propofol group (n=75)	Control group (n=481)	*p*-value	Propofol group (n=21)	Control group (n=35)	*p*-value
Pre-ovulatory follicle count	7.4 (1.9)	7.3 (1.8)	0.388	14.7 (2.8)	14.6 (2.5)	0.231	26.1 (5.8)	23.1 (2.2)	0.002
No. of oocytes retrieved	9.5 (3.1)	8.7 (3.0)	0.875	14.8 (4.9)	14.1 (3.5)	0.001	17.4 (4.4)	16.5 (3.7)	0.53
Oocytes retrieved rate (%)	131.7 (40.7)	122.0 (41.8)	0.641	103.4 (36.0)	97.9 (23.1)	0	68.3 (20.1)	72.0 (17.1)	0.567
No. of MII oocytes	8.5 (2.9)	7.2 (2.8)	0.485	13.0 (4.5)	12.1 (3.3)	0.01	14.9 (5.5)	14.3 (3.5)	0.006
MII oocytes rate (%)	89.0 (14.3)	84.0 (16.4)	0.261	87.9 (11.3)	86.7 (13.4)	0.087	84.0 (17.8)	87.0 (11.3)	0.029
No. of 2PN oocytes	7.1 (2.8)	5.8 (2.5)	0.259	10.2 (3.8)	9.8 (3.3)	0.112	10.8 (5.0)	11.2 (3.5)	0.021
2PN rate (%)	83.0 (15.9)	80.5 (18.2)	0.219	81.0 (14.5)	80.5 (14.6)	0.592	71.9 (15.2)	77.8 (14.6)	0.49
No. of cleavages	6.9 (2.7)	5.7 (2.4)	0.298	10.2 (3.8)	9.5 (3.3)	0.112	10.5 (5.0)	10.8 (3.4)	0.042
Cleavage rate (%)	97.5 (6.0)	98.3 (7.0)	0.44	97.4 (5.3)	97.4 (6.2)	0.794	97.7 (8.9)	96.8 (5.0)	0.984
No. of D3 high-quality embryo	3.0 (1.9)	1.8 (1.7)	0.092	4.0 (2.8)	3.5 (2.5)	0.058	4.0 (3.3)	4.0 (2.9)	0.294
High-quality embryo rate (%)	44.2 (27.2)	31.8 (26.5)	0.691	40.3 (24.1)	36.7 (22.6)	0.397	38.1 (28.1)	36.5 (19.9)	0.025
No. of frozen embryo	3.3 (2.5)	2.0 (2.0)	0.07	5.1 (3.3)	4.4 (3.0)	0.176	5.9 (4.0)	4.8 (3.1)	0.227
Frozen embryo rate (%)	59.1 (36.4)	49.0 (38.1)	0.356	59.4 (29.4)	54.6 (31.8)	0.924	67.0 (39.5)	50.9 (21.2)	0.187

Data are presented as mean (SD). No.: number; MII: metaphase II; 2PN: two pronucleus.

4.9 *vs.* 14.1 ± 3.5, p=0.001) and the oocyte retrieval rate (103.4 ± 36% *vs.* 97.9 ± 23.1%, p<0.001) were significantly higher in the propofol group than in the control group. Moreover, the number of MII oocytes was higher in the propofol group than in the control group (p=0.01). The MII oocyte rates, number of 2PN oocytes, 2PN oocyte rates, number of cleavage embryos, number of D3 high-quality embryos, high-quality embryo rates, number of frozen embryos, and the frozen embryo rates were higher in the propofol group, but the differences were not statistically significant in Groups 1 and 2. In Group 3, the pre-ovulatory follicle count was higher in the propofol group than in the control group (26.1 ± 5.8 *vs.* 23.1 ± 2.2, p=0.002); however, the number of oocytes retrieved was similar between the two groups (p=0.53).

Discussion

Herein, we aimed to evaluate the effect of propofol anesthetic used in IVF-ET on IVF outcomes, including oocyte retrieval parameters and clinical success. The results showed that oocyte retrieval under anesthesia with propofol in IVF-ET had no negative effects on the fertilization rate, cleavage rate, embryo quality, frozen embryo rate, clinical pregnancy rate, live birth, neonate weight, and sex ratio, and no statistically significant difference was detected between the propofol group and control group. Furthermore, the oocyte retrieval operation under anesthesia could get more oocytes when patients had a pre-ovulatory follicle count of 11–20. The current study revealed that the outcome for oocyte retrieval under anesthesia with propofol was safe with respect to IVF-ET parameter; thus, it might be a safe and helpful choice for patients to reduce the pain and fear while the embryo quality and clinical pregnancy may not be influenced.

For several years, propofol has been used as a kind of anesthesia in transvaginal oocyte retrieval for IVF, allowing a completely painless puncture on an outpatient basis. Previous studies have suggested that propofol may accumulate in follicular fluid (10), and dose-and time-dependent toxic effects of propofol on fertilization rates were reported in mice (19). However, no detrimental effects of propofol on fertilization and the quality of embryos were detected in humans (20). In the present study, the fertilization rate was similar between the propofol and the control groups, consistent with the previous study (11, 21). Unexpectedly, the number of oocytes retrieved, number of normally fertilized oocytes, number of D3 high-quality embryos, and number of frozen embryos in the propofol group were all higher than that in the control group. It seems that propofol used in oocyte retrieval was beneficial and harmless to IVF-ET, while a previous study showed no difference in the embryo quality between patients who received propofol or other anesthesia (21). However, the cited study's limitation is that it did not include or analyze patients who did not receive anesthesia. Considering that the control group was not subjected to any form of anesthesia, it would be advantageous to compare our findings with those from studies that have assessed different anesthetic agents. Consequently, our research would provide a more comprehensive context for understanding the relative safety of propofol.

One of the important aspects of this study that should be discussed is the relationship between age and oocyte retrieval rate. In the present study, the age in the propofol group was younger than that in the control group, and the number of AFC in the propofol group was more than in the control group, although not significantly. However, the level of E2 on the trigger day and the pre-ovulatory follicle count was significantly higher in the propofol group compared with the control group. Previous studies showed that age and AFC are related to fertility in domestic animals, younger with higher AFC (22). Furthermore, AFC was also correlated with oocyte count and the number of fresh and frozen embryos in the patients between 20-32 years old (23), which was consistent with the present study. However, the oocytes retrieved rate in the control group was higher than in the propofol group. In order to further analyze the relationship between the oocyte retrieval rate and oocyte retrieval operation with or without propofol, we divided the groups into three subgroups depending on the pre-ovulatory follicle count. The data showed that in Group 2, the number of oocytes retrieved, oocyte retrieval rate, and number of MII oocytes, were significantly higher in the propofol group compared to the control group; meanwhile, in Group 3, the propofol group had more mature oocytes and high-quality embryos than in the control group. The results indicated that propofol used during IVF-ET is beneficial for patients.

Another important aspect of this study that should be discussed is the effect of propofol on IVF-ET pregnancy outcomes. A non-significant difference in the biochemical pregnancy rate, the clinical pregnancy rate, early spontaneous abortion rate, multiplets pregnancy rate, ectopic pregnancy rate, and live birth was observed in the present study. However, the preterm delivery rate was significantly higher in the propofol group than in the control group, and the preterm delivery rate of twin pregnancy was also significantly higher in the propofol group. Twin pregnancies are at increased risk of preterm delivery and constitute at least 10% of cases of preterm delivery (24). The preterm birth risk was relatively low for women in their late 30s; however, a twin pregnancy is associated with an increased risk for most adverse perinatal outcomes (25). The patients in the propofol group were younger than those in the control group, and they may prefer to have twin pregnancies at one time and select two embryos for transfer; however, the risk of twin pregnancy could not be ignored. The results hint that fresh Single-blastocyst-transfer (SBT) may offer patients efficiency and security, as SBT can prevent high incidences of complications such as multiple pregnancies and ovarian hyperstimulation syndrome (26). On the other hand, some studies suggests that propofol could be associated with an elevated risk of preterm birth, possibly exerting its influence on pregnancy outcomes through several pathways (27). These include its possible effects on fetal organ development and alterations in maternal hemodynamics (28). Consequently, additional studies are imperative to elucidate the precise impact of propofol on preterm birth rates and to uncover the mechanisms at play.

However, it is important to acknowledge the limitations of retrospective studies, such as the risk of research biases, including recall bias and observer bias due to reliance on memory and self-reported data. Additionally, retrospective studies cannot establish

causality, leading to lower internal and external validity. Despite these limitations, this retrospective study can provide valuable preliminary data that can inform the design of larger, more rigorous prospective or randomized trials.

Owing to the constraints imposed by a limited sample size and potential selection bias inherent in a single-center retrospective study, future investigative efforts should prioritize multi-center collaborations to broaden and diversify the dataset. It is also imperative to incorporate regional patient demographics to facilitate a more nuanced and thorough assessment of propofol's safety during the oocyte retrieval process. Furthermore, the long-term safety for offspring must not be overlooked, necessitating additional clinical follow-up studies to thoroughly address this critical aspect.

Conclusion

The findings of this study indicate that the use of propofol during oocyte retrieval significantly enhanced the oocyte retrieval rate among patients with an estimated pre-ovulatory follicle count between 11 and 20. Importantly, propofol did not adversely affect key reproductive outcomes, including fertilization rate, embryo cleavage rate, embryo quality, frozen embryo rate, clinical pregnancy rate, biochemical pregnancy rate, early spontaneous abortion rate, multiplets pregnancy rate, ectopic pregnancy rate, live birth rate, and sex ratio. However, a notable increase in the preterm delivery rate was observed in the propofol group compared to the control group, suggesting that the potential risks associated with twin pregnancies should not be overlooked.

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 humans were approved by the Research Ethical Committee of the Second Affiliated Hospital of Wenzhou Medical University (2022-K-91-02). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

XL: Writing – review & editing, Writing – original draft. FZ: Writing – review & editing. XL: Writing – review & editing, Data curation. HX: Writing – review & editing, Formal analysis. JZ: Writing – original draft, Funding acquisition.

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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.2024.1497948/full#supplementary-material

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Effects of trigger-day progesterone in c-IVF/ICSI cycles on blastocyst culture outcomes

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Objective: To assess whether trigger-day progesterone (P) levels in conventional *in vitro* fertilization (c-IVF)/intracytoplasmic sperm injection (ICSI) cycles are associated with blastocyst culture outcomes.

Methods: In this retrospective analysis, 747 eligible patients (747 cycles) who adopted the gonadotropin-releasing hormone (GnRH) antagonist protocol and underwent c-IVF/ICSI between January 2021 to June 2024 were recruited. The P cutoff values were 1.0 and 1.5 ng/ml when trigger-day serum P was measured, and 4177 day3 (D3) embryos for blastocyst culture were grouped according to trigger-day P levels. Furthermore, the effects of trigger-day P on blastocyst culture outcomes were evaluated.

Results: In total, 747 cycles, 4177 D3 embryos for blastocyst culture were analyzed. After adjustments, multivariate logistic regression analysis revealed that compared with those in the normal level group, available blastocyst rate (adjusted OR, 0.780; 95% CI, 0.645-0.942; P=0.010) and D5 available blastocyst rate (adjusted OR, 0.736; 95% CI, 0.604-0.898; P=0.003) in the high level group were significantly reduced. Subgroup analysis showed that when female age was less than 35 years old, compared with that (36.30%) in the normal level group, the D5 available blastocyst rate (36.92%, adjusted OR, 0.744; 95% CI, 0.602-0.920; P=0.006) in the high level group was significantly reduced. In ICSI cycles, compared with that (28.69%) in the normal level group, the D5 available blastocyst rate (19.13%, adjusted OR, 0.369; 95% CI, 0.194-0.703; P=0.002) in the high level group was significantly decreased.

Conclusion(s): This study demonstrated that in the c-IVF/ICSI population, the trigger-day slightly elevated P (1.0-1.5ng/ml) was not related to blastocyst culture outcomes, while the trigger-day elevated P (>1.5ng/ml) was an important factor affecting D5 available blastocyst rate, especially when the woman was younger than 35 years old or insemination type was ICSI.

KEYWORDS

conventional *in vitro* fertilization (c-IVF), intracytoplasmic sperm injection (ICSI), trigger-day progesterone (P), gonadotropin-releasing hormone (GnRH) antagonist, available blastocyst rate, top-quality blastocyst rate

Introduction

With the maturity of embryo culture technology, many reproductive centers will carry out blastocyst culture for day 3 (D3) available cleavage-stage embryos cultured *in vitro*. By prolonging the embryo culture time, embryos with low developmental potential or genetic defects will be eliminated to a certain extent, and embryos with more developmental potential will be further screened out (1). Blastocyst transfer also improves the endometrial receptivity, increases the chance of implantation, improves the clinical pregnancy rate, and reduces the occurrence of multiple pregnancies, premature births and low birth weight infants (2–5). However, not all patients are suitable for blastocyst culture. Blastocyst culture has a certain risk that some embryos will be eliminated during the culture process, resulting in fewer or no available blastocysts for patients. At present, there is a lack of effective indicators to accurately predict the outcomes of blastocyst culture.

A large number of studies have confirmed that during conventional *in vitro* fertilization (c-IVF)/intracytoplasmic sperm injection (ICSI) controlled ovarian stimulation (COS), trigger-day elevated progesterone (P) not only reduces endometrial receptivity (6–8), advances the implantation window (9), and leads to lower clinical pregnancy rates, ongoing pregnancy rates, and live birth rates (10–21), but also affects embryo quality (6, 19, 22). However, there is still insufficient evidence on whether trigger-day elevated P level will also affect the outcomes of blastocyst formation on day 5 (D5) or day6 (D6) and increase the risk of no available blastocyst. The existing few studies are also partial, including only ICSI/preimplantation genetic testing (PGT) cycles or only top-quality blastocyst formation rate (6). Therefore, the purpose of this study was to evaluate the effects of trigger-day elevated P on available blastocyst rate, top-quality blastocyst rate and blastocyst formation time in c-IVF/ICSI cycles to provide reference for assisted reproductive technology (ART) clinical work.

Materials and methods

Study design and population

This retrospective cohort study included 1095 c-IVF/ICSI cycles of COS with gonadotropin-releasing hormone (GnRH) antagonist protocol from January 2021 to June 2024. The cycle exclusion criteria were as follows: The women over 40 years old; Chromosomal abnormalities in the male or female partners; More than 2 c-IVF/ICSI cycles; Missed relevant data; Low ovarian response. Oocytes or day3 cleavage-stage embryos (D3 embryos) those met any of the following criteria were excluded: Abnormal fertilized or unfertilized oocytes; Unavailable D3 embryos; Frozen or transplanted D3 embryos. Ultimately, **747** cycles (**747** eligible patients), with a total of 4177 D3 embryos for blastocyst culture were included in this study. Owing to the retrospective nature of the study, informed consent was waived. All operations were carried out in conformity with the applicable rules and regulations.

As described in a prior study (23), patients adopted the same protocol, namely, GnRH antagonist protocol. The dosage of gonadotropin (Gn) was individually coordinated according to the basic characteristics and responses of each patient. Continuous transvaginal ultrasound scans and serum estradiol (E_2), luteinizing hormone (LH) and P were used to track the cycles. Triggering was employed with 250 μg recombinant human chorionic gonadotropin (r-hCG, Merck Serono, Geneva, Switzerland) and 3,000 IU u-HCG (Livzon, Guangzhou, China). Thirty-six hours later, oocytes were retrieved under the guidance of transvaginal ultrasound and cultured in incubator for 2 ~ 4 hours prior to fertilization.

Laboratory procedures

Based on the total progressively motile sperm cell count (TPMC) after semen treatment on the day of oocyte retrieval, c-IVF (TPMC≥5 million) or ICSI (TPMC < 5 million) insemination method was selected. Fertilization was observed 20h after c-IVF or 17h after ICSI. The D3 embryos were scored on the third day after oocyte retrieval (24). Grade I, grade II and grade III embryos were defined as available embryos, Grade IV embryos were defined as unavailable embryos (discarded embryos). According to the condition of the patients, the D3 available embryos could be frozen, transferred or continued to culture until D5 or D6. If blastocyst culture was performed, the blastocysts were scored on D5 or D6 after oocyte retrieval based on previous literature (25, 26). The available blastocysts were considered as an expanded, hatching, or hatched blastocyst with ICM/TE grading (AA, AB, BA, BB, AC, CA, BC, CB, and CC) in this study. The top-quality blastocysts were considered as an expanded, hatching, or hatched blastocyst with high ICM/TE grading (AA, AB, BA, and BB).

P assessment

The sex hormones (E_2, LH, P) concentrations were measured from 8:00 am to 9:00 am on the trigger-day by siemens automatic chemiluminescence immunoanalyzer (ADVIA Centaur CP). The detection limit of chemiluminescence immunoassay was 0.03 ng/mL, the sensitivity was 0.15 ng/ml, the coefficient of variation within the group was 3.0%, and the coefficient of variation between the groups was 5.5%. The same detection method was used throughout the study and calibrated regularly to reduce unnecessary errors.

Main outcome measures

The key result of the study was D5 available blastocyst rate, with other indicators being D6 available blastocyst rate and top-quality blastocyst rate. Available blastocyst rate was defined as number of

available blastocysts per cultured, top-quality blastocyst rate was defined as number of top-quality blastocysts per cultured (11).

Statistical analysis

Statistical analysis was performed using SPSS Statistics for Windows version 26. Trigger-day P was regarded as a categorical variable, cycles were divided into the following three groups according to trigger-day P levels: The normal level group included cycles in which trigger-day P were less than 1.0ng/ml; The slightly elevated level group included cycles in which trigger-day P were between 1.0 ~ 1.5 ng/ml; The High level group included cycles in which trigger-day P were more than 1.50 ng/ml. Currently, there was no clear cut-off value for trigger-day P, so these cut-off values were chosen based on clinical practice. Trigger-day elevated P (>1.5 ng/ml) may affect embryo quality (11, 19). Additionally, the physiological P range is generally believed to be less than 1.0ng/ml. And in a retrospective analysis in 2022 (27), Wei et al. also set the threshold of slightly elevated P at 1.0ng/ml. Therefore, 1.0 ng/mL and 1.50 ng/ml were selected as the cut-off values of slightly elevated P level and high P level, respectively. After analysis by Kolmogorov-Smirnov method, all the continuous variables did not conform to normal distribution, which were presented as interquartile interval and were compared between groups using the Kruskal-Wallis test. We used frequencies (percentages) to represent categorical variables and used pearson chi-square test or likelihood ratio test to analyze the differences between groups. Multivariate logistic regression model was established to calculate the adjusted relative risks (RRs) and 95% confidence intervals (CIs). Potential confounders were selected according to routine clinical practice, existing literature reports (28, 29) and variables with significant differences (P<0.05) in the univariate analysis, and adjustments were made for these confounding variables in the analysis of changes in each outcome between groups to explore the relationship between trigger-day P and blastocyst culture outcomes. Adjusted confounding variables included female age, male age, infertility duration, infertility factors, female body mass index (BMI), total antral follicle count (AFC), cycle number, Gn total dosage, trigger-day E_2 level, trigger-day LH level, dominant follicle number, insemination type, D3 embryo numbers for blastocyst culture. All tests were bilateral, and statistical significance was defined as P<0.05.

Results

Patient demographics and general characteristics

A total of 1095 cycles (12209 oocytes) underwent GnRH antagonist protocol for controlled ovarian stimulation and adopted c-IVF/ICSI. Among them, 22 cycles (124 oocytes) with women over 40 years old, 79 cycles (831 oocytes) with male or female chromosomal abnormalities, 14 cycles (100 oocytes) with more than 2 cycles, 8 cycles (81 oocytes) with missed relevant data and 58 cycles (143 oocytes) with low response were excluded. In 10930 oocytes (914 cycles), 3 519 abnormal fertilized or unfertilized oocytes (3 cycles), 861 D3 unavailable embryos (6 cycles), 2373 frozen or transferred D3 embryos (158 cycles) were excluded. Finally, 4177 D3 embryos for blastocyst culture (**747** cycles) were included in the analysis (Figure 1).

Among them, There were 1008 D3 embryos (254 cycles) in normal trigger-day P group (P<1.0 ng/ml), 1411 D3 embryos (257 cycles) in slightly elevated trigger-day P group (1.0≤P ≤ 1.5 ng/ml), and 1758 D3 embryos (236 cycles) in high trigger-day P group (P>1.5 ng/ml) (Figure 1, Table 1). There were no significant differences in the following indicators grouped by trigger-day P: female age, male age, infertility duration, type of infertility, infertility factors, gravidity, parity and miscarriage (P>0.05). Female BMI (24.0 vs. 23.4 vs. 23.2, P=0.019) significantly decreased with increasing trigger-day P, total antral follicle count (AFC, 12 vs. 14 vs. 18) and the proportion of the first cycles (84.65% vs. 93.00 vs. 94.07, P<0.001) significantly increased with increasing trigger-day P (Table 1).

Evaluations of differences among groups

The results indicated that the dominant follicle number and trigger-day E_2 level were significantly increased respectively with increasing trigger-day P (P<0.05). Gn total dosage, trigger-day LH level, insemination type were no significant differences between groups (P>0.05, Table 1).

As shown in Table 1, There were no significant differences in the available blastocyst rate (47.42% vs. 48.05% vs. 49.37%, P=0.572), D5 available blastocyst rate (35.62% vs. 36.29% vs. 36.23%, P=0.933), D6 available blastocyst rate (11.80% vs. 11.76% vs. 13.14%, P=0.420), top-quality blastocyst rate (10.62% vs. 12.33% vs. 11.66%, P=0.430) and D5 top-quality blastocyst rate (10.62% vs. 11.83% vs. 10.98%, P=0.605) among the three groups. D6 top-quality blastocyst rate (0.00 vs. 0.50 vs. 0.68, P=0.036) were significantly increased respectively with increasing trigger-day P.

Association between trigger-day P level and blastocyst culture outcomes

In the multivariate logistic regression model, compared with those in the normal level group, available blastocyst rate (adjusted OR, 0.864; 95% CI, 0.726-1.029; P=0.100), D5 available blastocyst rate (adjusted OR, 0.845; 95% CI, 0.704-1.015; P=0.072), D6 available blastocyst rate (adjusted OR, 1.003; 95% CI, 0.768-1.311; P=0.980), top-quality blastocyst rate (adjusted OR, 1.004; 95% CI, 0.763-1.323; P=0.975), D5 top-quality blastocyst rate (adjusted OR, 0.937; 95% CI, 0.710-1.238; P=0.649) in the slightly elevated level group were no significant change; D6 available blastocyst rate (adjusted OR, 1.064; 95% CI, 0.799-1.417; P=0.670), top-quality blastocyst rate (adjusted OR, 0.859; 95% CI, 0.635-1.161; P=0.323) and D5 top-quality blastocyst rate (adjusted OR, 0.787; 95% CI, 0.579-1.069; P=0.126) in the high level group were also no significant change; available blastocyst rate (adjusted OR, 0.780;

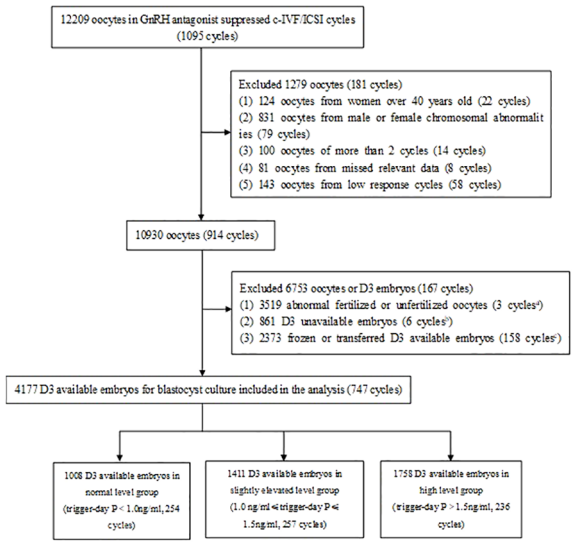

FIGURE 1
Study flowchart. GnRH, gonadotropin-releasing hormone; c-IVF, conventional in vitro fertilization; ICSI, intracytoplasmic sperm injection, D3, day 3; P, progesterone. 3 cycles in which all oocytes were abnormal fertilized or unfertilized were excluded. 6 cycles in which all D3 embryos were unavailable were excluded. 158 cycles in which all D3 available embryos were frozen or transferred were excluded.

95% CI, 0.645-0.942; P=0.010) and D5 available blastocyst rate (adjusted OR, 0.736; 95% CI, 0.604-0.898; P=0.003) in the high level group were significantly reduced (Table 2).

Then, we carried out subgroup analysis of adjusted D5 available blastocyst rate in c-IVF/ICSI cycles based on female age (<35 vs ≥35 years) and insemination type (c-IVF vs ICSI). When female age was less than 35 years old, the D5 available blastocyst rate was 36.69%. Compared with that (36.30%) in the normal level group, the D5 available blastocyst rate (36.67%, adjusted OR, 0.841; 95% CI, 0.693-1.021; P=0.081) in the slightly elevated level group had no significant change, which (36.92%, adjusted OR, 0.744; 95% CI, 0.602-0.920; P=0.006) in the high level group was significantly reduced. When female age was 35 years old or more, the D5 available blastocyst rate was 31.26%, which (33.70%, adjusted OR, 1.019; 95% CI, 0.556-1.867; P=0.951; 28.86%, adjusted OR, 0.772; 95% CI, 0.394-1.514; P=0.452) in the slightly elevated level group and the high level group had no significant change compared with that (30.58%) in the normal level group. In c-IVF cycles, the D5 available blastocyst rate was 38.77%, which (39.32%, adjusted OR, 0.916; 95% CI, 0.749-1.120; P=0.393; 38.81%, adjusted OR, 0.820; 95% CI, 0.662-1.015; P=0.069) in the slightly elevated level group and the high level group had no significant change compared with that (37.83%) in the normal level group. In ICSI cycles, the D5 available blastocyst rate was 22.98%. And compared with that (28.69%) in the normal level group, the D5 available blastocyst

TABLE 1 Patient and blastocyst characteristics in c-IVF/ICSI cycles included.

Characteristics	Trigger-day P level (ng/ml)			*P* value
	Normal level group (<1.0)	Slightly elevated level group (1.0-1.5)	High level group (>1.5)	
No. of cycles	254	257	236	
No. of D3 available embryos for blastocyst culture	1008	1411	1758	
Female age, median (IQR), y	30(28,33)	30(27,33)	29(27,32)	0.062a
Male age, median (IQR), y	31(29,34)	31(29,34)	30(28,34)	0.109a
Infertility duration, median (IQR), y	3(2,5)	3(2,5)	3(2,5)	0.841a
Type of Infertility, n(%)				0.378b
Primary	125(49.21)	134(52.14)	131(55.51)	
Secondary	129(50.79)	123(47.86)	105(44.49)	
Infertility factors, n(%)				0.272b
tubal factor	134(52.76)	157(61.09)	140(59.32)	
Ovulation disorders and low ovarian reserve	74(29.13)	51(19.84)	58(24.58)	
Male factor	25(9.84)	25(9.73)	17(7.20)	
Others	21(8.27)	24(9.34)	21(8.90)	
Gravidity, n(%)				0.405b
0	123(48.43)	126(49.03)	126(53.39)	
1	71(27.95)	70(27.24)	49(20.76)	

(Continued)

TABLE 1 Continued

Characteristics	Trigger-day P level (ng/ml)			*P* value
	Normal level group (<1.0)	Slightly elevated level group (1.0-1.5)	High level group (>1.5)	
2	60(23.62)	61(23.74)	61(25.85)	
Parity, n(%)				0.079c
0	204(80.32)	204(79.38)	194(82.20)	
1	44(17.32)	49(19.07)	42(17.80)	
2	6(2.36)	4(1.55)	0(0.00)	
Miscarriage, n(%)				0.278b
0	183(72.05)	181(70.43)	176(74.58)	
1	46(18.11)	60(23.35)	41(17.37)	
2	25(9.84)	16(6.22)	19(8.05)	
Female BMI, median (IQR), kg/m2	24.0(21.6,27.1)	23.4(21.5,26.0)	23.2(20.8,26.3)	0.019a,d
Total antral follicle count(AFC), median (IQR)	12(9,19)	14(10,20)	18(12,24)	<0.001a, d
Cycle number, n(%)				<0.001b, d
1	215(84.65)	239(93.00)	222(94.07)	
2	39(15.35)	18(7.00)	14(5.93)	
Gn total dose,median (IQR), U	2475(1800,3075)	2400(1725,3000)	2250(1650,3075)	0.277a
Dominant follicle number, median (IQR)	8(6,10)	9(7,13)	11.5(9,15)	<0.001a, d
Trigger-day E2 level, median (IQR), pg/ml	2895(2176,3769)	3697(2890,5188)	5748(4282,8209)	<0.001a, d
Trigger-day LH level, median (IQR), U/L	1.88(1.27,2.76)	1.92(1.09,3.25)	1.72(0.91,2.95)	0.176a
Trigger-day P level, median (IQR), ng/ml	0.75(0.59,0.87)	1.22(1.10,1.36)	1.90(1.69,2.35)	<0.001a, d
Insemination type, n(%)				0.059b
Conventional IVF	186(73.23)	199(77.43)	194(82.20)	
ICSI	68(26.77)	58(22.57)	42(17.80)	
Available blastocyst rate	478/1008(47.42)	678/1411(48.05)	868/1758(49.37)	0.572b
D5	359/1008(35.62)	512/1411(36.29)	637/1758(36.23)	0.933b
D6	119/1008(11.80)	166/1411(11.76)	231/1758(13.14)	0.420b
Top-quality blastocyst rate	107/1008(10.62)	174/1411(12.33)	205/1758(11.66)	0.430b
D5	107/1008(10.62)	167/1411(11.83)	193/1758(10.98)	0.605b
D6	0/1008(0.00)	7/1411(0.50)	12/1758(0.68)	0.036b,d

c-IVF, conventional in vitro fertilization; ICSI, intracytoplasmic sperm injection; D3, day 3; Others, uterine factors or unexplained infertility; BMI, body mass index; AFC, antral follicle count; Gn, gonadotropin; E2, estradiol; LH, luteinizing hormone; P, progesterone; D5, day 5; D6, day 6.
Continuous variables are presented as median and (inter quartiles range).
a Kruskal-Wallis test;
b Pearson Chi-Square test;
c Likelihood-ratio test;
d Statistically significant values.

TABLE 2 Adjusted blastocyst culture outcomes in c-IVF/ICSI cycles stratified by trigger-day P level (ng/ml).

Variables	Adjusted RR (95% CI)	*P* value
Available blastocyst rate		0.036a
Normal level group (<1.0)	Ref	Ref
Slightly elevated level group (1.0-1.5)	0.864(0.726-1.029)	0.100
High level group (>1.5)	0.780(0.645-0.942)	0.010a
D5 available blastocyst rate		0.010a
Normal level group (<1.0)	Ref	Ref
Slightly elevated level group (1.0-1.5)	0.845(0.704-1.015)	0.072
High level group (>1.5)	0.736(0.604-0.898)	0.003a
D6 available blastocyst rate		0.863
Normal level group (<1.0)	Ref	Ref
Slightly elevated level group (1.0-1.5)	1.003(0.768-1.311)	0.980
High level group (>1.5)	1.064(0.799-1.417)	0.670
Top-quality blastocyst rate		0.394
Normal level group (<1.0)	Ref	Ref
Slightly elevated level group (1.0-1.5)	1.004(0.763-1.323)	0.975
High level group (>1.5)	0.859(0.635-1.161)	0.323
D5 top-quality blastocyst rate		0.233
Normal level group (<1.0)	Ref	Ref
Slightly elevated level group (1.0-1.5)	0.937(0.710-1.238)	0.649
High level group (>1.5)	0.787(0.579-1.069)	0.126
D6b top-quality blastocyst rate	/	/

The multivariate regression model was adjusted for female age, male age, infertility duration, infertility factors, female BMI, AFC, cycle number, Gn total dosage, trigger-day E_2 level, trigger-day LH level, dominant follicle number, insemination type, D3 embryo numbers for blastocyst culture.
P, progesterone; RR, relative risk; CI, confidence interval; Ref, reference; BMI: body mass index; AFC, total antral follicle count; Gn, gonadotropin; E_2, estradiol; LH, luteinizing hormone; D3: day 3; D5, day 5; D6, day 6.
a Statistically significant values.
b D6 top-quality blastocyst numbers could not meet the requirement of sample size for multivariate regression analysis, so D6 top-quality blastocyst rate be unadjusted.

rate (20.78%, adjusted OR, 0.675; 95% CI, 0.413-1.105; P=0.118) in the slightly elevated level group had no significant change, which (19.13%, adjusted OR, 0.369; 95% CI, 0.194-0.703; P=0.002) in the high level group was significantly decreased (Table 3).

Discussion

The results of our study showed that the trigger-day slightly elevated P (1.0-1.5ng/ml) was not related to blastocyst culture outcomes, while the trigger-day elevated P (>1.5ng/ml) was an important factor affecting D5 available blastocyst rate, especially when the woman was younger than 35 years old or insemination type was ICSI.

This study showed that there was no statistical difference in the D6 available blastocyst rate between different trigger-day P level groups. And after adjusting for confounders, the increase of trigger-day P did not affect the D6 available blastocyst rate. Although there was no significant difference in the D5 available blastocyst rate among different trigger-day P level groups, after adjusting for confounders, it was shown that the trigger-day elevated P (>1.5ng/ml), not the trigger-day slightly elevated P (1.0-1.5ng/ml) would lead to a decrease in the D5 available blastocyst rate. In the subgroup analysis of female age (< 35 vs ≥35 years) and insemination type (c-IVF vs ICSI), it was concluded that when the trigger-day P was more than 1.5ng/ml, the adjusted D5 available blastocyst rate would decrease significantly in two subgroups of women younger than 35 years and ICSI cycles. To our knowledge, few studies to date had evaluated whether trigger-day P affect available blastocyst rate. Only a retrospective analysis by Racca et al. (19) reported 3400 ICSI cycles with GnRH antagonist protocol for COS. Grouping by trigger-day P values (≤0.50, 0.51-1.49, ≥1.50 ng/ml), and their results showed that the available blastocyst rate on day 5 decreased with the increase of trigger-day P (48.8%, 47.8%, and 38.8%, respectively). However, the P cut-off values and detection method of Racca et al. (19) were different from those in our study, and the study of Racca et al. only included ICSI cycles, only involved D5 available blastocyst rate, did not analyze D6 available blastocyst rate and adjust confounders.

Our data showed that in c-IVF/ICSI cycles, the trigger-day P level was irrelevant to top-quality blastocyst rate. A single-center retrospective cohort study by Turgut et al. (30) in 2020 included 1485 ICSI cycles with GnRH antagonist protocol for COS. They stratified according to serum P levels (< 0.8 ng/ml; 0.8-1.49 ng/ml; ≥1.5 ng/ml). Generalized estimating equations (GEE) analysis also did not show that the effect of P levels on top-quality blastocyst rate had any statistical significance [OR, 1.07; 95%CI, 0.98-1.16; P=0.113; OR, 0.93; 95%CI, 0.80-1.07; P=0.32]. In 2023, Li et al. (11) recruited 504 eligible patients who underwent PGT for retrospective analysis, and grouped according to trigger-day P levels (cut-off values were 0.5 and 1.5 ng/ml, respectively) to assess the effect of trigger-day P on embryo quality. Their results showed that there was no significant difference in top-quality blastocyst rate (8.71% vs. 8.24% vs. 7.94%) among different P levels (P>0.05). These findings are consistent with our conclusion. However, all patients included in Li et al. (11) adopted a gonadotropin-releasing hormone agonist long-acting protocol and underwent PGT cycles (aneuploid blastocysts were excluded). Moreover, all D3 available embryos were cultured to D5 or D6. The P cut-off values of (0.5 and 1.5 ng/ml) were also different from those in our study. Confounding variables (such as female age) were also not adjusted. Although Turgut et al. (30) analyzed various confounding factors and made adjustments through GEE, the inclusion of only ICSI cycles may limit the overall application of their conclusions, and their definition of top-quality blastocyst rate was inconsistent with the present study. However, the above results that there was no correlation between trigger-day P level and top-

TABLE 3 Subgroup analysis of D5 available blastocyst rate in c-IVF/ICSI cycles stratified by trigger-day P level (ng/ml).

Variables	n (%)	*P* value[a]	Adjusted	
			RR (95% CI)	*P* value
Women,s age				
<35y	1367/3726(36.69)	0.954		0.024[b]
Normal level group (<1.0)	322/887(36.30)		Ref	Ref
Slightly elevated level group (1.0-1.5)	451/1230(36.67)		0.841(0.693-1.021)	0.081
High level group (>1.5)	594/1609(36.92)		0.744(0.602-0.920)	0.006[b]
≥35y	141/451(31.26)	0.629		0.653
Normal level group (<1.0)	37/121(30.58)		Ref	Ref
Slightly elevated level group (1.0-1.5)	61/181(33.70)		1.019(0.556-1.867)	0.951
High level group (>1.5)	43/149(28.86)		0.772(0.394-1.514)	0.452
Insemination type				
Conventional IVF	1346/3472(38.77)	0.803		0.167
Normal level group (<1.0)	289/764(37.83)		Ref	Ref
Slightly elevated level group (1.0-1.5)	464/1180(39.32)		0.916(0.749-1.120)	0.393
High level group (>1.5)	593/1528(38.81)		0.820(0.662-1.015)	0.069
ICSI	162/705(22.98)	0.029[b]		0.010[b]
Normal level group (<1.0)	70/244(28.69)		Ref	Ref
Slightly elevated level group (1.0-1.5)	48/231(20.78)		0.675(0.413-1.105)	0.118
High level group (>1.5)	44/230(19.13)		0.369(0.194-0.703)	0.002[b]

The multivariate regression model was adjusted for female age, male age, infertility duration, infertility factors, female BMI, AFC, cycle number, Gn total dosage, trigger-day E_2 level, trigger-day LH level, dominant follicle number, insemination type, D3 embryo numbers for blastocyst culture.
P, progesterone; RR, relative risk; CI, confidence interval; Ref, reference; BMI: body mass index; AFC, total antral follicle count; Gn, gonadotropin; E_2, estradiol; LH, luteinizing hormone; D3: day3; D5, day 5.
[a]Pearson Chi-Square test;
[b]Statistically significant values.

quality blastocyst rate contradicted the studies of Kim et al. (6). In 2017, Kim et al. (6) found for the first time that the increase of trigger-day P in GnRH antagonist IVF/ICSI cycles was associated with a lower top-quality blastocyst rate. The reasons for the inconsistency between the findings of Kim et al. (6) and the conclusions of our study may be as follows: First, no embryos were transferred or frozen on D3 in the study of Kim et al. (6), all D3 available embryos were cultured to D5 or D6; Second, their definition of top quality blastocyst was also different from ours; Moreover, they statistically analyzed the top-quality blastocyst rate as a continuous variable, but, statistically, frequencies (such as top-quality blastocyst rate) should be described by categorical variables.

Animal studies had shown that lower P levels during the follicular phase can promote oocyte development *in vitro* (31). We conjecture that the high trigger-day P level delays embryo development or reduces embryo development potential by affecting oocyte quality, and large activation of the zygotic genome after the D3 embryonic phase cannot counteract the adverse effect of high trigger-day P level on embryo quality, leading to a decrease in D5 available blastocyst rate. However, the molecular mechanism by which high trigger-day P level affect blastocyst quality remains unclear. In addition, the underlying mechanism that causes trigger-day higher P level during ART is also unknown. Studies had shown that the number of follicles, the FSH drive and the LH activity were the three main factors that lead to increased P concentration during COS (32). According to the present study and earlier studies (13, 15, 20), trigger-day high P is usually accompanied by high E_2. We also noticed that with the increase of trigger-day P, the female BMI gradually decreased, and the number of AFC and dominant follicles gradually increased.

All patients in this single-center study received a uniform ovarian stimulation protocol. Morphological assessment of blastocyst was performed by an experienced embryologist under equivalent laboratory conditions. Multiple regression logic analysis model was also used to explain the influence of various confounding factors on blastocyst culture outcomes, and subgroup analysis was performed according to female age and insemination type to ensure the reliability of the results.

However, due to the limitations of retrospective study, there were inevitable biases even if the impact of potential confounders was minimized. First, we used c-IVF/ICSI as a model, and the results cannot be extrapolated to all infertile populations. Second,

trigger-day P does not have a definite cut-off value, which varies between studies at different centers and may vary depending on the detection method.

In summary, we used the c-IVF/ICSI cycles as a model to determine the relationship between trigger-day P and blastocyst culture outcomes. The results showed that the trigger-day slightly elevated P (1.0-1.5ng/ml) was not related to blastocyst culture outcomes, while the trigger-day elevated P (>1.5ng/ml) was an important factor affecting D5 available blastocyst rate, especially when the woman was younger than 35 years old or insemination type was ICSI. Because this study included c-IVF/ICSI patients treated with GnRH antagonist, it was unclear whether our findings can be extrapolated to populations using other protocols or to all infertility population. On the other hand, due to the small sample size and retrospective analysis in this study, the existence of bias cannot be ruled out, so it is recommended to carry out a randomized controlled trial with a larger sample size. In conclusion, in clinical practice, we should treat this result with caution. In order to predict the blastocyst culture outcomes, each center needs to evaluate the P threshold according to its own detection method when selecting D3 embryos for blastocyst culture.

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 humans were approved by Ethics Committee of Yuncheng Central Hospital, Shanxi Province. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

YS: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. JW: Data curation, Investigation, Writing – original draft. LZ: Data curation, Investigation, Writing – original draft. YC: Data curation, Investigation, Writing – original draft. AZ: Conceptualization, Formal analysis, Writing – review & editing, Resources.

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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.

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The transfer of double morphologically good Day 5 blastocysts increases the risk of clinical pregnancy loss in singleton pregnancies following frozen-thawed embryo transfer

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Background: To investigate whether double embryo transfer (DET) increases the risk of spontaneous clinical pregnancy loss (CPL) in singleton pregnancies following frozen-thawed embryo transfer (FET), compared to single embryo transfer (SET).

Methods: This retrospective cohort study included 2,448 females with singleton pregnancies (excluding vanishing twin cases) resulting from frozen-thawed single or double embryo transfers between January 2017 and September 2022. The CPL rate was the sole outcome measure. We compared CPL rates between SET and DET across three populations with increasing embryo developmental potential using binary logistic regression analysis: P1, comprising transfers of Day 3 cleavage-stage embryos; P2, comprising transfers of blastocysts; and P3, comprising transfers of top-quality blastocysts, defined as morphologically good Day 5 blastocysts.

Results: After adjusting for confounding factors, the comparison between SET and DET revealed the following findings: in P1, DET had a slightly higher CPL rate compared to SET [OR (95% CI): 1.18 (0.74-1.90), p=0.46]; In P2, DET showed a moderately higher CPL rate [OR (95% CI): 1.34 (0.96-1.87), p=0.08]; In P3, DET had a significantly higher CPL rate [OR (95% CI): 1.55 (1.02-2.37), p=0.04]. A combined analysis indicated that as the developmental potential of the transferred embryo increased (from P1 to P2 and further to P3), the impact of DET on CPL also increased, as reflected by the rising OR values and decreasing p-values. We proposed that in singleton pregnancies resulting from DET, the loss of a non-viable embryo at a later stage, when it has a larger cell mass, may trigger excessive intrauterine inflammation, thereby increasing the risk of CPL for the remaining full developmental potential embryo. In singleton pregnancies resulting from DET, a higher-quality embryo that fails is more likely to die at a later stage. This could explain why the impact of DET on CPL increases with the developmental potential of the embryo.

Conclusion: Since a significant difference in CPL between SET and DET was only observed in P3 population. Therefore, we concluded that compared to SET, the transfer of double morphologically good Day 5 blastocysts is associated with increased clinical pregnancy loss in singleton pregnancies following FET.

KEYWORDS

single embryo transfer, double embryo transfer, clinical pregnancy loss, intrauterine inflammation, developmental defect

Introduction

The number of embryos transferred is considered as a crucial factor influencing the clinical outcomes of *in vitro* fertilization-embryo transfer (IVF-ET). An increase in the number of embryos transferred has been found to significantly enhance the clinical pregnancy rate but also substantially increase the incidence of multiple pregnancies (1–4). Undoubtedly, multiple pregnancies significantly increase the risk of adverse gestational and perinatal outcomes, as well as short-term and long-term health complications for both mothers and babies (5–7). In natural pregnancies, the rate of multiple pregnancies is around 1%. By contrast, with assisted reproductive technology (ART) in China, the rate of multiple pregnancies exceeds 30% (8). To mitigate the high rate of multiple pregnancies, it is recommended that no more than two embryos be transferred per cycle in China. Consequently, most reproductive centers in China routinely transfer either one or two embryos per cycle.

Previous studies have reported a higher rate of adverse gestational and perinatal outcomes in singletons conceived by ART compared to those conceived spontaneously (9–12). The outcomes also differ between single embryo transfer (SET) and double embryo transfer (DET). Previous studies have indicated a higher risk of adverse outcomes in singletons from DET compared to SET, including the risk of neonatal death, low birthweight in frozen embryo transfer cycles, and very preterm birth and low birthweight in blastocyst transfer cycles (13–15). Theoretically, a singleton pregnancy from DET differs from a singleton pregnancy from SET. In a singleton pregnancy from DET, the surviving embryo could potentially be affected by the death of another embryo. It has been reported that embryonic apoptosis induces maternal sterile purulent inflammation, leading to the resorption of the dead embryo (16). It is possible that intrauterine inflammation resulting from the death of one embryo may adversely affect the surviving embryo, potentially leading to increased rates of adverse gestational and perinatal outcomes.

Here, we investigated whether singleton pregnancies from DET have a higher likelihood of spontaneous clinical pregnancy loss (CPL) compared to SET. A previous study has suggested a higher rate of missed abortion in patients with singleton pregnancies conceived after multiple embryo transfers (17). Nonetheless, the small sample size (only 195 singleton pregnancies) limits the conclusiveness of this finding. In the present study, we retrospectively analyzed frozen-thawed embryo transfer (FET) cycles with singleton pregnancies at our reproductive center from January 2017 to December 2022.

Materials and methods

Study design

This was a retrospective study. This study collected data from couples with confirmed singleton pregnancies via FET at our reproductive center from January 2017 to September 2022. This study was approved by the ethics committee of Changzhou Maternal and Child Health Care Hospital. Given the difference in embryo stage, the transfer cycles were initially divided into two groups: P1(Day 3 cleavage embryos) and P2 (blastocysts). Subsequently, each group was further subdivided based on the number of embryos transferred into SET and DET groups. In addition, data from SET and DET with top blastocyst (morphologically good Day 5 blastocysts, P3 group) were extracted for analysis. Baseline characteristics and the rate of CPL between SET and DET were compared in P1 and P2, respectively. Binary logistic regression analysis was used to evaluate the effect of DET on the occurrence of CPL in P1, P2 and P3, respectively. Further details of the study design were described in Figure 1.

Inclusion/exclusion criteria

FET cycles with a confirmation of singleton pregnancies (one heartbeat confirmed by the first ultrasonography) were included;

Females with a history of previous spontaneous abortion or uterine malformation were excluded;

Individuals with chromosomal disorders or a history of cancer were excluded;

Surgical sperm extraction and rescue-ICSI were excluded;

FET cycles resulting in ectopic pregnancies or induced abortions were excluded;

Cases lost to follow-up were excluded.

Embryo culture procedures

Females underwent controlled stimulation protocols to promote follicle growth. Oocytes were collected approximately 36 hours after the trigger under ultrasound guidance, and fertilization was performed with either conventional IVF or the ICSI method. Zygotes were evaluated on day 1, and embryos were morphologically scored on Day 3, 5, or 6. Day 3 cleavage embryos were frozen on Day 3, while blastocysts were frozen on Day 5 or 6.

Morphological score of embryos

The morphological scoring for Day 3 cleavage embryos and blastocysts was performed as previously described (18). Briefly, Day 3 embryos were classified into four grades based on their morphological appearance. Grades I and II were considered good embryos, grade III as non-good embryo, and grade IV embryos were discarded. For blastocysts, a morphologically good blastocyst was defined as having a blastocyst expansion grade over 3, with an A or B score for both the inner cell mass (ICM) and trophectoderm (TE). A morphologically non-good blastocyst was defined as having a blastocyst expansion grade over 3, a C score for either the ICM or TE, and an A or B score for the corresponding opposite component (TE or ICM).

Definitions

A singleton pregnancy is defined as a pregnancy where only one fetus is present, confirmed by the detection of a single heartbeat on the first ultrasonography.

Top blastocysts refer to morphologically good blastocysts that were formed on Day 5, characterized by a high degree of blastocyst expansion (over 3) and an A or B score for both the ICM and TE.

Clinical pregnancy loss refers to the spontaneous loss of a clinical pregnancy.

Embryo culture and transfer strategy

In late 2016, our reproductive center implemented blastocyst culture. To minimize the risk of having no embryos available for transfer, it's a common practice not to perform blastocyst culture for couples with a very limited number of Day 3 cleavage embryos (<=4). In the case of a moderate number of Day 3 cleavage embryos (>4 and <=7), the two to four best Day 3 embryos are frozen, while the remaining embryos undergo further culture. When the number of Day 3 cleavage embryos is =>7 and the number of Grade I and II embryos is =>4, all embryos are subjected to further culture. For embryo transfer priority, the order is as follows: morphologically good Day 5 blastocysts have the highest priority, followed by morphologically good blastocysts, then good Day 3 embryos, and finally non-optimal blastocysts.

Statistical analysis

Data were analyzed using SPSS software (Version 21, IBM). Continuous data were first examined by the Normality and Lognormality test. Data that were not normally distributed were compared using the Mann–Whitney U test. The constituent data were compared using the chi-square test. Binary logistic regression analysis was used to evaluate the impact of groups (DET vs. SET) on the occurrence of CPL, while adjusting for potential confounding factors including female age and BMI, semen DFI, number of oocytes retrieved, type of infertility, infertility duration, infertility cause, previous transfer cycle, endometrium preparation, and embryo quality. $p< 0.05$ was considered statistically significant.

Results

Characteristics of patients, ovarian stimulation, embryo transfer, and the occurrence of clinical pregnancy loss

In Day 3 cleavage embryo transfer cycles (P1), both the SET and DET groups displayed numerous similar baseline characteristics, including infertility duration, primary diagnoses, as well as BMI of both females and males, females and males with age over 35 years, semen DFI, males with semen DFI over 30, previously failed transfer cycles, ovarian stimulation protocols, fertilization methods, endometrial preparation protocols, endometrial thickness, and the proportion of transferred grade III embryos (Table 1). However, compared to the SET group, the DET group had a higher proportion of patients with primary infertility, younger ages for both females and males at oocyte retrieval or embryo transfer, a higher proportion of patients with at least one previous IVF cycle, a higher number of oocytes retrieved, and a higher proportion of transfer cycles with at least one grade III embryo (Table 1). The CPL rate was similar between the SET and DET groups (Table 1).

In blastocyst transfer cycles (P2), both the SET and DET groups displayed numerous similar baseline characteristics, including primary infertility, infertility duration, primary diagnoses, females and males with age over 35 years, BMI of both males and females, males with semen DFI over 30, previous IVF cycles, ovarian stimulation protocols, oocytes retrieved, fertilization method, and endometrial thickness (Table 1). However, compared to the SET group, the DET group had younger ages for both females and males at oocyte retrieval or embryo transfer, less females with BMI over 30, lower semen DFI, more patients with at least a previous failed transfer cycles, a higher percentage of endometrial preparation with ovarian stimulation protocol, fewer Day 5 or morphologically good blastocysts transferred, and more transfer cycles with at least one day 6 embryo or one morphologically non-good blastocyst (Table 1). The CPL rate was higher in the DET group than in the SET group, although the difference was not significant (Table 1).

The effect of DET on the occurrence of CPL among groups

Here, we evaluated the effect of DET on the occurrence of CPL in three distinct groups, P1, P2, and P3. The developmental potential of embryos in these three groups gradually increased from P1 (Day 3 cleavage embryos) to P2 (blastocysts) and to P3 (top blastocysts).Variables including the number of oocytes retrieved, female age (at oocyte retrieval), infertility duration, infertility cause, type of infertility, female BMI, semen DFI, previous transfer cycle, protocols of endometrial preparation, and embryo quality were considered as confounding factors. After adjusting for confounding factors, compared to SET, DET showed a slightly higher rate of CPL in P1[OR (95% CI): 1.18 (0.74-1.90), p=0.46] (Table 2); a moderate higher rate of CPL in P2 [1.34 (0.96-1.87), p=0.08] (Table 3); a significant higher rate of CPL in P3 [1.55 (1.02-2.37), p=0.04] (Table 4).

Proposed mechanism

A combined analysis showed that DET progressively increases the risk of CPL as the developmental potential of the embryos rises (from P1 to P2, and further to P3) (Figure 2). We proposed that in singleton pregnancies following DET, the loss of one embryo at a later developmental stage (potentially after biochemical pregnancy) may trigger excessive intrauterine inflammation, leading to the loss of the clinically established pregnancy of the remaining full potential embryo (Figure 3). In singleton pregnancies after DET, as the developmental potential of the transferred embryos increases, the likelihood of later-stage embryo loss also rises, which explains this observation. Due to being unaffected by the loss of another embryo, an embryo with full developmental potential may result in a live birth using the SET strategy (Figure 3).

Discussion

In the present study, we investigated whether DET increases the risk of CPL in singleton pregnancies, compared to SET. Previous studies have shown adverse outcomes in singleton births following DET compared to SET, suggesting that the death of one embryo indeed influences the remaining surviving embryo with full potential (13, 19, 20). In the present study, we found that as the potential of transferred embryos increased (from Day 3 cleavage embryos to blastocysts to top blastocysts), the effect of DET on the occurrence of CPL became progressively more evident in singleton pregnancies. More importantly, we found that transferring two morphologially good Day 5 blastocyst significantly increases the CPL rate in singleton pregnancies following FET, compared to the transfer of single morphologially good Day 5 blastocyst.

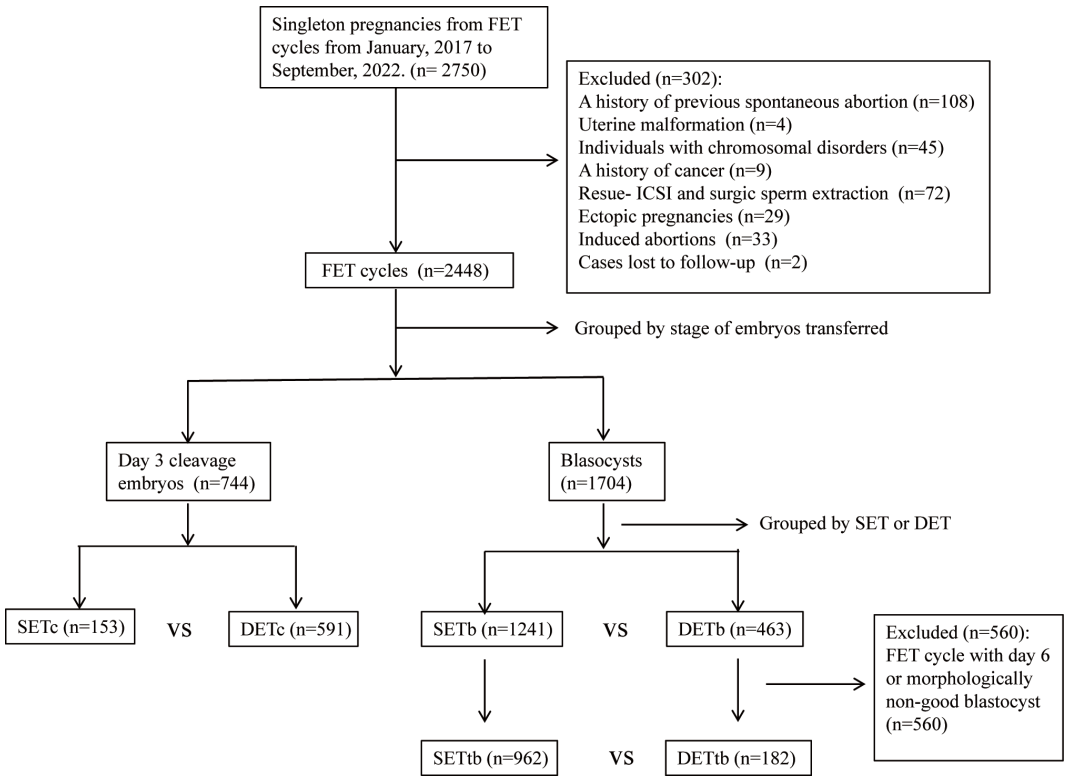

FIGURE 1
Study flow diagram. SET, single embryo transfer; DET, double embryo transfer; SETc, SET with Day 3 cleavage embryo; DETc, DET with Day 3 cleavage embryo; SETb, SET with blastocyst; DETb, DET with blastocyst; SETtb, SET with top blastocyst; DETtb, DET with top blastocyst.

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TABLE 1 Description of cohort.

Characteristics	Day 3 cleavage embryo			Blastocyst		
	SETc	DETc	*p*	SETb	DETb	*p*
FET cycles	153	591		1241	463	
Primary infertility (%)	68 (44.4)	357 (60.4)	0	701 (56.5)	259 (55.9)	0.84
Infertility duration	3 [1.5, 5]	3 [2,4]	0.77	3 [2, 4]	3 [2, 4]	0.55
Primary diagnosis (%)						
Tubal factor	59 (38.6)	263 (44.5)		620 (50.0)	229 (49.5)	
DOR	38 (24.8)	103 (17.4)	0.23	40 (3.2)	13 (2.8)	0.95
Ovulatory dysfunction	13 (8.5)	39 (6.6)		178 (14.3)	73 (15.8)	
Male factor	18 (11.8)	79 (13.4)		171 (13.8)	64 (13.8)	
Others	25 (16.3)	107 (18.1)		232 (18.7)	84 (18.1)	
Female Age						
at oocyte retrieval	32 [29, 35]	31 [28, 35]	0.04	30 [28, 33]	29 [27, 32]	0.01
>35 years old	36 (23.5)	127 (21.5)	0.58	133 (10.7)	46 (9.9)	0.64
at embryo transfer	33 [30, 36]	31 [29, 35]	0.04	31 [28, 33]	30 [28,33]	0.01
>35years old	41 (26.8)	137 (23.2)	0.35	154 (12.4)	52 (11.2)	0.51
Male age						
at oocyte retrieval	33 [29, 37]	31 [29,35]	0.03	31 [29, 34]	30 [28,33]	0
at embryo transfer	33 [30, 37]	32 [29, 36]	0.02	32 [29, 35]	31 [29,34]	0
BMI						
Female	22.3 [20.13, 25.3]	22 [20, 24.4]	0.39	22 [19.9, 25.0]	21.8[19.9, 24.8]	0.47
>30	12 (7.8)	20 (3.4)	0.02	65 (5.2)	13 (2.8)	0.03
Male	24.6 [22.6,27.7]	24.4 [22.4,27]	0.21	24.6 [22.4, 27.1]	24.3 [22.3, 27.2]	0.36
Semen DFI	14.0 [8.73,21.84]	12.7 [8.27, 20.19]	0.33	12.8 [8.2, 19.1]	11.1 [7.1,17.7]	0.01
>30	9 (5.88)	27 (4.57)	0.5	79 (6.4)	25 (5.4)	0.46
Previous IVF cycles (%)						
0	119 (77.78)	443 (74.96)		1134 (91.4)	420 (90.7)	
1	11 (7.19)	100 (16.92)	0.01	92 (7.4)	38 (8.2)	0.84
=>2	23 (15.03)	48 (8.12)		15 (1.2)	5 (1.1)	
Previous transfer cycles (%)						
0	102 (66.7)	389 (65.8)		937 (75.5)	302 (65.2)	
1	36 (23.5)	141 (23.9)	0.99	222 (17.9)	112 (24.2)	0
=>2	15 (9.8)	56 (10.3)		82 (6.6)	49 (10.6)	
GnRH analogues						
Agonist	56 (63.6)	264 (44.7)		906 (73.0)	363 (78.4)	
Antagonist	26 (17.0)	106 (17.9)	0.11	274 (22.1)	83 (17.9)	0.07
No analogues	71 (46.4)	221 (37.4)		61 (4.9)	17 (3.7)	
Oocyte retrieved	5 [3, 10]	7 [5, 11]	0	13 [10, 17]	13 [11-17]	0.11

(Continued)

TABLE 1 Continued

Characteristics	Day 3 cleavage embryo			Blastocyst		
	SETc	DETc	*p*	SETb	DETb	*p*
Fertilization methods						
IVF	120 (78.4)	473 (80.0)	0.66	1060 (85.4)	411 (88.8)	0.07
ICSI	33 (21.6)	118 (20.0)	0.66	181 (14.6)	52 (11.2)	0.07
Endometrium preparation						
Artificial cycle	114 (74.5)	436 (73.8)		997 (80.3)	342 (73.9)	
Ovarian stimulation cycle	29 (19.0)	122 (20.6)	0.83	208 (16.8)	107 (23.1)	0.01
Modified natural cycle	10 (6.5)	33 (5.6)		36 (2.9)	14 (3.0)	
Thickness of endometrium	9.75 [8.63, 11.0]	9.3 [8.1, 10.7]	0.12	9 [8, 10]	9 [8, 10]	0.83
Embryo quality (%)						
I/II	144 (94.1)	1061 (89.8)	0.09	/	/	
Cycle with at least one grade III embryo	9 (5.9)	112 (19.0)	0	/	/	
Morphologically good blastocyst	/	/		1204 (97.0)	753 (81.3)	0
Cycle with at least one morphologically non-good blastocyst	/	/		37 (3.0)	148 (32.0)	0
Day 5 blastocyst	/	/		936 (75.4)	508 (54.9)	0
Cycles with at least one day 6 blastocyst	/	/		268 (21.6)	252 (54.4)	0
CPL (%)	30 (19.1)	127 (21.5)	0.61	200 (16.1)	93 (20.1)	0.05

FET, frozen-thawed embryo transfer; declined ovarian reserve; DFI, DNA fragmentation index; CPL, clinical pregnancy loss.
Data are presented as the median [the first quartile, the third quartile] or count (percentage).

For Day 3 cleavage embryo transfer, it was common practice to transfer two embryos (if available). Owing to the higher developmental potential of blastocyst, SET was more commonly used for blastocyst transfer, particularly for morphologically good Day 5 blastocysts. Consequently, in the Day 3 cleavage embryo transfer group, most singleton pregnancies were achieved thorough DET, while in the blastocyst transfer group, most singleton pregnancies were achieved through SET. Our embryo culture and transfer strategy, as outlined in the Methods section, may contribute to the older age and lower ovarian reserve in couples receiving SET in the present study. For DET, transferring one good embryo and one non-good embryo is more likely to result in a singleton pregnancy, compared to transferring two good or two non-good embryos. The morphological score of the blastocyst also determines whether one or two embryos are transferred. Therefore, the embryo quality in the DET group was lower compared to SET in the present study. Additionally, a failed transfer cycle with a good embryo often leads to the decision to transfer two embryos in the subsequent cycle. Consequently, the DET group had more couples with at least one failed transfer cycle, particularly in blastocyst transfer. The heterogeneity between the SET and DET populations could potentially introduce bias into the entire study. However, variables such as age, embryo quality, infertility duration and previous transfer cycles were considered as confounding factors in the analysis, which help to mitigate potential bias.

In natural conception, the zygote develops into a Day 3 cleavage embryo and progresses further into a blastocyst within the oviduct (21–23). Then, the blastocyst moves into the uterine cavity, hatches from the zona pellucida, and initiates the process of implantation (21). The interaction between the blastocyst and the endometrium, along with the rapid growth of the blastocyst, stimulates the secretion of β-HCG, leading to a biochemical pregnancy (24, 25). Following this, the embryo continues to grow, developing a fetalheart, which leads to a clinical pregnancy (26). In ART, the processes that occur in the oviduct are replicated *in vitro*, within a laboratory dish (27). In humans, embryonic loss is common and can occur at any stage of development, including before or at the blastocyst stage, after a biochemical pregnancy (biochemical pregnancy loss), or after the establishment of clinical pregnancy (clinical pregnancy loss) (28–30).

It has been reported that the embryonic apoptosis can directly trigger maternal sterile purulent inflammation, leading to the resorption of the dead embryo (16). It is well established that some degree of systemic or uterine inflammation is necessary both for normal implantation and pregnancy while excessive inflammation can increase the risk of miscarriage (31). Evidence from ART indicates that higher levels of serum C-reactive protein, which reflects inflammation, are associated with pregnancy loss (32). A recent prospective study showed a significantly higher of pro-inflammatory cytokines at day 16 following embryo transfer

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TABLE 2 The effect of DET on the occurrence of CPL for Day 3 cleavage embryo transfer.

	OR (95% CL)	*p*
Occytes retrieved (n=744)	1.00 (0.95-1.05)	0.97
Female age (n=744)	1.12 (1.07-1.18)	0.00
Type of infertility		
Primary infertility (n=429)	1 (ref)	
Secondary infertility (n=315)	0.84 (0.56-1.23)	0.39
Infertility duration (n=744)	0.96 (0.89-1.04)	0.31
Infertility causes		
Male factor (n=97)	1 (ref)	
Ovarian function decline (n=141)	0.76 (0.38-1.53)	0.45
*Others (n=506)	0.62 (0.36-1.08)	0.09
BMI		
<=30 (n=712)	1 (ref)	
>30 (n=32)	0.64 (0.26-1.54)	0.49
DFI		
<=30 (n=708)	1 (ref)	
>30 (n=36)	0.50 (0.19-1.35)	0.17
Previous transfer cycle		
0 (n=497)	1 (ref)	
1 (n=177)	1.17 (0.75-1.83)	0.50
>1 (n=70)	1.11 (0.59-2.10)	0.74
Endometrium preparation		
Ovarian stimulation cycle (n=148)	1 (ref)	
Artificial cycle (n=552)	0.76 (0.46-1.24)	0.27
Modified natural cycle (n=44)	0.96 (0.44-2.11)	0.92
Embryo quality		
Good embryos (n=624)	1 (ref)	
Containing at least one grade III embryo (n=120)	1.13 (0.68-1.86)	0.64
Group		
SET (n=153)	1 (ref)	
DET (n=591)	1.18 (0.74-1.90)	0.46

Data are presented as the OR (95% CL).*Others includes tubal factor, ovulatory dysfunction and other factor. DFI, DNA fragmentation index; BMI, body mass index; SET, single embryo transfer; DET, double embryo transfer.

(during biochemical pregnancy, but before clinical pregnancy) in women who experienced subsequent miscarriage (33), indicating that an excess of pro-inflammatory factors during implantation increases the risk of CPL. Based on these studies, it is reasonable to assume that in singleton pregnancies following DET, the death of an

TABLE 3 The effect of DET on the occurrence of CPL for blastocyst transfer.

	OR (95% CL)	*p*
Occytes retrieved (n=1704)	0.99 (0.96-1.01)	0.36
Female age (n=1704)	1.06(1.00-1.10)	0.00
Type of infertility		
Primary infertility (n=960)	1 (ref)	
Secondary infertility (n=744)	1.00 (0.76-1.32)	0.99
Infertility duration (n=1704)	1.00 (0.94-1.07)	0.93
Infertility causes		
Male factor (235)	1 (ref)	
Declined ovarian reserve (53)	1.06 (0.50-2.27)	0.88
*Others (1416)	1.02 (0.69-1.51)	0.92
BMI		
<=30 (n=1626)	1 (ref)	
>30 (n=78)	1.56 (0.90-2.70)	0.12
DFI		
<=30 (n=1600)	1 (ref)	
>30 (n=104)	1.03 (0.60-1.79)	0.91
Previous transfer cycle		
0 (n=1239)	1 (ref)	
1 (n=334)	0.74 (0.51-1.06)	0.10
>1 (n=131)	1.45 (0.93-2.25)	0.10
Endometrium preparation		
Ovarian stimulation cycle (n=315)	1 (ref)	
Artificial cycle (n=1339)	1.27 (0.88-1.82)	0.20
Modified natural cycle (n=50)	0.98 (0.42-2.26)	0.96
Embryo morphology		
Good (n=1519)	1 (ref)	
One non-good embryo (n=122)	1.23 (0.72-2.11)	0.44
Non-good (n=63)	1.97 (1.13-3.50)	0.02
Days of embryo		
Day 5 (n=1183)	1 (ref)	
Day 5+ day 6 (n=86)	0.55 (0.27-1.13)	0.10
Day 6 (n=435)	1.39 (1.03-1.89)	0.03
Group		
SET (n=1241)	1 (ref)	
DET (n=463)	1.34 (0.96-1.87)	0.08

Data are presented as the OR (95% CL).*Others includes tubal factor, ovulatory dysfunction and other factor. DFI, DNA fragmentation index; BMI,: body mass index; SET, single embryo transfer; DET, double embryo transfer.

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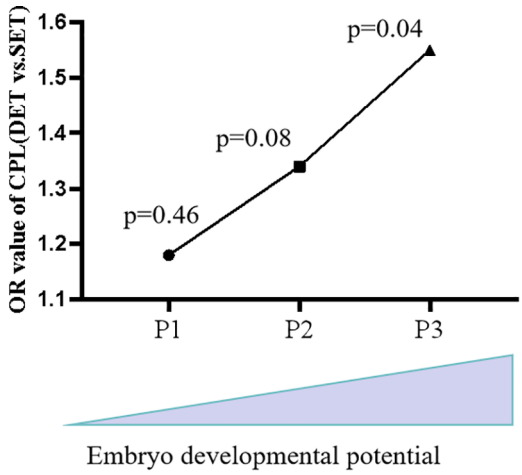
TABLE 4 The effect of DET on the occurrence of CPL for top blastocyst transfer.

	OR (95% CL)	p
Occytes retrieved (n=1144)	0.98 (0.95-1.01)	0.15
Female age (n=1144)	1.05 (1.00-1.11)	0.04
Type of infertility		
Primary infertility (n=635)	1 (ref)	
Secondary infertility (n=509)	1.09 (0.76-1.55)	0.65
Infertility duration (n=1144)	1.01 (0.93-1.09)	0.85
Infertility causes		
Male factor (150)	1 (ref)	
Declined ovarian reserve (34)	1.04 (0.93-2.80)	0.94
*Others (960)	0.85 (0.52-1.40)	0.53
BMI		
<=30 (n=1091)	1 (ref)	
>30 (n=53)	0.91 (0.40-2.08)	0.83
DFI		
<=30 (n=1078)	1 (ref)	
>30 (n=66)	1.36 (0.70-2.67)	0.37
Previous transfer cycle		
0 (n=907)	1 (ref)	
1 (n=182)	0.65 (0.39-1.10)	0.11
>1 (n=55)	1.11 (0.52-2.34)	0.80
Endometrium preparation		
Ovarian stimulation cycle (n=203)	1 (ref)	
Artificial cycle (n=901)	1.64 (0.99-2.72)	0.05
Modified natural cycle (n=30)	0.79 (0.22-2.85)	0.71
Group		
SET (n=962)	1 (ref)	
DET (n=182)	1.55 (1.02-2.37)	0.04

Data are presented as the OR (95% CL).*Others includes tubal factor, ovulatory dysfunction and other factor. DFI, DNA fragmentation index; BMI, body mass index; SET, single embryo transfer; DET, double embryo transfer.



P1: Transfer of Day 3 cleavage embryos
P2: Transfer of blastocysts
P3: Transfer of top blastocysts (morphologically good Day 5 blastocysts)

FIGURE 2
Combined analysis of DET on the occurrence of CPL across groups. As the potential of transferred embryos increases (from Day 3 cleavage embryos to blastocysts and further to top blastocysts), the impact of DET on the occurrence of CPL became progressively evident. CPL, Clinical pregnancy loss; SET, single embryo transfer; DET, double embryo transfer.

embryo at a later developmental stage, when it has a larger cell mass, may trigger an excess inflammatory response, significantly increasing the risk of loss of the remaining full potential embryo. It is well established that embryo developmental potential is tightly associated with clinical outcomes. There should be an order of embryo developmental potential, live birth embryos > clinical pregnancy embryos > biochemical pregnancy embryos > blastocysts > Day3 cleavage embryos. In the setting of singleton pregnancies following DET, the likelihood of the decreased embryo dying at later stage should follow this order, morphologically good Day 5 embryos > blastocysts > Day3 cleavage embryos. This well explain the observation in the present study that, as the developmental potential of transferred embryos increases, the effect of DET on the occurrence of CPL becomes more pronounced.

Our findings are supported by several studies. A recent study analyzing data from 4232 women showed that DET had a lower cumulative live birth rate compared to SET [OR (95% CL): 0.76 (0.53–1.07)] (34). Another study, including data from 49,333 patients, demonstrated that iSET is associated with a significantly higher cumulative livebirth rate [OR (95% CL): 1.32 (1.26 –1.38)], compared to iDET (35). Clua et al. analyzing data from 1139 oocyte donation cycles, revealed that the cumulative pregnancy and livebirth rates of SET vs. DET is 82.8% vs. 77.2% and 76.4% vs. 63.7% respectively (36). This indicates a higher rate of CPL in the DET group (SET vs DET: 6.4% vs. 12.7%). A more recent study has shown that DET increases the rate of CPL for females receiving euploid frozen blastocyst transfer compared to SET (37). More importantly, the results from these studies can be well explained by the present study, which suggests that the DET strategy may result in the loss of full-potential embryos in singleton pregnancies.

The findings of this study are clinically significant. While DET significantly increases the risk of twin pregnancies, it remains prevalent in reproductive centers. One key reason for this is that many patients consider twin pregnancies acceptable. However, this study revealed that transferring two morphologically good Day 5 blastocysts increased the risk of CPL in singleton pregnancies, a risk that would be unacceptable to most patients. These findings may encourage the broader adoption of the SET strategy. A recently published committee opinion on embryo transfer limits suggests that for women aged 38 years or older, transferring two or more embryos may be considered (38). Full-potential embryos are particularly valuable for patients with poor prognoses. Therefore,

based on our findings, SET should be prioritized to avoid the possible loss of full potential embryos. Additionally, our study provides novel evidence that supports the most recent ESHRE guidelines on the number of embryos to transfer during IVF/ICSI, which emphasize that no clinical or embryological factors alone justify recommending DET over eSET (39).

The present study has several strengths. Firstly, its design is robust, as we analyzed the impact of DET on the occurrence of CPL in singleton pregnancies across three groups: the Day 3 cleavage embryo group, the blastocyst group, and the top blastocyst group, each representing progressively increased potential of the dead embryo. Secondly, we proposed a theory that helps explain our observations effectively. Thirdly, the primary findings of our study are supported by a large cohort study involving 49,333 patients (35). However, owing to the retrospective nature of the study, there is a possibility that important confounding factors were not fully accounted for. Moreover, the sample size of our study may have been insufficient to achieve statistical significance for certain comparisons, such as transfer of Day 3 cleavage embryos. Additionally, the data utilized in the present study were derived from a single reproductive center. We encourage reproductive centers, particularly large ones, to repeat our analysis using their own data to test our findings. Furthermore, since our study was based on data from FET cycles, the applicability of our conclusions to fresh embryo transfer cycles warrants further investigation.

Conclusion

Since a significant difference in CPL between SET and DET was only observed in the population with the transfer of morphologically good Day 5 blastocysts, we concluded that, compared to SET, the transfer of double morphologically good Day 5 blastocysts is associated with an increased risk of clinical pregnancy loss in singleton pregnancies following FET. Whether double transfers of Day 3 cleavage embryos or non-morphologically

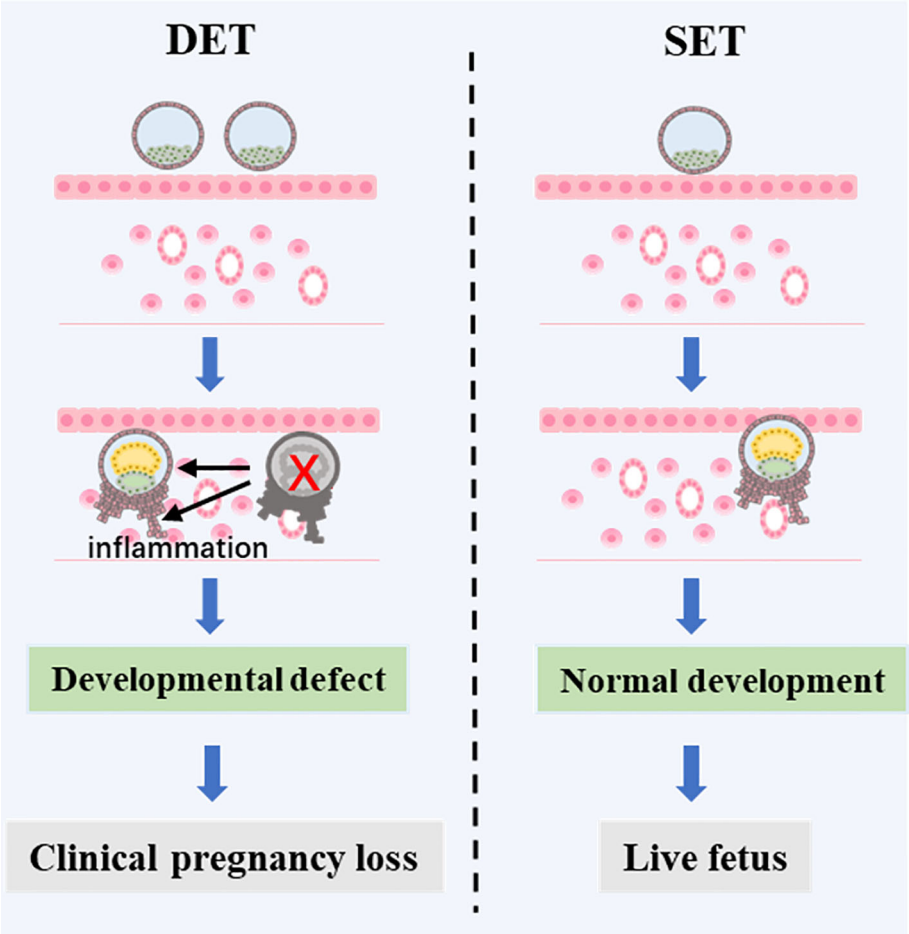


FIGURE 3
A proposed mechanism of CPL caused by DET. In DET, two embryos are transferred, one with full developmental potential and one with limited developmental potential. The embryo with limited potential may establish a biochemical pregnancy but then fail to progress to a clinical pregnancy. This failure triggers an inflammatory response in the uterus, which negatively affects the development of the surviving embryo with full developmental potential, ultimately leading to the loss of the clinical pregnancy. However, if the embryo with full developmental potential were transferred alone, a live birth could be achieved.

good Day 5 blastocysts increase the risk of CPL in singleton pregnancies, compared to single transfers of Day 3 cleavage embryos or non-morphologically good Day 5 blastocysts, still needs to be tested with large datasets.

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 studies involving humans were approved by the Ethics Committee of Changzhou Maternal and Child Health Care Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

YW: Data curation, Formal Analysis, Funding acquisition, Investigation, Writing – original draft. QW: Data curation, Formal Analysis, Investigation, Writing – original draft. XL: Data curation, Formal Analysis, Investigation, Writing – original draft. LL: Data curation, Formal Analysis, Investigation, Writing – original draft. HW: Conceptualization, Writing – review & editing. LC: Conceptualization, Funding acquisition, Writing – review & editing. XD: Conceptualization, Data curation, Funding acquisition, Writing – review & editing.

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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.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

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Oocytes with aggregates of smooth endoplasmic reticulum may not affect reproductive outcomes in split IVF-ICSI insemination: a retrospective study

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Objective: To investigate the impact of smooth endoplasmic reticulum aggregates (SERa) in oocytes on embryological outcomes and clinical and neonatal outcomes during split IVF-ICSI cycles.

Methods: A retrospective analysis was conducted using clinical data from January 2020 to December 2023 at the Reproductive Medicine Center of Hainan Women and Children's Medical Center. Patients were divided into SERa+ and SERa- cycles based on the visibility of SERa after the removal of cumulus cells. Basic patient characteristics, embryological outcomes, clinical and neonatal outcomes were compared between the two groups.

Results: Compared to the SERa- cycles, the SERa+ cycles showed significantly higher levels of E_2 on the day of hCG administration ($P<0.01$) and a significantly increased number of retrieved oocytes ($P<0.01$). In terms of embryological outcomes, the total D3 high-quality embryo rate was significantly higher in the SERa+ cycles ($P<0.01$). There was a significant increase in the D3 high-quality embryo rate for ICSI, but no difference in the D3 high-quality embryo rate for IVF. No significant differences were observed between the SERa+ and SERa- cycles in terms of βhCG positivity rate, clinical pregnancy rate, implantation rate, early miscarriage rate, live birth rate, preterm birth rate, newborn height, and weight ($P>0.05$). No congenital birth defects were found in either group.

Conclusion: The occurrence of SERa in split IVF-ICSI cycles may be associated with increased E_2 levels on hCG day, and the presence of SERa does not appear to affect *in vitro* fertilization, embryological, clinical, or neonatal outcomes.

KEYWORDS

split IVF-ICSI, oocyte, SERa, embryological outcomes, clinical and neonatal outcomes

1 Introduction

In assisted reproductive technology, the quality of the oocyte directly influences the quality of the embryo and its subsequent developmental potential (1, 2). The smooth endoplasmic reticulum aggregate (SERa), first identified in 1997 (3), is a cytoplasmic anomaly characterized by a central, round, transparent, and flat disc within the oocyte's cytoplasm. SERa has attracted considerable attention in reproductive medicine. The incidence of SERa varies widely, with reported rates ranging from 4.0% to 23.1% in cycles and 17.6% to 34.4% in individual oocytes (4). The release of calcium from the SER plays a critical role in oocyte maturation, fertilization, and early embryonic development (5, 6). Although the precise mechanisms underlying SERa formation remain unclear, ongoing research and data collection are essential for understanding its impacts and mechanisms. In 2004, a case was reported where a baby diagnosed with Beckwith-Wiedemann syndrome was born following a cycle involving SERa+ oocytes (7). Subsequent studies have indicated a significant decrease in live birth rates in cycles with SERa+ oocytes, along with a relatively higher incidence of congenital anomalies (8–10). Given these potential negative effects, the 2011 Istanbul Consensus recommended against using SERa+ oocytes (11). However, other studies have not observed an increased risk of congenital anomalies in embryos derived from SERa+ oocytes, nor have they found reduced pregnancy rates (12–14). It is reported that only 14% of centers discard SERa+ oocytes (15). Due to these inconsistent findings, the revised Vienna consensus by Alpha/ESHRE reconsidered this recommendation in 2017, advising a case-by-case approach (16). Therefore, in clinical IVF practice, the lack of consistent guidelines has led to varying attitudes among clinicians and embryologists regarding the handling of SERa+ oocytes, highlighting the urgent need for extensive clinical data to inform decision-making in embryo transfer.

Currently, clinical studies on SERa are expanding, primarily focusing on either intracytoplasmic sperm injection (ICSI) or conventional *in vitro* fertilization (IVF) cycles involving SERa-positive oocytes. However, there is a paucity of research investigating the impact of SERa in split IVF-ICSI cycles on embryonic development and clinical outcomes. This study aims to conduct a comprehensive analysis of clinical data from patients with SERa-positive oocytes undergoing split IVF-ICSI cycles at the Hainan Women and Children's Medical Center between January 2020 and December 2023. By exploring the effects of SERa on early embryological, clinical, and neonatal outcomes, this research seeks to provide scientific guidance for managing SERa-positive oocytes in assisted reproductive treatments while optimizing embryo transfer strategies. Ultimately, this study aspires to enhance both the success rate and safety of clinical applications.

2 Materials and methods

2.1 Patients and study design

This study selected infertile couples undergoing *in vitro* fertilization-embryo transfer (IVF-ET) treatment at the Hainan Provincial Women's and Children's Medical Center from January 2020 to December 2023 as research subjects. Inclusion criteria: suitability for split IVF-ICSI treatment; fresh oocyte retrieval cycles; age ≤ 40 years. Exclusion criteria: age > 40 years; patients with≤ 3 oocytes retrieved; patients utilizing vitrified/thawed or donated oocytes; male patients with testicular issues, percutaneous epididymal sperm aspiration, or severe teratozoospermia;patients experiencing total fertilization failure (TFF); preimplantation genetic testing (PGT) cycles; and those lacking clinical baseline data or follow-up. According to the presence or absence of SERa in oocytes, participants were divided into two groups: SERa+ cycles (at least one oocyte testing positive for SERa) and SERa- cycles (no oocytes with SERa). The flow chart illustrating patient inclusion in this study is presented in Figure 1.

2.2 Research methodology

2.2.1 Ovulation induction and oocyte retrieval

All patients underwent ovulation induction and follicular monitoring in accordance with the standard clinical protocols established at our center. The dosage of gonadotropins was tailored to each patient, taking into consideration factors such as age, body mass index (BMI), antral follicle count (AFC), and their response to previous ovarian stimulation cycles. Oocyte retrieval was conducted 34–37 hours post-triggering, once dominant follicles reached a diameter of 17–18 mm, utilizing transvaginal ultrasound guidance for precision in the procedure, during which the oocytes were meticulously recorded.

2.2.2 ICSI, IVF fertilization, embryo culture, and morphological observation

The retrieved cumulus-oocyte complexes (COCs) in ICSI insemination were maintained in G-IVF PLUS medium (Vitrolife, Sweden) for 3~4 hours prior to cumulus cell removal. ICSI was conducted 1 to 2 hours after denudation, with careful attention taken to avoid injecting sperm into the SERa. Only MII oocytes were utilized for ICSI. Comprehensive records of SERa+ oocytes were meticulously maintained during the ICSI procedure and subsequently entered into the system. IVF insemination occurred 3 to 4 hours following oocyte retrieval, ensuring that the concentration of progressively motile sperm (PR) was controlled at a range of 100,000-150,000/ml. The remaining COCs were fertilized by IVF using overnight fertilization and degranulated 16~17 hours after insemination. Fertilization assessment took place approximately 16 to 18 hours later under a magnification of 400×using an inverted microscope, focusing on the identification of pronuclei. Embryos were cultured *in vitro* for a duration of 3 to 7 days under controlled conditions of 37°C, with an atmosphere comprising 5% O2 and 6% CO2 in Vitrolife culture media. Observations and detailed records regarding fertilization outcomes, subsequent embryonic development, and pregnancy results post-transfer were systematically documented.

2.2.3 SERa evaluation

ICSI insemination was conducted 1–2 hours post oocyte denudation, during which SERa were also evaluated. For IVF

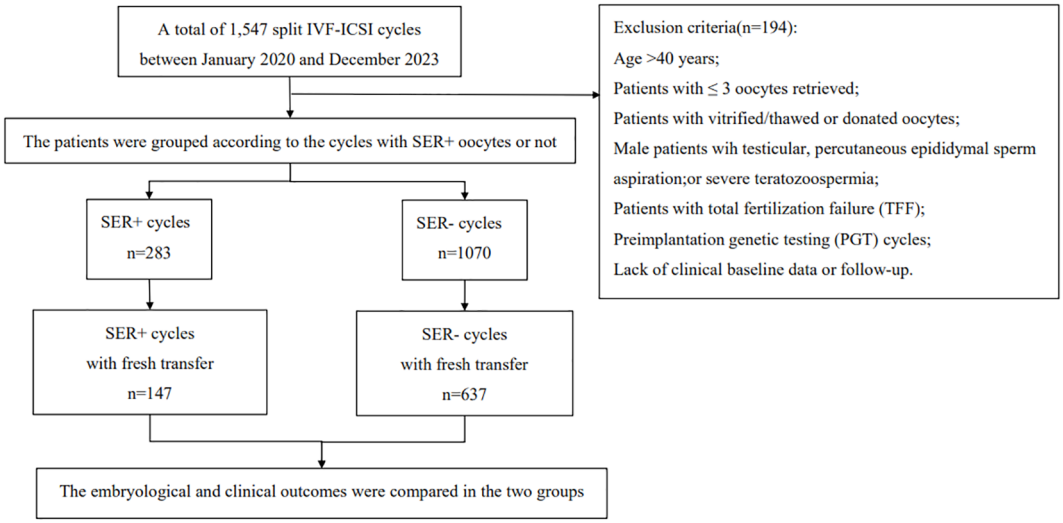

FIGURE 1
Flow chart of the study.

fertilization, SER observation was performed concurrently with pronuclear assessment on the following day after denudation. Oocytes were examined under high magnification (400×). Normal oocytes exhibit a uniform distribution of cytoplasm. The presence of large, round, flat, semi-transparent discoid structures within the cytoplasm indicates the occurrence of SERa, as illustrated in Figure 2 with a red arrow (Figure 2).

2.2.4 Embryo quality assessment

Embryo assessment time points were determined according to the standardized criteria outlined in the Istanbul Consensus Protocol, while prokaryotic scoring was performed utilizing the Scott-Z assessment. Embryos were comprehensively evaluated at the cleavage stage following our center's established protocol, which takes into account embryo morphology, developmental rate, and blastomere count. Scoring of blastocysts was conducted according to the Gardner system.

2.2.5 Primary observational indicators

The formulas for laboratory and clinical observational indicators are presented in Tables 1 and 2, respectively (Tables 1, 2).

2.2.6 Statistical methods

The one-sample Kolmogorov-Smirnov test was employed to evaluate the normality of continuous data. Continuous variables were expressed as mean ± standard deviation (SD) when they followed a normal distribution. Differences in continuous variables were analyzed using the t-test. Mann-Whitney U test was applied for non-normally distributed data. Categorical variables were presented as percentages, with differences assessed using either the chi-squared test or Fisher's exact test, depending on appropriateness. Statistical analyses were conducted using the Statistical Program for Social Sciences (SPSS Inc., Version 27.0,

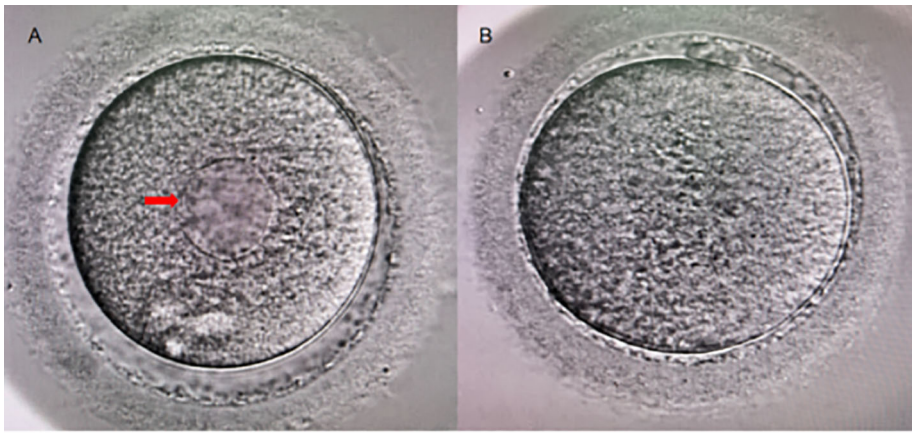

FIGURE 2
Human Metaphase II Oocytes (400×). **(A)** A metaphase II oocyte exhibiting SERa (indicated by the red arrow); **(B)** A normal metaphase II oocyte.

TABLE 1 Laboratory observational indicator.

Term	Formula
MII oocyte rate	Total number of MII oocytes/Total number of retrieved oocytes ×100%
Total fertilization rate	Total number of fertilized oocytes/Total number of MII oocytes ×100%
Normal fertilization rate	Number of normally fertilized oocytes/Total number of MII oocytes ×100%
Cleavage rate	Number of cleaved embryos/Number of fertilized oocytes×100%
High-quality Day 3 embryo rate	High-quality Day 3 embryos/Number of normally fertilized cleaved embryos ×100%
Blastocyst formation rate	Stage 2 and above blastocysts/Total number of cleavage-stage embryos cultured for blastocyst × 100%
High-quality blastocyst rate	Number of high-quality blastocysts/Number of stage 2and above blastocysts × 100%
Available blastocyst rate	Number of usable blastocysts/Number of stage 2 and above blastocysts × 100%

(i) High-quality Day 3 embryo: Normally fertilized and has 7–9 cells with ≤10% fragmentation on Day 3.
(ii) High-quality blastocyst: Stage 3 and above blastocysts with inner cell mass and trophectoderm cells not containing grade C.
(iii) Available blastocyst: Stage 3 and above blastocysts where neither the inner cell mass nor trophectoderm cells simultaneously contain grade C.

Chicago, IL, USA). A p-value of less than 0.05 in a two-tailed test was deemed statistically significant.

3 Results and analysis

This study collected data from 1,547 fresh oocyte retrieval cycles that underwent split IVF-ICSI at the Hainan Provincial Women's and Children's Medical Center between January 2020 and December 2023. Among these cycles, 309 were identified as SERa+, resulting in a SERa positivity rate of 19.97%. After applying the inclusion and exclusion criteria, a total of 1,353 split IVF-ICSI cycles (including 283 SERa+ and 1,070 SERa- cycles) were included in the final analysis. Among SERa+ cycles, 588 SERa+ oocytes were identified, with 34 (5.8%) derived from conventional IVF and 554 (94.2%) from ICSI. Each SERa+ cycle contained an average of 2.08 ± 1.74 SERa+ oocytes. Notably, SERa+ oocytes represented a substantial proportion (13.73%, 588/4283) of all retrieved oocytes.

TABLE 2 Clinical observational indicators.

Term	Formula
β-hCG positivity rate	Total number of β-hCG positive individuals/Total number of transfer patients ×100%
Clinical pregnancy rate	Total number of clinical pregnancies/Total number of transfer patients ×100%
Implantation rate	Number of implanted embryos/Number of transferred embryos ×100%
Early miscarriage rate	Number of early miscarriages/Total number of clinical pregnancies ×100%
Live birth rate	Number of live births/Number of transfer cycles ×100%
Preterm birth rate	Number of preterm deliveries/Number of transfer cycles ×100%

3.1 Comparison of general clinical data between the two groups

As summarized in Table 3, no statistically significant differences were observed between the two groups regarding age, duration of infertility, type of infertility, body mass index, and baseline levels of FSH, LH, E_2, P, and AMH ($P > 0.05$). In terms of ovulation induction protocols, there were no differences noted in LH and P levels or the number of follicles on the day of hCG administration ($P > 0.05$). However, the levels of E_2 on the day of hCG administration were significantly elevated in the SERa+ cycles ($P < 0.01$). Additionally, the average E_2 level per oocyte on hCG day was notably higher in the SERa+ cycles ($P < 0.05$).With respect to medication usage, there were no significant differences between the groups concerning the total amount of Gonadotropin used or the duration for which Gn was administered ($P > 0.05$); nevertheless, the SERa+ cycles demonstrated a significant increase in the number of oocytes retrieved ($P < 0.01$).

3.2 Comparison of embryological outcomes between the two groups

In terms of embryological outcomes, as presented in Table 4, the SERa+ cycles exhibited no significant differences compared to the SERa- cycles regarding total fertilization rate, normal fertilization rate, cleavage rate, blastocyst formation rate, high-quality blastocyst rate, and available blastocyst rate ($P > 0.05$). However, the incidence of high-quality Day 3 embryos was significantly greater in the SERa+ cycles ($P < 0.01$).

3.3 Comparison of embryological outcomes in ICSI insemination between the two groups

As illustrated in Table 5, compared to the SERa- cycles, no significant differences were observed in the SERa+ cycles concerning the rate of MII mature oocytes, fertilization rate, normal fertilization rate, cleavage rate, blastocyst formation rate, high-quality blastocyst rate, and available blastocyst rate ($P > 0.05$). However, there was a significant increase in the rate of high-quality Day 3 embryo within the SERa+ cycles ($P < 0.01$).

3.4 Comparison of embryological outcomes in IVF insemination between the two groups

In the context of IVF insemination, no significant differences were observed between the SERa+ and SERa- cycles regarding

TABLE 3 Comparison of general clinical data.

Item	SERa+ cycles (n=283)	SERa- cycles (n=1070)	*P*
Female age (years)	32.33 ± 3.82	31.96 ± 3.78	0.187
Duration of infertility (years)	4.72 ± 3.18	4.69 ± 2.95	0.629
Infertility types			0.155
Primary,% (n)	54.42 (154)	59.25 (634)	
Secondary,% (n)	45.58 (129)	40.75 (436)	
Female BMI (kg/m^2)	21.95 ± 3.48	22.01 ± 6.78	0.918
Basal FSH level (IU/L)	6.38 ± 1.93	6.57 ± 2.12	0.109
Basal LH level (IU/L)	6.14 ± 3.55	6.13 ± 5.53	0.436
Basal E_2 level (pg/mL)	67.61 ± 285.47	83.64 ± 487.48	0.213
Basal P level (ng/mL)	0.42 ± 1.84	0.40 ± 1.76	0.421
Basal AMH level (ng/mL)	4.14 ± 3.23	4.17 ± 3.09	0.743
E_2 on hCG day (pg/mL)	3189.99 ± 1846.86	2764.97 ± 1515.84	0.001
Average E_2 level per oocyte on HCG day	261.79 ± 119.82	243.70 ± 106.77	0.025
LH on hCG day (IU/L)	3.58 ± 9.12	3.29 ± 2.52	0.360
P on hCG day (ng/mL)	0.62 ± 0.35	0.58 ± 0.75	0.404
Mean no. of Follicle on hCG day	12.81 ± 5.52	12.06 ± 5.67	0.057
Mean no. of oocytes retrieved	15.50 ± 6.44	13.90 ± 6.35	0.001
Total gonadotrophins dose (U)	1865.70 ± 568.27	1849.26 ± 635.79	0.352
Duration of Gn (days)	9.88 ± 1.47	9.81 ± 1.87	0.106

TABLE 4 Comparison of embryological outcomes.

Item	SERa+ cycles (n=283)	SERa- cycles (n=1070)	*P*
Total fertilization rate, % (n)	82.83 (3474/4194)	82.26 (11643/14154)	0.406
Total normal fertilization rate,% (n)	70.43 (2954/4194)	70.92 (10038/14154)	0.549
Total cleavage rate, % (n)	97.58 (3390/3474)	97.67 (11372/11643)	0.754
Total high-quality Day 3 embryo rate,% (n)	42.68 (1227/2875)	38.37 (3742/9752)	0.001
Total blastocyst formation rate,% (n)	68.48 (1671/2440)	68.41 (5476/8005)	0.960
Total high-quality blastocyst rate,% (n)	68.04 (1137/1671)	67.71 (3708/5476)	0.811
Total available blastocyst rate,% (n)	83.90 (1402/1671)	84.02 (4601/5476)	0.909

TABLE 5 Comparison of embryological outcomes in ICSI insemination.

Item	SERa+ cycles (n=283)	SERa- cycles (n=1070)	*P*
MII oocyte rate,% (n)	96.04 (2161/2250)	94.71 (7067/7462)	0.011
ICSI fertilization rate, % (n)	86.81 (1876/2161)	87.18 (6161/7067)	0.660
ICSI normal fertilization rate,% (n)	78.90 (1705/2161)	79.92 (5648/7067)	0.299
ICSI cleavage rate,% (n)	98.67 (1851/1876)	98.46 (6066/6161)	0.587
ICSI-D3 high-quality embryo rate,% (n)	46.38 (774/1669)	40.11 (2215/5523)	0.001
ICSI blastocyst formation rate,% (n)	69.96 (983/1405)	71.03 (3136/4415)	0.459
ICSI high-quality blastocyst rate,% (n)	69.68 (685/983)	69.71 (2186/3136)	0.999
ICSI available blastocyst rate,% (n)	85.25 (838/983)	85.33 (2676/3136)	0.959

fertilization rate, normal fertilization rate, cleavage rate, high-quality Day 3 embryo rate, high-quality blastocyst rate, and available blastocyst rate (P > 0.05)(As illustrated in Table 6).

3.5 Comparison of clinical and neonatal outcomes between the two groups

There were 147 fresh transfer cycles in the SERa+ cycles and 637 fresh transfer cycles in the SERa- cycles. No significant differences were observed between the SERa+ and SERa- cycles regarding β-hCG positivity rate, clinical pregnancy rate, implantation rate, early miscarriage rate, live birth rate, preterm birth rate, as well as the heights and weights of newborns (P > 0.05). The SERa+ cycles recorded 68 live births while the SERa- cycles had 274 live births; notably, no congenital birth defects were identified in either cohort (As illustrated in Table 7).

3.6 Comparison of clinical and neonatal outcomes between the two groups in SERa+ cycles

Among the 147 SERa+ cycles, a total of 23 embryos derived from ICSI were transferred across 15 cycles. This included 16 embryos originating from SERa+ oocytes. No significant differences were observed between the SERa+ and SERa- oocytes in terms of β-hCG positivity rate, clinical pregnancy rate, implantation rate, early miscarriage rate, live birth rate, preterm birth rate, as well as the heights and weights of newborns (P > 0.05). The embryos derived from SERa+ oocytes resulted in 10 live births, while those from SERa- oocytes yielded 58 live births; notably, no congenital birth defects were identified in either cohort(As illustrated in Table 8).

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TABLE 6 Comparison of embryological outcomes in IVF insemination.

Item	SERa+ cycles (n=283)	SERa- cycles (n=1070)	*P*
IVF fertilization rate, % (n)	78.60 (1598/2033)	77.35 (5482/7087)	0.239
IVFnormal fertilization rate,% (n)	61.44 (1249//2033)	61.94 (4390/7087)	0.679
IVF cleavage rate,% (n)	96.31 (1539/1598)	96.79 (5306/5482)	0.342
IVF-D3 high-quality embryo rate,% (n)	37.56 (453/1206)	36.11 (1527/4229)	0.360
IVF blastocyst formation rate,% (n)	66.47 (688/1035)	65.18 (2340/3590)	0.458
IVF high-quality blastocyst rate,% (n)	65.70 (452/688)	65.04 (1522/2340)	0.785
IVF available blastocyst rate,% (n)	81.98 (564/688)	82.26 (1925/2340)	0.865

TABLE 7 Comparison of clinical and neonatal outcomes.

Item	SERa+ cycles (n=147)	SERa- cycles (n=637)	*P*
Female age (years)	32.90 ± 3.83	32.35 ± 3.71	0.112
Average number of embryos transferred	1.17 ± 0.38	1.23 ± 0.42	0.123
proportions of transferred embryos			0.994
IVF-derived embryos, % (n)	52.91 (91/172)	52.94 (414/782)	
ICSI-derived embryos, % (n)	47.09 (81/172)	47.06 (368/782)	
β-hCG positivity rate, % (n)	58.50% (86/147)	60.44% (385/637)	0.709
clinical pregnancy rate, % (n)	54.42% (80/147)	53.06% (338/637)	0.784
implantation rate,% (n)	50.58% (87/172)	46.55% (364/782)	0.354
early miscarriage rate, % (n)	13.75% (11/80)	21.30% (72/338)	0.160
live birth rate,% (n)	41.50% (61/147)	39.56% (252/637)	0.709
preterm birth rate,% (n)	11.48% (7/61)	12.70% (32/252)	0.999
Total number of live infants (n)	68	274	–
Birth weight (kg)	2.99 ± 0.41	2.91 ± 0.57	0.182
Birth heights (cm)	48.93 ± 1.81	48.86 ± 2.08	0.797
Birth defects	0	0	–

TABLE 8 Comparative analysis of clinical and neonatal outcomes in SERa+ cycles.

Item	SERa+ Oocytes in SERa+ cycles (n=15)	SERa- Oocytes in SERa+ cycles (n=132)	*P*
Female age (years)	33.13 ± 3.20	32.87 ± 3.90	0.803
No. of embryos transferred (n)	23	149	–
Average number of embryos transferred	1.53 ± 0.52	1.13 ± 0.34	<0.001
IVF-derived embryos transferred proportion,% (n)	0 (0/23)	66.44 (99/149)	–
ICSI-derived embryos transferred proportion,% (n)	100 (23/23)	33.56 (50/149)	–
No. of SERa+ oocytes derived embryos transferred (n)	16	–	–
β-hCG positivity rate,% (n)	66.67% (10/15)	56.82% (75/132)	0.464
clinical pregnancy rate,% (n)	66.67% (10/15)	53.03% (70/132)	0.315
implantation rate, % (n)	52.17% (12/23)	50.34% (75/149)	0.870
early miscarriage rate,% (n)	10.00% (1/10)	15.71% (11/70)	1.000
live birth rate,% (n)	60.00% (9/15)	39.39% (52/132)	0.125
Total number of live infants (n)	10	58	–
Birth weight (kg)	3.04 ± 0.21	2.98 ± 0.44	0.494
Birth heights (cm)	49.70 ± 0.67	48.79 ± 1.91	0.141
Birth defects	0	0	–

4 Discussion

Fertilization of oocytes is a multifaceted process influenced by various factors, including the maturity of both the oocyte and sperm, as well as the vitality and fusion of genetic material. These elements are critical in assisted reproductive technology (ART). Certain infertility treatment cycles may experience low fertilization rates or even complete fertilization failure, with incidence rates ranging from 10% to 20%. Such challenges not only lead to repeated failures in subsequent assisted pregnancy attempts but also impose significant psychological and economic stress on individuals undergoing these treatments (17). The split IVF-ICSI technique plays a pivotal role in ART and serves as an effective strategy to mitigate low fertilization rates (18, 19). In our study, we observed the occurrence rate of SERa

at 19.97% in split IVF-ICSI cycles; however, there is limited research on the impact of SERa on embryological, clinical, or neonatal outcomes within these cycles. This study focused on patients undergoing treatments involving split IVF-ICSI cycles, analyzing the effects of SERa on the developmental potential and clinical outcomes of sibling embryos resulting from both IVF and ICSI fertilization methods. These findings hold significant clinical implications and provide a foundation for strategic adjustments when managing SERa+ oocytes.

Our comparison of general clinical data between the two groups revealed no significant differences in age, duration of infertility, type of infertility, body mass index, and baseline levels of FSH, E_2, LH, and P. This indicates that these baseline variables had a negligible impact on our study findings. In contrast to the SERa- cycles, the SERa+ cycles exhibited a tendency towards elevated E_2 levels and an increased total number of oocytes retrieved on hCG day, which is consistent with previous studies (20). The occurrence of ovarian hyperstimulation may be associated with SERa since research has shown that SERa is not present in oocytes from unstimulated patients (21).The occurrence of SERa is positively correlated with E_2 levels on the day of hCG administration, and it is widely accepted that the emergence of SERa is associated with elevated E_2 levels (22). However, current research investigating whether increased E_2 levels directly lead to the occurrence of SERa remains limited. A recent study examining the potential impact of aromatase inhibitor protocols on reducing SERa incidence in oocytes (23) found that these inhibitors did not significantly decrease the occurrence of SERa.

This suggests that elevated E_2 levels may not be the primary cause of SERa; rather, its occurrence could result from a combination of inherent patient factors and ovarian stimulation. Consequently, further investigation into possible predictive factors for SERa occurrence is warranted. The primary function of smooth endoplasmic reticulum involves calcium storage and release, which are essential during processes such as oocyte activation, fertilization, and energy accumulation (24).The cytoplasmic anomaly SERa may disrupt calcium storage and oscillation during fertilization. Previous studies have reported significantly reduced fertilization rates in SERa + cycles (25). However, other investigations, including our own, found no significant differences in total fertilization rates or normal fertilization rates between SERa+ and SERa- cycles (26). Notably, the SERa+ cycles exhibited a trend toward increased rates of high-quality Day 3 embryos, particularly ICSI, while no such differences were observed in conventional IVF. A cohort study (27) has indicated significantly lower rates of high-quality embryos in SERa+ cycles compared to their SERa- counterparts, although another study reported no differences (26). The inconsistencies among these studies regarding high-quality embryo rates may stem from non-uniform definitions of what constitutes a high-quality embryo or from small sample sizes, thus necessitating further research. Concerning the impact of SERa on blastocyst development, existing literature suggests that SERa significantly influences both blastocyst quality and developmental speed, leading to a reduction in the blastocyst formation rate (28). Our study did not reveal any significant differences between the two groups concerning overall blastocyst formation rates, high-quality blastocyst rates, or available blastocyst rates—regardless of whether IVF or ICSI was employed—consistent with recent findings (26). Some studies propose that the presence of SERa does not hinder ongoing blastocyst development nor interfere with the formation rates of high-quality embryos or affect euploidy and aneuploidy ratios (20, 30).

Our findings are consistent with several studies, indicating no significant differences between the two groups in terms of βhCG positivity rates, clinical pregnancy rates, implantation rates, early miscarriage rates, live birth rates, preterm birth rates, as well as the heights and weights of newborns (29). Embryos derived from SERa+ oocytes have the potential to develop into normal and healthy newborns. Furthermore, there is no definitive negative correlation observed between SERa+ oocytes and cycles concerning embryology, clinical outcomes, or newborn results. The question of whether SERa adversely affects embryonic developmental potential and clinical outcomes remains a topic of debate. Additionally, while our study did not identify any birth defects among live births in either group, recent meta-analyses (31) suggest that SERa+ cycles/oocytes may carry a potential risk for an increased incidence of major birth defects.

This study presents several limitations. As a retrospective analysis, it is inherently subject to biases and cannot adequately control for participant heterogeneity. The number of embryos derived from SERa+ oocytes in this study was relatively small, resulting in a limited sample size. Our investigation concentrated on SERa+ cycles rather than SERa+ oocytes; therefore, caution should be exercised when interpreting the results of this study. Future research with larger sample sizes and prospective designs is essential for validating our findings. Additionally, further long-term follow-up regarding clinical outcomes and newborns resulting from embryos derived from SERa+ oocyte transfers is necessary to evaluate the potential for developmental abnormalities.

Given the clinical significance of SERa+ oocytes, we propose establishing an international multicenter registry to systematically track outcomes of embryos derived from these oocytes. Such a registry would enable: (1) standardized data collection on fertilization rates, embryo quality, and pregnancy outcomes; (2) correlation of SERa+ morphology with genetic and epigenetic profiles; and (3) development of evidence-based guidelines for clinical management.

In conclusion, this study indicates that SERa is associated with hormone levels in patients undergoing assisted reproductive technology; however, it does not appear to influence embryonic development or clinical outcomes. Consequently, discarding SERa oocytes may not represent the most ethical approach. The avoidance of wastage of oocytes and embryos remains a persistent concern in daily IVF practice. Nevertheless, current conclusions regarding the developmental and clinical outcomes of embryos derived from SERa are inconsistent. Caution is warranted when transferring embryos originating from SERa oocytes in assisted reproductive treatments, highlighting the need for large-scale, multicenter data studies. Further investigations into the causes and mechanisms underlying SERa formation in oocytes are essential to provide evidence that supports decision-making during clinical embryo transfers, ultimately enhancing clinical outcomes for patients experiencing infertility.

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 humans were approved by Medical Ethics Committee of Hainan Provincial Women's and Children's Medical Center. The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because In accordance with national legislation and institutional guidelines, written informed consent for participation was not required from the participants or their legal guardians/next of kin.

Author contributions

YL: Formal analysis, Funding acquisition, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. J-JH: Data curation, Formal analysis, Writing – review & editing. HL: Data curation, Formal analysis, Writing – review & editing. Z-YL: Data curation, Formal analysis, Writing – review & editing. J-JZ: Data curation, Formal analysis, Funding acquisition, Methodology, Writing – review & editing. L-SS: Project administration, Supervision, Writing – review & editing.

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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.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

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The effect of overnight culture after thawing of D3 cleavage-stage embryos on clinical pregnancy outcomes: focus on embryo development to day 4

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Objective: This study aims to investigate the impact of day-3 (D3) cleavage-stage embryo thawing with immediate transfer versus thawing and overnight culture before transfer on clinical outcomes. It also examines the relationship between cleavage-stage embryo developmental speed after overnight culture and clinical pregnancy outcomes, as well as factors influencing clinical pregnancy in frozen embryo transfer (FET).

Methods: A retrospective analysis was conducted on 1,040 patients who underwent D3 cleavage-stage frozen embryo transfer at Yulin City Maternal and Child Health Hospital between July 2022 and December 2023. Patients were divided into two groups based on embryo culture time after thawing: control (same-day transfer, 2-3 hours) and experimental (overnight culture, 18-20 hours). Clinical pregnancy rates, embryo implantation rates, early miscarriage rates, and multiple pregnancy rates were compared between groups. The experimental group was further subdivided based on the number of cleavage blastomeres increased after culture: A1 (≥4 blastomeres), A2 (1-3 blastomeres), and A3 (no increase). A binary logistic regression analysis identified independent factors affecting clinical pregnancy outcomes in FET.

Results: No significant differences were found between the control and experimental groups in clinical pregnancy rate (37.2% vs. 40.2%), embryo implantation rate (24.9% vs. 26.4%), early miscarriage rate (13.1% vs. 18.8%), or multiple pregnancy rate (9.2% vs. 10.2%) ($P > 0.05$). In the experimental group, clinical pregnancy rates for A1, A2, and A3 subgroups were 44.2%, 29.8%, and 25.5%, respectively. Early miscarriage rates were 18.6%, 10.7%, and 38.5%, showing statistically significant differences ($P < 0.05$). Female age, endometrial thickness, embryo morphology, and the number of cleavage blastomeres were identified as independent factors influencing clinical pregnancy rate.

Conclusion: This study indicates that D3 embryos with an increase in the number of blastomeres to more than four or entering the compaction stage after overnight culture have better pregnancy outcomes. Female age and endometrial thickness are important factors influencing clinical pregnancy

rates. Optimizing culture conditions and ensuring optimal endometrial thickness may help improve the success rate of frozen-thawed embryo transfer.

KEYWORDS

D3 cleavage-stage embryo, frozen embryo transfer (FET), overnight culture, embryo development, clinical pregnancy outcomes

1 Introduction

With advancements in assisted reproductive technology (ART), vitrification techniques have become increasingly refined, and the application of cryopreservation in *in vitro* fertilization (IVF) labs has improved survival rates of oocytes, cleavage-stage embryos, and blastocysts after thawing. This has led to the widespread adoption of frozen-thawed embryo transfer (FET) (1). Data indicates that over the past decade, the number of FET cycles has increased significantly, accounting for more than 30%-40% of all transfer cycles in many regions worldwide (2).

FET not only allows patients to choose an optimal time for embryo transfer, helps prevent ovarian hyperstimulation syndrome, and reduces embryo waste, but it also plays a key role in decreasing multiple pregnancy rates and increasing cumulative live birth rates (3, 4). Furthermore, compared to fresh embryo transfer, FET has been shown to improve implantation, clinical pregnancy, and ongoing pregnancy rates in ART cycles (5), while posing lower risks of placenta previa, placental abruption, low birth weight, very low birth weight, preterm birth, small-for-gestational age, and perinatal mortality (6).

Factors influencing clinical outcomes of FET include maternal age, BMI, embryo quality, endometrial thickness on the day of transfer, number of embryos transferred, and post-thaw recovery of embryos (7). However, optimizing post-thaw outcomes for cleavage-stage embryos remains controversial, with no consensus yet reached. In clinical practice, the *in vitro* culture duration after D3 cleavage-stage embryo thawing varies, and its impact on pregnancy outcomes is still debated (8).

Our study is novel in that it provides a detailed analysis of the clinical outcomes of Day 3 cleavage-stage embryos subjected to overnight culture (to Day 4) after thawing, compared to embryos transferred shortly after thawing. Specifically, we assessed the developmental potential of embryos during the overnight culture and categorized them based on the extent of blastomere division into subgroups (A1, A2, and A3). This level of granularity in embryo classification, combined with a large sample size, allows for a nuanced understanding of how post-thaw developmental progression impacts clinical outcomes.

Therefore, this study retrospectively analyzed 1040 FET cycles at our center, all involving D3 cleavage-stage embryo transfers after thawing, to investigate the relationship between *in vitro* culture duration after D3 embryo thawing and clinical pregnancy outcomes, as well as the impact of different embryo developmental rates following overnight culture. This analysis also considers additional factors influencing clinical pregnancy outcomes in FET.

2 Materials and methods

2.1 Study subjects and grouping

A retrospective analysis was conducted on medical records of patients who underwent D3 cleavage-stage frozen-thawed embryo transfer (FET) at our center from July 2022 to December 2023, encompassing a total of 1040 cycles. Ethical approval for this study was obtained from the ethics committee, and the approval number was YLSFYLLKY2025-02-05-1.Inclusion criteria were female age <50 years and male age <60 years, with infertility factors such as female pelvic/tubal or ovulatory disorders, and male oligoasthenoteratozoospermia. IVF or ICSI was performed. Exclusion criteria included FET cycles with sequential cleavage-stage and blastocyst transfer, egg recipients, and patients who underwent oocyte vitrification followed by embryo transfer.

The cycles were grouped based on the timing of thawing. The control group (470 cycles) involved thawing in the morning with embryo transfer approximately 2 hours later. The experimental group (570 cycles) underwent thawing in the afternoon of the previous day, followed by an 18–20 hour overnight culture prior to transfer.

2.2 Methods

2.2.1 Embryo cryopreservation and thawing

The KITAZATO cryopreservation and thawing kits (Japan) and Vitrolife sequential culture media were used.

2.2.1.1 Embryo vitrification

At room temperature, embryos were transferred from G1 medium to the equilibration solution (ES) and equilibrated for 10 minutes until approximately 90% of blastomeres recovered. Embryos were then transferred to vitrification solution (VS) and, within 60 seconds, loaded onto cryo-straws and preserved in liquid nitrogen.

2.2.1.2 Embryo thawing

Cryo-straws containing embryos were transferred directly from liquid nitrogen to WS1 thawing solution prewarmed to 37°C, shaken gently to ensure release of the embryo into the solution, and kept in the solution for 1 minute. The embryos were subsequently moved through WS2, WS3, and WS4 solutions sequentially (3 min, 5 min, 5 min), with WS2 and WS3 at room temperature and WS1 and WS4 at 37°C. Embryos were then cultured overnight in microdrops of G2 medium within a tri-gas incubator (6% CO_2, 5% O_2, balanced with N_2) to await transfer.

2.2.2 Endometrial preparation

Endometrial preparation includes the natural cycle, ovulation induction cycle, and rtificial cycle.

2.2.2.1 Natural cycle

Starting from day 10 of menstruation, monitor follicle development with ultrasound until the dominant follicle diameter is ≥16mm. At this point, begin monitoring blood levels of estradiol, progesterone, and luteinizing hormone. On the day of ovulation, administer oral dydrogesterone 10mg tid (Duphaston, 10mg/tablet, Abbott Biologicals B.V.) or progesterone capsules 200mg bid (Yimaixin, 50mg/pill, Zhejiang Xianju) for luteal support (D0). Embryo transfer on day 3.

2.2.2.2 Ovulation induction cycle

Start oral administration of letrozole (2.5 mg per tablet, Jiangsu Hengrui) at 2.5 mg to 5 mg once daily for 5 days starting from day 2 to day 5 of menstruation. Begin monitoring ovulation 5 days later. If two monitored follicles are ≤10 mm, add HMG 75-150 IU until the leading follicle diameter is ≥16 mm. Then start monitoring blood E2, P, and LH levels. Once natural ovulation occurs or if the follicle does not rupture but P exceeds 1 ng/ml, administer orally: 1. Dydrogesterone 10 mg three times daily (Duphaston, 10 mg per tablet, Abbott Biologicals B.V., Netherlands) 2. Progesterone capsules 200 mg twice daily (Yimaixin, 50 mg per tablet, Zhejiang Xianju) for luteal support (Day 0). Transfer on Day 5.

2.2.2.3 Artificial cycle

Start oral administration of estradiol valerate (Juvacon, 1 mg/tablet, Bayer France, DELPHARM Lille S.A.S.) or Femilon red tablets (each red tablet equivalent to 1 mg of estradiol valerate, Abbott Biologicals B.V., Netherlands) from the second to third day of menstruation. Once the endometrial thickness reaches ≥8 mm, administer progesterone injection 40 mg daily (20 mg/vial, Zhejiang Xianju) for luteal support (D0). Embryo transfer is performed on the D3.

2.2.3 Embryo transfer

D3 cleavage-stage embryos were considered viable for transfer if at least half of the blastomeres survived; embryos with more than half of the blastomeres degenerated were not transferred.

2.2.3.1 Control group

Embryos were thawed in the morning and transferred into the G2 medium culture dish for 2 hours before transfer.

2.2.3.2 Experimental group

Embryos were thawed the previous afternoon and cultured in G2 medium for 18–20 hours overnight, and the development status was observed the following morning before transfer.

2.3 Outcome measures

2.3.1 D3 embryo assessment

Grade I: Derived from normal fertilization, 8 cells of uniform size with ≤5% fragmentation; Day 2 (D2) embryo quality is Grade I or II, with 4 cells. Grade II: Derived from normal fertilization, 7–9 cells, with a cell count increase of more than 2 cells from D2, uniform or slightly uneven cell size, and ≤10% fragmentation; D2 embryo quality is Grade I or II. Grade III: ≥5 cells with a cell count increase of at least 2 cells from D2, moderate cell uniformity (≤++), and fragmentation between 5% and 50%. Grade IV: ≤4 cells, or severe cell size unevenness, with ≥50% fragmentation. Grades I and II are collectively classified as high-quality embryos; Grade III is classified as non-high-quality, and Grades I, II, and III are considered usable embryos.

2.3.2 Thawing and culture protocol

The cycles were grouped based on the timing of embryo thawing. The control group (470 cycles) involved thawing in the morning with embryo transfer approximately 2 hours later. The experimental group (570 cycles) underwent thawing in the afternoon of the previous day, followed by an 18–20 hour overnight culture prior to transfer. In the experimental group, embryos were further divided into three subgroups based on the number of blastomeres observed the next morning:

Group A1: Embryos with more than 4 cells were assessed for further cleavage and developmental potential. For embryos entering the compaction stage, individual cell counts were not distinguishable due to the tightly packed arrangement of cells. In this study, compacted embryos were evaluated as part of the cleavage-stage developmental continuum, without being categorized separately. The primary focus was on assessing overall morphological development and clinical outcomes rather than subdividing specific morphological stages.

Group A2: Embryos with 1–3 new blastomeres.

Group A3: Embryos with no increase in blastomeres.

2.3.3 Other metrics and pregnancy determination

Post-Thaw Embryo Survival Rate: Number of surviving thawed embryos / total number of thawed embryos. Post-Thaw Intact Embryo Rate: Number of intact thawed embryos / total number

of thawed embryos. Fourteen days post-transfer, blood hCG is measured, and clinical pregnancy is confirmed via ultrasound at 28 days if a gestational sac is observed. Clinical Pregnancy Rate: Number of clinical pregnancy cycles / total transfer cycles × 100%. Embryo Implantation Rate: Number of implanted embryos / total number of transferred embryos × 100%. Early Miscarriage Rate: Natural miscarriages within 12 weeks of gestation / number of clinical pregnancy cycles × 100%. Multiple Pregnancy Rate: Number of multiple pregnancies / number of clinical pregnancies × 100%.

2.4 Statistical methods

Data were analyzed using SPSS 25.0 software. Continuous variables were presented as mean ± standard deviation (x ± s) and analyzed with the t-test. Categorical variables were expressed as percentages (%) and analyzed with the χ^2 test. A p-value <0.05 was considered statistically significant. Binary logistic regression was conducted to assess the correlation between the increase in blastomere number and clinical pregnancy rate, with odds ratios (OR) and 95% confidence intervals (CI) calculated.

3 Results

3.1 Comparison of basic characteristics between the control and experimental groups

There were no statistically significant differences between the control and experimental groups in terms of the average age of the female participants, duration of infertility, body mass index (BMI), endometrial thickness on the day of embryo transfer, average number of embryos transferred, embryo survival rate and integrity after thawing, endometrial preparation protocol, and embryo morphology ($P > 0.05$), (Table 1).

3.2 Comparison of cinical outcomes between the control and experimental groups

In the experimental group (570 cycles), the clinical pregnancy rate was 40.20%, the embryo implantation rate was 26.40%, the early miscarriage rate was 18.80%, and the multiple pregnancy rate was 10.18%. In the control group (470 cycles), the clinical pregnancy rate was 37.20%, the embryo implantation rate was 24.90%, the early miscarriage rate was 13.10%, and the multiple pregnancy rate was 9.15%. The clinical pregnancy rate, implantation rate, early miscarriage rate, and multiple pregnancy rate were slightly higher in the experimental group compared to the control group, but the differences were not statistically significant ($P > 0.05$) (Table 2).

3.3 Relationship between embryo development speed and clinical pregnancy outcomes in the pre-embolization thaw group

The results showed that there were no statistically significant differences in basic characteristics such as female age, duration of infertility, body mass index, endometrial thickness on the day of embryo transfer, and infertility type between the A1, A2, and A3 groups ($P > 0.05$). However, there were statistically significant differences between the groups in terms of average number of embryos transferred ($P = 0.048$), endometrial preparation protocol ($P = 0.019$), and embryo morphology ($P = 0.00$), while the difference in multiple pregnancy rates was not statistically significant ($P > 0.05$).

Regarding the clinical pregnancy rate, the A1, A2, and A3 groups had rates of 44.2%, 29.8%, and 25.5%, respectively, with significant differences between the three groups ($P = 0.003$). Further pairwise comparisons showed that the differences in clinical pregnancy rates between the A1 and A2 groups ($\chi2 = 6.613$, $p = 0.01$) and between the A1 and A3 groups ($\chi2 = 6.559$, $P = 0.01$) were statistically significant, but the difference between the A2 and A3 groups was not significant ($\chi2 = 0.301$, $P = 0.583$).

For embryo implantation rates, the A1, A2, and A3 groups had rates of 29.3%, 18.3%, and 17.9%, respectively, with significant differences between the three groups ($P = 0.002$). Further pairwise comparisons revealed that the differences in implantation rates between the A1 and A2 groups ($\chi2 = 8.859$, $P = 0.003$) and between the A1 and A3 groups ($\chi2 = 5.442$, $P = 0.02$) were statistically significant, while the difference between the A2 and A3 groups was not significant ($\chi2 = 0.008$, $P = 0.929$).

As for early miscarriage rates, the A1, A2, and A3 groups had rates of 18.6%, 10.7%, and 38.5%, respectively, with no statistically significant differences between the three groups ($P = 0.129$). Pairwise comparisons showed that there were no significant differences in early miscarriage rates between the A1 and A2 groups, and between the A1 and A3 groups ($P > 0.05$), but the difference between the A2 and A3 groups was statistically significant ($\chi2 = 4.352$, $P= 0.037$) (Table 3).

3.4 Multivariate logistic regression analysis of factors influencing clinical pregnancy in the experimental group

In the multivariate logistic regression analysis, female age and endometrial thickness were found to have a significant impact on clinical pregnancy. Increasing female age significantly decreased the likelihood of pregnancy (OR = 0.892, $P = 0.000$), while higher endometrial thickness significantly increased the pregnancy rate (OR = 1.174, $P = 0.002$). Other factors, such as duration of infertility, body mass index (BMI), number of embryos transferred, endometrial preparation protocol, and embryo morphology (good or poor quality), did not show a significant impact on clinical pregnancy (Table 4).

TABLE 1 Comparison of basic characteristics between the control and experimental groups.

	Control group	Experimental group	t/χ^2	*P*
Number of cycles	470	570		
Average age of female partner (years)	36.80 ± 5.08	37.25 ± 5.19	-1.413	0.856
Infertility duration (years)	4.89 ± 3.46	4.83 ± 3.69	0.542	0.430
body mass index (kg/m^2)	22.56 ± 3.21	22.72 ± 3.22	-0.802	0.515
Inner membrane thickness (mm)	9.80 ± 1.72	9.97 ± 1.83	-1.555	0.155
Average number of embryos transferred	1.87 ± 0.34	1.87 ± 0.32	-0.626	0.211
Survival rate of revived embryos (%)	98.88	98.69	0.138	0.710
Recovery embryo integrity rate (%)	97.08	96.77	0.151	0.697
Infertility type			2.031	0.154
Primary infertility	139	146		
Secondary infertility	331	424		
Endometrial preparation plan			7.550	0.056
Natural cycle	89	100		
Ovulation induction cycle	70	82		
Hormone replacement cycle (GnRH-α+HRT)	91	151		
Hormone replacement cycle (HRT)	220	237		
Transplanted embryo morphology			4.148	0.126
Excellent embryo	275	333		
Excellent embryo +non excellent embryo	114	161		
non excellent embryo	81	76		

4 Discussion

Optimizing embryo freezing, thawing, and transfer protocols is crucial for improving clinical outcomes in frozen embryo transfer (FET). The *in vitro* culture t duration of D3 cleavage-stage embryos post-thawing influences cryoprotectant removal, developmental potential, and activation. However, the optimal parameters for this process remain undetermined. Some studies suggest that pre-thawing and overnight culture of D3 cleavage-stage embryos do not significantly increase implantation or clinical pregnancy rates (9, 10). Conversely, other studies propose that restoring embryo developmental potential requires mitotic recovery, which may necessitate a longer duration, and that overnight culture could improve clinical pregnancy outcomes (11, 12). Our findings indicate that, compared to the same-day thawing group, overnight culture of D3 embryos post-thawing did not significantly enhance clinical pregnancy or implantation rates, nor did it affect early miscarriage or multiple pregnancy rates.

This outcome may be attributed to the restoration of amino acid metabolism to pre-freezing levels within the first hour after embryo thawing. In FET cycles, embryos typically recover within 2 to 3 hours post-thawing and subsequently develop normally. Thus, prolonging *in vitro* culture duration did not significantly impact embryo quality (13, 14). Moreover, prolonged *in vitro* culture may negatively affect embryos. Research indicates that an imbalance between reactive oxygen species (ROS) and antioxidants, resulting in oxidative stress (OS), is a critical factor influencing assisted reproductive technology (ART) outcomes (15). External factors, including oxygen levels, CO2 concentration, and temperature, may trigger excessive ROS production, compromising embryonic cell functions and ultimately impairing embryo development and quality. Prolonged *in vitro* culture time may also impair the embryo's DNA repair mechanisms. While the *in vitro* environment partially replicates conditions within the fallopian tube and uterus, the *in vivo* setting remains more favorable for embryo development. Thus, timely embryo-maternal interaction may better support embryo development and enhance endometrial receptivity.

Although this study did not demonstrate that overnight culture improved FET clinical pregnancy outcomes for D3 embryos, an extended culture period of 18-20 hours led to more than 90% of embryos exhibiting varying degrees of cleavage ball proliferation. In the experimental group, embryos displaying an increase of ≥4 cleavage balls had higher clinical pregnancy and implantation rates, along with a significantly lower early miscarriage rate compared to embryos without cleavage ball proliferation. This finding suggests that embryos with restored cleavage ball division may possess greater developmental potential and higher pregnancy

TABLE 2 Comparison of clinical outcomes between the control and experimental groups.

	Clinical pregnancy rate (%)	Implantation rate (%)	Early miscarriage rate (%)	Multiple birth rate (%)
control group	37.20 (175/470)	24.90 (218/877)	13.10 (23/175)	9.15 (43/470)
Experimental group	40.20 (229/570)	26.40 (283/1071)	18.80 (43/229)	10.18 (58/570)
χ^2	0.938	0.619	2.304	0.310
p	0.333	0.431	0.129	0.578

success rates. Previous studies suggest that embryos with at least two cleavage balls restored after overnight culture are more likely to achieve favorable clinical pregnancy outcomes (13). Following D3 embryo thawing, overnight culture Following survival rate, mitotic recovery, cleavage ball number, symmetry, and fragmentation, aiding in the assessment of developmental potential and prediction of FET outcomes (16). An increase in cleavage ball count indicates greater embryonic developmental potential, making embryo selection based on this criterion beneficial for enhancing clinical pregnancy and implantation rates while reducing early miscarriage rates (17). Embryos failing to exhibit cleavage ball proliferation post-thawing often present chromosomal abnormalities, with only a 20% likelihood of normal chromosomal composition. The transfer of such embryos may result in implantation failure or early miscarriage (18).

A study on the transfer of frozen embryos thawed on Day 2 and Day 3 showed that for embryos frozen on Day 2, there was no statistically significant difference in implantation rate, clinical pregnancy rate, or live birth rate between immediate transfer after thawing and transfer after overnight culture to Day 3 (19). Our study similarly found that for cleavage-stage embryos frozen on Day 3, whether they were transferred immediately after thawing or after

TABLE 3 Relationship between embryo development speed and clinical pregnancy outcomes in the pre-embolization thaw group.

	A1 group (≥4 cells)	A2 group (1-3 cells)	A3 group (No increase)	F/χ^2	*p*
Number of cycles	425	94	51		
Average age of female partner (years)	37.09 ± 5.19	38.07 ± 4.76	37.12 ± 5.81	1.388	0.251
Infertility duration (years)	4.83 ± 3.70	4.75 ± 3.63	4.35 ± 3.36	1.148	0.318
body mass index (kg/m²)	22.72 ± 3.31	22.85 ± 3.07	22.54 ± 2.78	0.456	0.634
Inner membrane thickness (mm)	10.03 ± 1.83	9.89 ± 1.83	9.55 ± 1.82	0.522	0.593
Average number of embryos transferred	1.87 ± 0.33	1.91 ± 0.28	1.86 ± 0.35	3.047	0.048
Infertility type				1.417	0.492
Primary infertility	109	21	16		
Secondary infertility	316	73	35		
Endometrial preparation plan				15.191	0.019
Natural cycle	67	24	9		
Ovulation induction cycle	65	12	5		
Hormone replacement cycle (GnRH-α+HRT)	126	17	8		
Hormone replacement cycle (HRT)	167	41	29		
Transplanted embryo morphology				36.149	0.00
Excellent embryo	273	49	11		
Excellent embryo +non excellent embryo	103	30	28		
non excellent embryo	49	15	12		
Multiple birth rate (%)	11.53	5.32	7.84	3.581	0.167
Clinical pregnancy rate (%)	44.2 (188/425)	29.8 (28/94)	25.5 (13/51)	11.711	0.003
Implantation rate (%)	29.3 (233/796)	18.3 (33/180)	17.9 (17/95)	12.935	0.002
Early miscarriage (%)	18.6 (35/188)	10.7 (3/28)	38.5 (5/13)	4.099	0.129

TABLE 4 Multivariate logistic regression analysis of factors influencing clinical pregnancy in the experimental group.

	Non pregnancy group	Pregnancy group	OR (95%CI)	*P*
Number of cycles	341	229		
Average age of female partner (years)	38.466 ± 5.188	35.450 ± 4.639	0.892 (0.859-0.927)	0.000
Infertility duration (years)	4.829 ± 3.770	4.688 ± 3.478	1.011 (0.961-1.064)	0.676
Body mass index (kg/m^2)	22.951 ± 3.046	22.380 ± 3.447	0.950 (0.898-1.005)	0.076
Average age of female partner (years)	9.770 ± 1.815	10.259 ± 1.822	1.174 (1.060-1.299)	0.002
Average number of embryos transferred	1.874 ± 0.332	1.877 ± 0.318	0.984 (0.558-1.737)	0.957
Endometrial preparation plan n (%)				
Natural cycle	60 (17.5)	40 (17.4)	–	–
Ovulation induction cycle	41 (12.0)	41 (17.9)	1.235 (0.651-2.342)	0.519
Hormone replacement cycle (GnRH-α+HRT)	95 (27.8)	56 (24.4)	0.906 (0.518-1.586)	0.730
Hormone replacement cycle (HRT)	145 (42.5)	92 (40.1)	1.126 (0.671-1.891)	0.653
Transplanted embryo morphology n (%)				
non excellent embryo	56 (16.4)	20 (8.7)	–	–
Excellent embryo +non excellent embryo	100 (29.3)	61 (26.6)	1.589 (0.831-3.038)	0.161
Excellent embryo	185 (54.2)	148 (64.6)	1.889 (1.046-3.404)	0.034
Number of blastomeres growing n (%)				
No growth	38 (11.1)	13 (5.6)	–	–
1-3 cells	66 (19.3)	28 (12.2)	1.316 (0.571-3.031)	0.519
≥4 cells	237 (69.5)	188 (82.0)	2.208 (1.064-4.581)	0.033

overnight culture, there was no statistically significant difference in clinical pregnancy outcomes. This suggests that extending the *in vitro* culture time in frozen-thawed embryo transfer (FET) cycles may not significantly improve pregnancy outcomes, and the optimal timing of embryo transfer should be determined based on individualized clinical factors.

Furthermore, in FET cycles, blastocyst transfer is generally associated with higher clinical pregnancy and live birth rates compared to cleavage-stage embryo transfer (20). However, blastocyst transfer has also been linked to an increased incidence of pregnancy-induced hypertension, placental abnormalities, perinatal mortality, preterm birth, and large-for-gestational-age infants (21). A potential mechanism for these adverse effects is that prolonged *in vitro* culture alters the epigenetic programming of the embryo due to changes in culture medium composition and oxygen tension, potentially impacting neonatal outcomes. Additionally, during assisted reproductive technology (ART) procedures, suboptimal culture conditions may prevent some genetically normal embryos from developing into blastocysts. Consequently, extended culture may lead to the loss of potentially viable embryos that could have been cryopreserved and used clinically.

This study offers a novel perspective by analyzing the clinical outcomes of Day 3 cleavage-stage embryos cultured overnight (to Day 4) after thawing. While previous studies have shown that Day 4 morula-stage embryos have higher implantation potential than embryos transferred immediately after thawing, our findings add depth by identifying subgroups based on blastomere division during overnight culture. Embryos with ≥4 divisions showed better clinical pregnancy rates and lower miscarriage rates, emphasizing the value of morphological assessment. Although some studies report no significant differences between short-term and long-term culture (8, 10), our results align with research indicating that extended culture enhances embryo selection by identifying those with greater developmental potential (16).

In this study, the correlation between factors such as female age, duration of infertility, and endometrial thickness with clinical pregnancy rate was analyzed. Previous studies have pointed out that factors such as female age, endometrial thickness on the day of transfer, body mass index (BMI), and the number of embryos transferred can all influence the outcomes of FET (22). The results of this study suggest that female age is negatively correlated with clinical pregnancy rate. As age increases, the probability of embryo-endometrial synchrony decreases, the likelihood of chromosomal abnormalities in the embryos increases, and the clinical pregnancy rate decreases. Endometrial thickness is a commonly used indicator for predicting endometrial receptivity. Studies have shown that in FET cycles, when the endometrial thickness is less than 7mm, the

clinical pregnancy rate and live birth rate significantly decrease (23). Infertility duration, endometrial thickness and number of embryos transferred might affect the live birth rate after frozen embryo transfer among young women. This result could help inform clinical decisions and counseling to increase the live birth rate after frozen embryo transfer among young women (24). Therefore, although endometrial thickness has some impact on FET outcomes, its role in different age groups still requires further research.

This study has several limitations. First, its retrospective design and single-center setting may introduce selection bias and limit generalizability. Second, some confounding variables, such as embryo genomic stability and hormone levels, were not fully controlled, which could influence the results. Third, grouping embryos based on blastomere division provided useful insights but was somewhat subjective and did not fully assess long-term developmental potential. Additionally, the study lacked long-term follow-up data, leaving the extended impact of embryo thawing and culture on pregnancy outcomes unexamined. Lastly, compacted embryos were included in the cleavage-stage analysis without separate classification to focus on overall developmental potential and clinical outcomes. While the compaction stage could provide additional insights, this study aimed to evaluate the broader implications of extended culture. Future research should consider detailed classifications to explore this further.

In conclusion, this study indicates that D3 embryos with an increase in the number of blastomeres to more than four or entering the compaction stage after overnight culture have better pregnancy outcomes. Female age and endometrial thickness are important factors influencing clinical pregnancy rates. Optimizing culture conditions and ensuring optimal endometrial thickness may help improve the success rate of frozen-thawed embryo transfer.

Data availability statement

All relevant data is contained within the article: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving humans were approved by Ethics Committee of Yulin Maternal and Child Health Hospital (Ethical approval number: YLSFYLLKY2025-02-05-1). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

LL: Writing – original draft, Data Curation, Validation. XL: Conceptualization, Writing – original draft, Writing – review & editing, Formal analysis, Software. BL: Methodology, Investigation, Writing – review & editing, Visualization. WL: Resources, Project administration, Writing – review & editing. LZ: Conceptualization, Writing – original draft, Writing – review & editing. JZ: Validation, Writing – review & editing. JW: Investigation, Data Curation, Writing – review & editing. KF: Software, Formal analysis, Writing – review & editing. DL: Funding acquisition, Supervision, Writing – review & editing. FL: Writing – original draft, Writing – review & editing, Visualization. GD: Methodology, Resources, Writing – review & editing. YL: Writing – original draft, Writing – review & editing, Supervision, Funding acquisition. ZY: Conceptualization, Writing – original draft, Writing – review & editing, Supervision.

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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.

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Clinical outcomes of frozen–thawed single blastocyst transfer derived from low-quality day 3 embryos: A retrospective cohort study

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Purpose: Our aim was to explore the clinical outcomes of a single blastocyst frozen–thawed transfer (single blastocyst frozen–thawed transfer (singleton frozen embryo transfer, sFET) derived from low-quality day 3 (D3) embryos.

Methods: This retrospective cohort study was conducted at the Reproductive Health Center of the Third Affiliated Hospital of Zhengzhou University. All data on sFET were collected between March 2016 and September 2022. Blastocysts derived from good-quality and low-quality D3 embryos were designated as the good-quality group and the low-quality group, respectively. Patients were divided into three groups according to age: <35 group, 35–39 group, and ≥40 group. Based on whether preimplantation genetic testing (PGT) was performed or not, the blastocysts derived from low-quality embryos were divided into the PGT group and the non-PGT group, respectively.

Results: After adjusting for female age, male age, infertility duration, and other potential confounders, the difference in the clinical pregnancy rate and the live birth rate in the good quality and low-quality groups maintained statistical significance [adjusted odds ratio adjusted odds ratio (aOR) = 0.32 and 0.35, $p < 0.001$]. When adjusting for embryo quality, the clinical pregnancy rate and the live birth rate in the <35 and 35–39 groups were significantly higher than those in the ≥40 group (OR = 3.02 and 3.56, $p < 0.001$; OR = 1.89 and 1.84, $p < 0.001$). Embryo quality significantly affected the clinical pregnancy rate and the live birth rate ($p < 0.001$). The clinical pregnancy rate and the live birth rate in the PGT group were higher than those in the non-PGT group (40.0% *vs*. 29.3% and 40.0% *vs*. 22.0%, respectively).

Conclusion: D3 embryos with low score/low quality can still obtain a certain live birth rate after further culturing to blastocysts with PGT.

KEYWORDS

low-quality embryos, morphological score, frozen-thawed transfer, single blastocyst, clinical outcomes

Introduction

In the process of *in vitro* fertilization embryo transfer (IVF-ET) treatment, the majority of reproductive centers select high-quality or high-morphological-score embryos for transplantation or freezing after performing morphological scoring on the third day (D3) after fertilization. Low-quality embryos with poor developmental potential would be discarded after informed consent. However, it is still controversial whether low-quality D3 embryos have clinical value or not (1). Emerging evidence indicates that vitrified–thawed blastocysts originating from poor-quality D3 embryos are capable of establishing viable pregnancies and delivering healthy offspring (2, 3). Stecher et al. demonstrated that culturing low-quality D3 embryos to blastocysts prior to vitrification could improve the utilization rate of embryos and the cumulative pregnancy rate of cycles (4). The above studies indicate that even low-quality D3 embryos may still show better developmental potential during blastocyst culture.

Studies have shown that a large proportion of embryos with high morphological scores may be aneuploid, while some low-quality D3 embryos may also be euploid (5, 6). However, in the majority of cases, the correlations between aneuploidy and the morphologies of embryos have been weak (5).

Clinically, due to advanced age, decreased ovarian reserve, and other reasons, some patients do not have high-quality embryos. For these patients, the use of embryos with low quality and poor development potential will be of great significance. This study aimed to explore the clinical pregnancy outcomes of a single blastocyst frozen–thawed transfer derived from low-quality D3 embryos.

Materials and methods

Participants

This study was conducted at the Reproductive Health Center of the Third Affiliated Hospital of Zhengzhou University. All data on single blastocyst frozen–thawed transfer (singleton frozen embryo transfer, sFET) were collected between March 2016 and September 2022.

The inclusion criteria were as follows: 1) infertile couples who had experienced IVF-ET due to female tubal factors and male factors, among others, and 2) sFET. The exclusion criteria were as follows: 1) patients who donated sperm or oocytes; 2) patients with incomplete medical records; and 3) patients who had experienced recurrent implantation failure.

All sFETs derived from low-quality D3 embryos were divided into the pre-implantation genetic testing (PGT) group and the non-PGT group based on whether PGT was performed.

Methods

Ovarian stimulation program

Based on the woman's age and ovarian reserve function, the clinician would formulate an appropriate scheme for ovulation promotion (7). Oocyte retrieval was performed under ultrasound guidance at 36–38 h after human chorionic gonadotropin (hCG) injection.

In vitro fertility and embryo culture

Based on oocyte maturity and sperm quality on the day of oocyte retrieval, the oocytes were inseminated via *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) at 38–40 h after hCG injection. After 16–18 h, the appearance of two evident pronuclei indicates fertility. The zygotes were cultured in the cleavage medium (G-1 PLUS; Vitrolife, Gothenburg, Sweden).

The embryos were transferred from the cleavage medium into the blastocyst medium (G-2; Vitrolife, Gothenburg, Sweden) on day 3 after insemination for development into blastocysts. Subsequently, they were cultured until day 7 after insemination in humidified air maintained at 37°C under a 6% CO_2 and 5% O_2 atmosphere. The development of blastocysts was observed and scored during this period.

In non-PGT treatment cycles, the blastocysts were either transferred in the fresh cycle or cryopreserved for subsequent FET based on the clinical indications and patient-specific factors. For the PGT cycles, once the blastocysts had developed, three to five trophoblast cells were taken for genetic testing, and then the blastocysts were cryopreserved. The decision to perform the transplantation.

Embryo score

D3 embryos were scored according to the following criteria: grade I—blastomere number (BL) of 6–10, of equal size, and fragmentation (FR) = 0%-5%; grade II—BL = 6–10, slightly equal in size, and FR = 5%–24%; grade III—BL = 6–10, unequal in size, and FR = 25%–49% or BL = 4–5 or >10; and grade IV—severely unequal-sized blastomeres, or FR > 50%, or embryo arrest. Grades I, II, and III indicate good-quality embryos, of which grades I and II are top-quality, and grade IV indicates low-quality embryos.

In our center, dependent on the situation of the patients, one or two good-quality embryos on D3 were chosen for freezing or transfer, while the others were cultured and frozen when they developed into blastocysts.

The blastocysts were observed and scored according to Gardner (8) on D5, D6, and D7 after insemination. Blastocysts at stage 3 or higher with an inner cell mass (ICM) score ≥B were considered for transfer or freezing. Blastocysts that scored 4BB or higher were considered top-quality blastocysts. Blastocysts derived from good-quality D3 embryos and bad-quality D3 embryos were defined as the good-quality group and the low-quality group, respectively.

Vitrification and warming of blastocysts

Vitrification and warming of the blastocysts were carried out according to the instructions in the Vit Kit (Kitazato Biopharma, Shizuoka, Japan). Before vitrification, the Vit Kits were stored at room temperature for at least 30 min. First, the blastocysts were incubated for 10 min in an equilibration solution, followed by a vitrification solution for 60 s. Subsequently, the blastocysts were placed in a carrier before being loaded into a cannula in liquid nitrogen. During warming, the cannula was taken off, the carrier end was rapidly immersed in a thawing solution (TS) at 37°C, and the

blastocyst was kept there for 1 min. Then, the blastocyst was transferred to a diluent solution for 3 min, followed by washing solutions 1 and 2 for 3 min. Finally, the blastocyst was placed in blastocyst medium (G-2; Vitrolife, Gothenburg, Sweden) for transfer.

Endometrial preparation

The routine scheme of our center (9) was adopted for the endometrial preparation scheme of the FET cycle, which is selected according to the specific situation of the patient. Currently, the natural cycle, artificial cycle, and stimulation cycle are often used. For patients with regular menstruation and normal ovulation, natural cycles are adopted. Artificial cycles were used for patients with anovulation, luteal insufficiency, and a thin endometrium. Stimulation cycles are used for patients with follicular dysplasia, ovulation disorders, polycystic ovarian syndrome (PCOS), or contraindications to estrogen use.

Evaluation of pregnancy outcome

A serum β-hCG level ≥50 IU/L on day 14 after transfer, along with a gestational sac observed in the intrauterine cavity on day 35 after transfer, indicated clinical pregnancy. According to the American Society for Reproductive Medicine (ASRM), a miscarriage is defined as a termination of pregnancy at <20 weeks of gestation with a fetal weight of less than 500 g. A live birth is defined as a pregnancy reaching 28 weeks of gestation and resulting in the delivery of a live neonate.

Methods for calculating clinical indicators

The clinical indicators were determined as follows: Clinical pregnancy rate = count of clinical pregnancy cycles/count of transfer cycles × 100%; live birth rate = count of live birth cycles/count of transfer cycles × 100%; Abortion rate = count of abortion cycles/count of transfer cycles × 100%; and euploidy rate = count of euploid embryos/count of embryos with PGT.

Statistical analysis

All data were analyzed using SPSS 25.0. Measurement data are indicated as mean ± standard deviation (SD), and continuous variables were analyzed using a *t*-test. Count data are shown as percentages. Chi-square analysis was used to compare the rates between groups. Logistic regression was applied to control for confounding factors. A *p*-value less than 0.05 was considered statistically significant.

Results

Comparison of the basic clinical data and the clinical outcomes between the two groups

A total of 10,146 sFET cycles were compared in this study, of which 9,842 were in the good quality group and 304 were in the low-quality group. Female age, male age, and infertility duration in the good-quality group were all significantly lower than those in the low-quality group ($p < 0.05$). In addition, compared with the good-quality group, the clinical pregnancy rate and the live birth rate were significantly lower in the low-quality group ($p < 0.001$). After adjusting for female age, male age, infertility duration, and other potential confounders, the difference maintained statistical significance [adjusted odds ratio (aOR) = 0.32 and 0.35, $p < 0.001$] (Table 1).

Effect of age on the pregnancy outcomes from sFET in the good-quality and low-quality groups

Patients were divided into three different age groups: <35 years old (the <35 group), 35–39 years old (the 35–39 group), and ≥40 years old (the ≥40 group). The effects of age on the pregnancy outcomes from sFET in the good-quality and low-quality groups were compared.

A comparative analysis of the blastocyst quality scores between the two groups was performed. The analysis revealed a significantly higher proportion of top-quality blastocysts in the good-quality group compared with the low-quality group (57.1% *vs.* 12.5%, $p < 0.01$). Moreover, the rate of bad-quality blastocysts in the low-quality group was higher than that of the good-quality group (42.9% *vs.* 87.5%, $p < 0.01$) (Figure 1).

In the same age group, the clinical pregnancy rate and the live birth rate of the good quality group were higher than those in the low-quality group, and the difference between the <35 group and the 35–39 group was statistically significant ($p < 0.05$) (Table 2).

Multivariate regression analysis was performed to control for confounding factors (embryo quality and age). After adjusting for embryo quality, the clinical pregnancy rate and the live birth rate in the <35 group were significantly higher than those in the ≥40 group (OR = 3.02 and 3.56, $p < 0.001$). The pregnancy and live birth rates in the 35–39 group were still higher than those in the ≥40 group, but the odds ratios decreased (OR = 1.89 and 1.84, $p < 0.001$). Embryo quality significantly affected the clinical pregnancy rate and the live birth rate ($p < 0.001$) (Table 3; Supplementary Table S1).

Euploidy rates of the good-quality group and the low-quality group in the PGT cycles

The results of the 650 cycles of sFET with PGT from March 2016 to September 2022 were included. The euploidy rate of the blastocysts from the low-quality group was higher than that of the good-quality group; however, the difference was not statistically significant ($p = 0.561$) (Table 4).

Basic clinical data and outcomes of the sFET cycles derived from low-quality embryos in the PGT and non-PGT groups

There were 15 and 413 sFET cycles derived from low-quality embryos in the PGT and non-PGT groups, respectively. Compared

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TABLE 1 Comparison of basic clinical data and clinical outcomes among the two groups.

Basic clinical data and outcomes	Good-quality group (*n* = 9,842)	Low-quality group (*n* = 304)	*p*-value	aOR (95%CI)[a]	a*p*-value[a]
Female age (years)	31.7 ± 4.6	32.9 ± 5.1	<0.001	–	–
Male age (years)	32.5 ± 5.2	33.8 ± 5.8	<0.001	–	–
Female body mass index (kg/m^2)	24.0 ± 3.4	23.8 ± 3.1	0.35	–	–
Infertility type (%)			0.52	1.05 (0.83–1.33)	0.68
Primary infertility	3,584 (36.4%)	116 (38.2%)			
Secondary infertility	6,258 (63.6%)	188 (61.8%)			
Main cause of infertility (%)			<0.001		
Female subjects	6,082 (61.8%)	227 (74.7%)			
Male subjects	1,575 (16.0%)	33 (11.0%)			
Mixed	2,126 (21.6%)	41 (13.2%)			
Other	59 (0.6%)	3 (1.1%)			
Infertility duration (years)	3.2 ± 2.6	3.7 ± 3.4	0.001	1.04 (1.01–1.08)	0.02
Endometrial preparation method			0.032		
Artificial cycle	4,652 (47.3%)	328 (50.1%)		1.00 (reference)	–
Natural cycle	3,997 (40.6%)	271 (41.4%)		0.98 (0.82–1.17)	0.82
Stimulated cycle	1,193 (12.1%)	56 (8.5%)		0.79 (0.58–1.08)	0.14
Clinical pregnancy rate	5,721 (58.0%)	83 (27.3%)	<0.001	0.32 (0.25–0.41)	<0.001
Miscarriage rate	1,059 (10.7%)	20 (6.6%)	0.021	0.72 (0.45–1.16)	0.18
Live birth rate	4,662 (47.2%)	63 (20.7%)	<0.001	0.35 (0.26–0.46)	<0.001

[a]Adjusted for female age, male age, infertility duration, infertility type, and endometrial preparation method.

with the non-PGT group, the clinical pregnancy rate and the live birth rate in the PGT group were higher; moreover, the miscarriage rate in the PGT group was lower. No significant differences were found ($p > 0.05$) (Table 5).

Discussion

Morphological assessment continues to serve as the primary method for evaluation of embryo development potential. However, it has several limitations. Morphological scoring relies on visual assessment, which can be subjective. Additionally, morphology does not detect chromosomal abnormalities (aneuploidy), which are a major cause of implantation failure and miscarriage (10). Furthermore, high morphological scores do not always correlate with successful implantation or live birth (11, 12). In addition, many embryos may receive the same high score, making it difficult to choose the single best one for transfer.

In assisted reproductive technology, embryos with poor morphological scores are generally considered to have lower developmental potential. However, emerging research has demonstrated that some low-morphological-score embryos may undergo self-repair mechanisms to restore normal development and even achieve successful pregnancies (13, 14). Embryos with significant fragmentation (>25%) can undergo intrinsic repair processes during blastocyst development. These self-repair mechanisms, including lysosomal degradation of cellular fragments, enable certain fragmented embryos to achieve morphological normalization and to develop into viable blastocysts for transfer (15). It has been demonstrated that, during the development of D3 embryos into blastocysts, with the activation of the embryo genome, embryos with genetic and metabolic defects will be naturally eliminated, and a portion of these embryos appear to be able to repair themselves and eventually develop into blastocysts (16–18). Furthermore, with the development of blastocyst culturing and freezing technology, low-quality embryos will still have the potential to develop into blastocysts, even into high-quality blastocysts when cultured

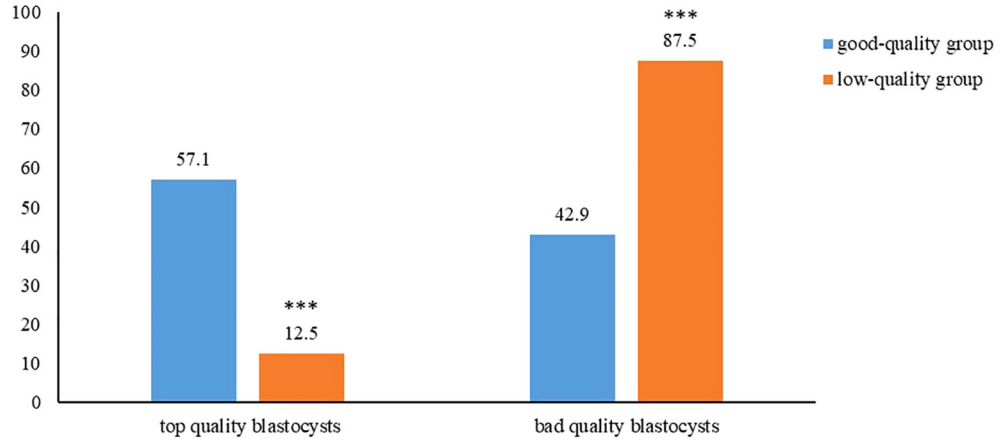

FIGURE 1
The comparison of the proportions of high-quality blastocysts and low-quality blastocysts between the two groups. Top quality blastocysts shows 57.1% in the good-quality group (blue) and 12.5% for the low-quality group (orange). Bad quality blastocysts shows 42.9% for good-quality and 87.5% for low-quality.

TABLE 2 Comparison of pregnancy outcomes between good-quality and low-quality groups within the same age range.

Group	Good-quality group (*n* = 9,842)	Low-quality group (*n* = 304)	*p*-value
<35 years			
ET cycles	7,409	205	
Clinical pregnancy rate	4,548 (61.4%)	55 (26.8%)	0.000
Miscarriage rate	697 (9.4%)	9 (4.4%)	<0.001
Live birth rate	3,829 (38.9%)	46 (22.4%)	0.000
35–39 years			
ET cycles	1,809	64	
Clinical pregnancy rate	957 (52.9%)	20 (31.3%)	0.000
Miscarriage rate	259 (14.3%)	7 (10.9%)	0.416
Live birth rate	689 (38.1%)	13 (20.3%)	0.000
≥40 years			
ET cycles	624	35	
Clinical pregnancy rate	216 (34.6%)	8 (22.9%)	0.153
Miscarriage rate	70 (11.2%)	3 (8.6%)	0.591
Live birth rate	144 (23.1%)	5 (14.3%)	0.226

ET, embryo transfer.

in vitro (18). Studies have shown that the blastocysts derived from low-quality embryos have the potential to deliver healthy babies successfully after freezing and thawing (3). In this study, it was found that the clinical pregnancy rate and the live birth rate were significantly lower in the low-quality group than in the high-quality group. Although the results revealed that embryo quality is an independent predictor of pregnancy outcomes, live birth rates in patients who underwent freeze–thaw transfer of single blastocysts derived from low-quality D3 embryos accounted for 20.7%, which may be related to the self-repair function of the embryo (19).

TABLE 3 Comparison of pregnancy outcomes of blastocyst transfer from the good-quality group and the low-quality group in different age groups.

Variable	Clinical pregnancy rate: aOR (95%CI)	*p*-value	Live birth rate: aOR (95%CI)	*p*-value
Age group				
<35 years	3.15 (2.67–3.72)	<0.001	3.62 (2.99–4.38)	<0.001
35–39 years	1.91 (1.58–2.31)	<0.001	1.87 (1.51–2.32)	<0.001
Embryo quality				
Good-quality (*vs.* low-quality)	2.92 (2.24–3.80)	<0.001	2.98 (2.23–3.98)	<0.001

Adjusted for embryo quality. Reference group: ≥40 years
aOR, adjusted odds ratio.

TABLE 4 Comparison of the euploidy rates in the good-quality group and the low-quality group in pre-implantation genetic testing (PGT) cycles.

	High-quality group (*n* = 635)	Low-quality group (*n* = 15)	*p*-value
Euploidy rate			0.561
No	73 (11.5%)	1 (6.7%)	
Yes	562 (88.5%)	14 (93.3%)	

TABLE 5 Basic clinical data and outcomes of single blastocyst frozen–thawed transfer (sFET) cycles derived from low-quality embryos of pre-implantation genetic testing (PGT) and non-PGT.

Basic clinical data and outcomes	PGT cycles (*n* = 15)	Non-PGT cycles (*n* = 413)	*p*-value
Female age (years)	31.1 ± 4.7	32.3 ± 5.0	0.27
Male age (years)	33.5 ± 5.5	33.3 ± 5.5	0.58
Female body mass index (kg/m^2)	23.9 ± 3.1	23.7 ± 3.0	0.87
Infertility type (%)			0.55
Primary infertility	7 (46.7%)	161 (39.0%)	
Secondary infertility	8 (53.3%)	252 (61.0%)	
Infertility duration (years)	3.0 ± 2.6	3.7 ± 3.2	0.41
Main cause of infertility (%)			0.39
Female subjects	13 (86.7%)	314 (76.5%)	
Male subjects	1 (6.7%)	37 (8.9%)	
Mixed	1 (6.7%)	62 (15.0%)	
Endometrial preparation method			0.05
Artificial cycle	11 (73.3%)	202 (48.9%)	
Natural cycle	3 (20.0%)	184 (44.6%)	
Stimulated cycle	1 (6.7%)	27 (6.5%)	
Clinical pregnancy rate	6 (40.0%)	121 (29.3%)	0.37
Miscarriage rate	0 (0%)	26 (6.3%)	0.28
Live birth rate	6 (40.0%)	91 (22.0%)	0.10

Age is another independent factor affecting assisted reproductive technology pregnancy outcomes. In this study, it was found that the parental ages in the low-quality group were significantly higher than those in the high-quality D3 group, suggesting that the advanced age of couples can affect the embryo quality in the cleavage stage. Consistent with a previous study, we found that whether the transplanted blastocysts were from the good-quality or the low-quality D3 group, the clinical pregnancy rate and the live birth rate

in the <35-year-old group were highest (20). In the same age group, the live birth rate of the good-quality D3 group was higher than that of the low-quality group. In addition, age is an independent factor affecting live birth rates. With advancing age, the aneuploidy rate of embryos increases by 10%, which is also the main cause of embryo implantation failure and abortion in elderly (≥40 years old) patients during IVF cycles (21, 22).

Aneuploidy is the main cause of spontaneous abortion. Munne et al. reported that the aneuploidy rate of embryos was 63% and that the chromosome aneuploidy rate of embryos in the low-quality group was higher than that of the good-quality group in women aged 35–37 years (23). However, Lee et al. reported that the most obvious association between chromosomes and morphology concerned embryo gender rather than aneuploidy (24). In this study, there was no statistically significant association between the morphological score and the euploidy rate ($p = 0.561$). Nevertheless, the small sample size in the low-quality PGT group limited the robustness of our findings; thus, this analysis should be considered hypothesis-generating rather than conclusive. More studies with larger sample sizes are needed to confirm these findings. Our findings were also consistent with the conclusion of Lee et al., who used a different scoring system.

For those patients who do not have any embryos, low-quality embryos can be cultured into blastocysts, which can give them a chance for transfer and even a successful pregnancy. Moreover, we compared the clinical outcomes in PGT and non-PGT cycles of the blastocysts from the low-quality group. It was found that the clinical pregnancy rate and the live birth rate of the blastocysts from the low-quality group in the PGT cycles were higher than those in the non-PGT cycles. These results indicate that biopsy and PGT significantly enhance blastocyst utilization efficiency in the low-quality group while reducing unnecessary embryo transfers.

This study did not evaluate neonatal outcomes (such as birth defects and preterm birth), which is a significant limitation. Although there is controversy in the existing literature (25) regarding the association between embryo quality and perinatal outcomes, low-quality D3 embryos may be subjected to additional stress when cultured *in vitro* to the blastocyst stage, theoretically increasing the risk (26, 27). Future research should verify this hypothesis through the design of birth cohort studies (such as follow-up until infancy).

In summary, although embryos with development potential can be screened by further cultivation to blastocysts, the aneuploidy rate of blastocysts from low-quality embryos is still high. Thus, for infertile couples without good-quality D3 embryos, blastocyst culture of low-quality embryos and PGT can be performed to obtain euploid blastocysts, which can improve the clinical pregnancy and live birth rates.

Data availability statement

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

Ethics statement

This research was approved by the Ethics Committee of the Third Affiliated Hospital of Zhengzhou University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

XZ: Data curation, Funding acquisition, Writing – original draft. QZ: Supervision, Writing – review & editing. YG: Supervision, Validation, Writing – review & editing.

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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.

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The author(s) declare that no Generative AI was used in the creation of this manuscript.

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Supplementary material

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Comparison of clinical outcomes between flexible antagonist protocol and long luteal phase protocol in patients with normal ovarian reserve function: a prospective cohort study

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Objective: Ovarian stimulation protocols play a pivotal role in the success of *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) treatments. This study compares the clinical outcomes of the long luteal phase GnRH agonist protocol and the flexible GnRH antagonist protocol in patients with normal ovarian reserve.

Methods: This prospective cohort study was conducted at the Reproductive Medicine Center, Sanmenxia Hospital, Yellow River, from March 2021 to September 2023. Patients with normal ovarian reserve were enrolled and randomly assigned by a 1:3 ratio to either the long luteal phase protocol (Group A, n=42) or the flexible antagonist protocol (Group B, n=118). Data on patient characteristics, ovarian response, and embryological outcomes were collected and analyzed. Clinical outcomes, including clinical pregnancy, live birth rates, and ovarian hyperstimulation syndrome (OHSS) incidence, were assessed. Multivariate logistic regression was conducted to identify risk factors associated with clinical pregnancy.

Results: There were no significant differences in baseline characteristics between the two groups ($P>0.05$). In terms of primary clinical outcomes, there were no significant differences in clinical pregnancy rate (54.8% *vs*. 56.8%, $P=0.092$), live birth rate (47.6% *vs*. 52.5%, $P=0.278$), or incidence of OHSS (0% *vs*. 2.5%, $P=0.055$) between Group A and Group B. Multivariable logistic regression analysis identified significant predictors of clinical pregnancy, including younger age (OR = 0.956, $P = 0.042$), higher AFC (OR = 1.127, $P = 0.018$), higher AMH levels (OR = 1.357, $P = 0.005$), greater endometrial thickness (OR = 1.162, $P = 0.021$), higher number of oocytes retrieved (OR = 1.234, $P = 0.023$), and better embryo quality (Grade I-II) (OR = 1.485, $P = 0.002$). No significant differences were observed between age-related subgroups ($P>0.05$), but success rates decreased with increasing age, highlighting age as a key factor influencing IVF/ICSI outcomes.

Conclusion: The study found no significant differences in primary clinical outcomes between the two groups. However, younger age, higher AFC, higher AMH levels, greater endometrial thickness, higher number of oocytes retrieved, and better embryo quality were significant predictors of clinical pregnancy.

KEYWORDS

ovarian stimulation protocols, clinical pregnancy, GnRH agonist, GnRH antagonist, IVF/ICSI outcomes, normal ovarian reserve

Introduction

Infertility is a common and distressing condition affecting approximately 10-15% of reproductive-aged couples worldwide. Assisted reproductive technologies (ART), particularly *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI), have revolutionized the management of infertility. Central to the success of IVF/ICSI is controlled ovarian hyperstimulation (COH), which aims to recruit multiple follicles to maximize the number of mature oocytes for fertilization (1). Optimal ovarian stimulation protocols are particularly crucial for achieving successful pregnancy outcomes, especially in patients with normal ovarian reserve, who typically have a favorable response to stimulation protocols (1, 2). However, the selection of the most appropriate stimulation protocol for this patient population remains a subject of ongoing debate.

The long luteal phase gonadotropin-releasing hormone agonist (GnRH-a) protocol has long been regarded as the standard approach for ovarian stimulation. This protocol involves the administration of a GnRH-a during the luteal phase of the preceding cycle to downregulate the hypothalamic-pituitary-ovarian axis, suppressing premature luteinizing hormone (LH) surges and allowing for more controlled follicular development. Ovarian stimulation is initiated after sufficient downregulation has been achieved, usually after 14 days of GnRH-a administration (3). Although this protocol has been associated with favorable clinical outcomes, including higher pregnancy and live birth rates, its extended duration and the potential for side effects such as ovarian hyperstimulation syndrome (OHSS) and flare-ups during downregulation pose challenges for some patients (4, 5). In contrast, the flexible GnRH antagonist protocol, developed as an alternative to the traditional GnRH-a protocol, offers a shorter and more patient-friendly approach. This protocol involves the administration of a GnRH antagonist during the follicular phase when the leading follicle reaches a diameter of 12–14 mm, effectively suppressing premature LH surges without the need for extended downregulation. The shorter duration of treatment and reduced risk of OHSS make the antagonist protocol an attractive option for both patients and clinicians (6). Additionally, the antagonist protocol has demonstrated comparable pregnancy outcomes to the agonist protocol in several studies (7, 8). However, concerns remain regarding its effectiveness in specific patient populations, such as those with normal ovarian reserve, where a more aggressive stimulation approach may be beneficial.

Patients with normal ovarian reserve, characterized by adequate antral follicle counts (AFC) and anti-Müllerian hormone (AMH) levels, typically respond well to ovarian stimulation. However, the optimal stimulation protocol for this population remains contentious. While the long luteal phase protocol may offer advantages in terms of controlled follicular development and a more predictable response, it is associated with higher risks of OHSS due to the recruitment of a larger number of follicles. The flexible antagonist protocol, on the other hand, may reduce the risk of OHSS but could lead to suboptimal ovarian response and lower pregnancy rates in some patients (9). As such, there is a need for robust comparative studies to determine which protocol is more effective in achieving successful pregnancy outcomes while minimizing adverse effects in patients with normal ovarian reserve. Recent studies have begun to shed light on this issue (10–13). This study aims to address these gaps by conducting a comprehensive comparison of clinical outcomes between the flexible antagonist protocol and the long luteal phase protocol in patients with normal ovarian reserve. Specifically, we seek to evaluate clinical pregnancy rates, live birth rates, and the incidence of OHSS, as well as secondary outcomes such as embryo quality, fertilization rates, and the number of oocytes retrieved.

Patients and methods

Study design

This prospective cohort study was conducted between March 2021 and September 2023 at the Reproductive Medicine Center, Sanmenxia Hospital, Yellow River. The study was complied with the guidelines of the Declaration of Helsinki and relevant national regulations. The study protocol has been reviewed and approved by the hospital's institutional ethics committee (No: 2024LLS20240914043), and all participants have provided written informed consent.

Population

A total of 341 patients were initially assessed for eligibility. Following the application of inclusion and exclusion criteria, 172 patients met the eligibility requirements and were included in the

study. Participants were then allocated into two groups using a 1:3 ratio: 43 patients were assigned to Group A (Long Luteal Phase Protocol) and 129 to Group B (Flexible Antagonist Protocol). During follow-up, 1 patient in Group A and 11 patients in Group B were lost to follow-up. As a result, 42 patients in Group A and 118 patients in Group B were included in the final analysis (Figure 1). The 1:3 allocation ratio was based on the proportional use of protocols in clinical practice at our center during the study period. The flexible antagonist protocol (Group B) was more commonly utilized due to its shorter duration and lower risk of OHSS, whereas the long agonist protocol (Group A) was reserved for specific cases.

Inclusion Criteria were as follow: (1)Women included in this study were aged less than 40 years, with normal menstrual cycles (25–35 days) in the preceding three months, and AFC ranging from 8 to 15. (2)Baseline serum (FSH levels were ≤10 U/L, and AMH levels were >1.1 ng/mL. (3)All patients met the established criteria for IVF/ICSI treatment. Exclusion Criteria were as follow: (1) presence of chromosomal abnormalities in either partner, uterine abnormalities (e.g., unicornuate uterus, bicornuate uterus, or septate uterus), (2) any contraindications for IVF-embryo transfer (IVF-ET).

Data collection

Data collection was meticulously carried out to record both biochemical and clinical outcomes following embryo transfer, including key demographic, clinical, and outcome variables. Baseline variables included patient age, body mass index (BMI), duration of infertility, AFC, AMH levels, and baseline serum FSH. During ovarian stimulation, data on gonadotropin dose, stimulation duration, endometrial thickness, estradiol and progesterone levels on trigger day, and the number of follicles ≥18 mm were recorded. Embryology-related variables included the number of oocytes retrieved, mature oocyte count, fertilization rate, and embryo quality on day 3.

Treatment protocols

Long Luteal Phase Protocol: this protocol involved the initiation of a GnRH agonist in the mid-luteal phase of the preceding menstrual cycle to achieve downregulation of the hypothalamic-pituitary-ovarian axis. After 14 days of downregulation, COH was commenced using recombinant human FSH (rFSH) or urinary-derived gonadotropins. The gonadotropin dose was adjusted based on patient age, body weight, and AFC. Ovulation was triggered with human chorionic gonadotropin (hCG) once at least three follicles reached a diameter of ≥18 mm, and oocytes were retrieved 36 hours after the trigger.

Flexible Antagonist Protocol: this protocol began with COH initiated on day 2 or 3 of the menstrual cycle. The starting dose of gonadotropins was individualized according to ovarian response as assessed by transvaginal ultrasound (B-ultrasound) and serum estradiol (E2) levels. When the leading follicle reached 12 mm in

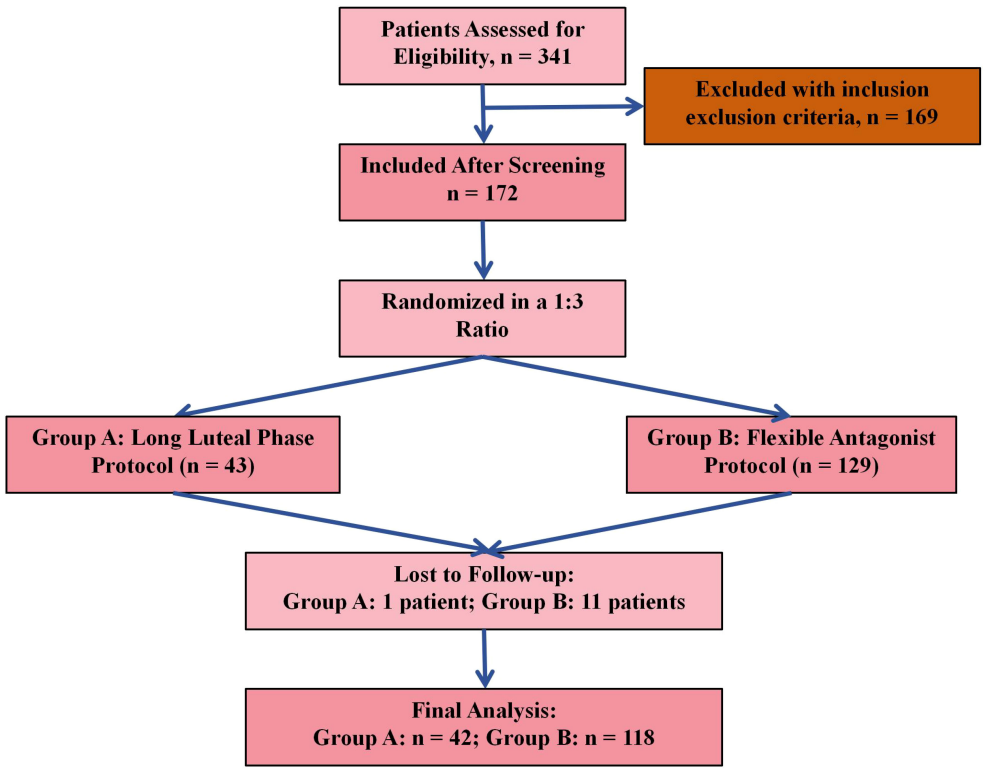

FIGURE 1
Flow chart.

diameter, a GnRH antagonist was administered to prevent premature LH surges. Ovulation was triggered using a dual trigger (hCG combined with a GnRH agonist) once two or more follicles reached ≥18 mm, and oocyte retrieval occurred 36 hours post-trigger.

Embryo assessment and transfer

Embryos were assessed on day 3 post-fertilization according to standard morphologic criteria. Grading was based on the number of blastomeres, degree of fragmentation, and uniformity of blastomere size, with grades I and II considered high-quality embryos. Embryos of higher quality were prioritized for transfer. In cases of a high risk for OHSS— characterized by serum E2 levels exceeding 4000 pg/mL or retrieval of more than 15 oocytes— a freeze-all strategy was employed. This involved cryopreservation of all viable embryos and postponement of the embryo transfer to a subsequent frozen embryo transfer (FET) cycle to minimize the risk of OHSS.

Study outcome

Patients with confirmed clinical pregnancies were followed at regular intervals to ensure continued pregnancy viability and to monitor for complications. The first follow-up was conducted at 12 weeks of gestation, focusing on the viability of the pregnancy and the assessment of early fetal development. A second follow-up was carried out at 24 weeks of gestation to further assess fetal growth and maternal health. Finally, all patients were followed up 2 weeks postpartum to document delivery outcomes, including birth weight, gestational age at delivery, and any neonatal complications.

The primary outcomes included: (1) Clinical pregnancy rate: defined as the number of clinical pregnancy cycles per number of transfer cycles × 100%. Clinical pregnancy was confirmed by the presence of at least one gestational sac with a fetal heartbeat detected via transvaginal ultrasound at 6–8 weeks of gestation. This calculation includes both fresh embryo transfer cycles and the first frozen embryo transfer (FET) cycle for patients undergoing a freeze-all strategy, with each patient contributing one transfer cycle. (2) Live birth rate: defined as the number of live births per number of transfer cycles × 100%. A live birth is defined as the delivery of at least one living infant at or beyond 24 weeks of gestation. This calculation includes both fresh embryo transfer cycles and the first FET cycle for patients undergoing a freeze-all strategy, with each patient contributing one transfer cycle, representing a single-cycle liver birth rate. (3) Incidence of moderate to severe OHSS: classified based on standard criteria including symptom severity, ovarian enlargement, and biochemical markers.

The secondary outcomes included: (1) Oocyte metrics: total number of oocytes retrieved, mature oocyte rate (number of mature oocytes per total oocytes retrieved), and fertilization rate. (2) Blastocyst formation rate: The percentage of embryos progressing to the blastocyst stage. (3) Embryo quality: proportion of high-quality embryos (grades I and II). (4) Implantation rate: the number of gestational sacs per number of embryos transferred × 100%. (5) Miscarriage rates: early miscarriage rate (spontaneous loss of pregnancy within 12 weeks) and overall miscarriage rate (spontaneous loss of pregnancy at any gestational age).

Sample size calculation

The sample size was calculated by Pass 11.0 and aimed to detect a 15% difference in clinical pregnancy rate (50% for the long luteal phase protocol *vs.* 35% for the flexible antagonist protocol) based on prior studies (14, 15). Using a two-proportion z-test with 80% power, a two-sided $\alpha = 0.05$, and a 1:3 allocation ratio, 144 patients (36 in Group A and 108 in Group B) were required. Accounting for a 10% dropout rate, the target enrollment was increased to 160 patients (40 in Group A and 120 in Group B).

Statistical analysis

Continuous variables were described as mean ± standard deviation (SD) for normally distributed data. For data that were not normally distributed, variables were expressed as the median and interquartile range (IQR). Comparisons between the two groups were conducted using independent t-tests for normally distributed variables and the Mann-Whitney U test for non-normally distributed variables. Categorical variables were expressed as percentages or proportions. Comparisons between groups were made using chi-square tests or Fisher's exact test.

Multivariable logistic regression analysis was conducted to identify independent predictors of clinical pregnancy, adjusting for potential confounders. Variables included in the model (age, BMI, AFC, AMH, gonadotropin dose, number of oocytes retrieved, and embryo quality [Grade I-II]) were selected *a priori* based on their established clinical relevance to IVF/ICSI outcomes, and their potential associations in univariate analyses ($P < 0.10$). The grouping factor (long luteal phase *vs.* flexible antagonist protocol) was excluded from the model because the primary study objective was to compare protocol outcomes directly, and the exploratory analyses confirmed that the protocol type was not a significant predictor ($P > 0.05$). Adjusted odds ratios (ORs) with 95% confidence intervals (CIs) were reported.

A subgroup analysis by age (>20–25, 30–35, and 35–<40 years) was performed to assess clinical pregnancy and live birth rates within each age stratum, comparing Group A and Group B using chi-square tests or Fisher's exact test. All statistical analyses were performed using the latest version of SPSS (IBM, Chicago, USA). A two-tailed *P* value of less than 0.05 was considered statistically significant.

Results

Comparison of basic characteristics

Table 1 showed no significant differences in baseline characteristics between Group A and Group B. Age, BMI,

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duration of infertility, AFC, AMH, FSH levels, gonadotropin dose, stimulation duration, number of follicles ≥18 mm, oocytes retrieved, mature oocyte count, fertilization rates, and day 3 embryo quality were comparable across both groups (P>0.05).

Comparison of clinical outcomes

The clinical pregnancy rate was similar between groups (54.8% *vs.* 56.8%, P=0.092), with fresh cycle rates of 53.3% *vs.* 56.5% and FET cycle rates of 58.3% *vs.* 57.6%. Likewise, the live birth rate showed no significant difference (47.6% *vs.* 52.5%, P=0.278), with fresh cycle rates of 46.7% *vs.* 52.9% and FET cycle rates of 50.0% *vs.* 51.5%. These findings indicate comparable efficacy of the two protocols in achieving clinical pregnancy and live births. Additionally, the incidence of OHSS was low, with no cases in Group A and 2.5% in Group B, but this difference did not reach statistical significance (P=0.055) (Table 2, Figure 2).

For the secondary outcomes, there were no significant differences in the number of embryos transferred (1.8 ± 0.4 *vs.* 1.9 ± 0.3, P=0.093) (Figure 3), implantation rate (45.2% *vs.* 42.4%, P=0.447), or early miscarriage rate (4.8% *vs.* 7.6%, P=0.329) (Figure 3). Similarly, the overall miscarriage rate (7.1% *vs.* 12.7%, P=0.127), the number of blastocysts formed (5.2 ± 2.0 *vs.* 5.4 ± 1.8, P=0.549), and the day 5 blastocyst formation rate (38.1% *vs.* 39.8%, P=0.643) were all comparable between the two groups (Table 2).

Logistic regression analysis of risk factors for clinical pregnancy

The univariate logistic regression analysis (Table 3) revealed several significant predictors of clinical pregnancy (P < 0.05). Younger age (OR = 0.940, P = 0.035), lower BMI (OR = 0.950, P = 0.045), higher AFC (OR = 1.150, P = 0.015), higher AMH levels (OR = 1.400, P = 0.008), greater endometrial thickness (OR = 1.180, P = 0.018), higher gonadotropin dose (OR = 1.003, P = 0.044), higher estradiol on trigger day (OR = 1.002, P = 0.050), lower progesterone on trigger day (OR = 0.820, P = 0.031), greater number of oocytes retrieved (OR = 1.250, P = 0.020), higher mature oocyte count (OR = 1.220, P = 0.025), and better embryo quality (Grade I-II) (OR = 1.600, P = 0.010) were significantly associated with increased odds of clinical pregnancy. Additionally, duration of infertility (OR = 0.900, P = 0.090), FSH levels (OR = 0.920, P = 0.080), and fertilization rate (OR = 1.030, P = 0.070) showed marginal associations (P < 0.10).

Variables with P < 0.10 in the univariate logistic regression analysis were included in the multivariable logistic regression analysis, and the results (Table 4) demonstrated that younger age (OR = 0.956, P = 0.042), higher AFC (OR = 1.127, P = 0.018), higher AMH levels (OR = 1.357, P = 0.005), greater endometrial thickness (OR = 1.162, P = 0.021), greater number of oocytes retrieved (OR = 1.234, P = 0.023), and better embryo quality (Grade I-II) (OR = 1.485, P = 0.002) were significant independent predictors of clinical pregnancy (P < 0.05). Gonadotropin dose (OR = 1.002, P = 0.050)

TABLE 1 Comparison of basic characteristics between the two groups.

Variables	Group A (n=42)	Group B (n=118)	P value
Age (years)	31.2 ± 3.5	30.9 ± 3.6	0.641
BMI (kg/m²)	24.6 ± 2.3	24.8 ± 2.2	0.618
Duration of infertility (years)	3.8 ± 1.7	3.6 ± 1.6	0.495
AFC	10.8 ± 2.1	11.0 ± 2.2	0.609
AMH (ng/mL)	3.4 ± 0.7	3.5 ± 0.8	0.474
FSH (U/L)	7.5 ± 1.2	7.6 ± 1.3	0.663
Gonadotropin dose (IU)	2108.3 ± 350.4	2028.7 ± 316.2	0.175
Stimulation duration (days)	10.2 ± 1.3	9.8 ± 1.5	0.127
Estradiol on trigger day (pg/mL)	3021.6 ± 508.7	2954.7 ± 460.2	0.433
Endometrial thickness (mm)	10.5 ± 1.8	10.7 ± 1.7	0.723
Progesterone on trigger day (ng/mL)	1.0 ± 0.3	1.1 ± 0.3	0.689
Number of follicles ≥18 mm	9.4 ± 2.1	9.6 ± 2.0	0.584
Number of oocytes retrieved	11.8 ± 2.7	12.0 ± 2.5	0.664
Mature oocyte count	10.2 ± 2.3	10.4 ± 2.1	0.606
Fertilization rate (%)	75.6 ± 10.1	74.2 ± 11.5	0.485
Day 3 embryo quality (Grade I-II) (%)	30 (71.4%)	87 (73.7%)	0.774

TABLE 2 Comparison of clinical outcomes between the two groups.

Clinical outcome	Group A (n=42)	Group B (n=118)	P value
Primary outcome			
Clinical pregnancy rate (%)	23 (54.8%)	67(56.8%)	0.092
Fresh cycles (n)	16/30 (53.3%)	48/85 (56.5%)	
FET cycles (n)	7/12 (58.3%)	19/33 (57.6%)	
Live birth rate (%)	20 (47.6%)	62 (52.5%)	0.278
Fresh cycles (n)	14/30 (46.7%)	45/85 (52.9%)	
FET cycles (n)	6/12 (50.0%)	17/33 (51.5%)	
OHSS incidence (%)	0	3 (2.5%)	0.055
Secondary outcome	**Group A (n=42)**	**Group B (n=118)**	
Embryos transferred	1.8 ± 0.4	1.9 ± 0.3	0.093
Implantation rate (%)	19 (45.2%)	50 (42.4%)	0.447
Early miscarriage rate (%)	2 (4.8%)	9(7.6%)	0.329
Overall miscarriage rate (%)	3 (7.1%)	15 (12.7%)	0.127
Blastocysts formed	5.2 ± 2.0	5.4 ± 1.8	0.549
Day 5 blastocyst formation rate (%)	16 (38.1%)	47 (39.8%)	0.643

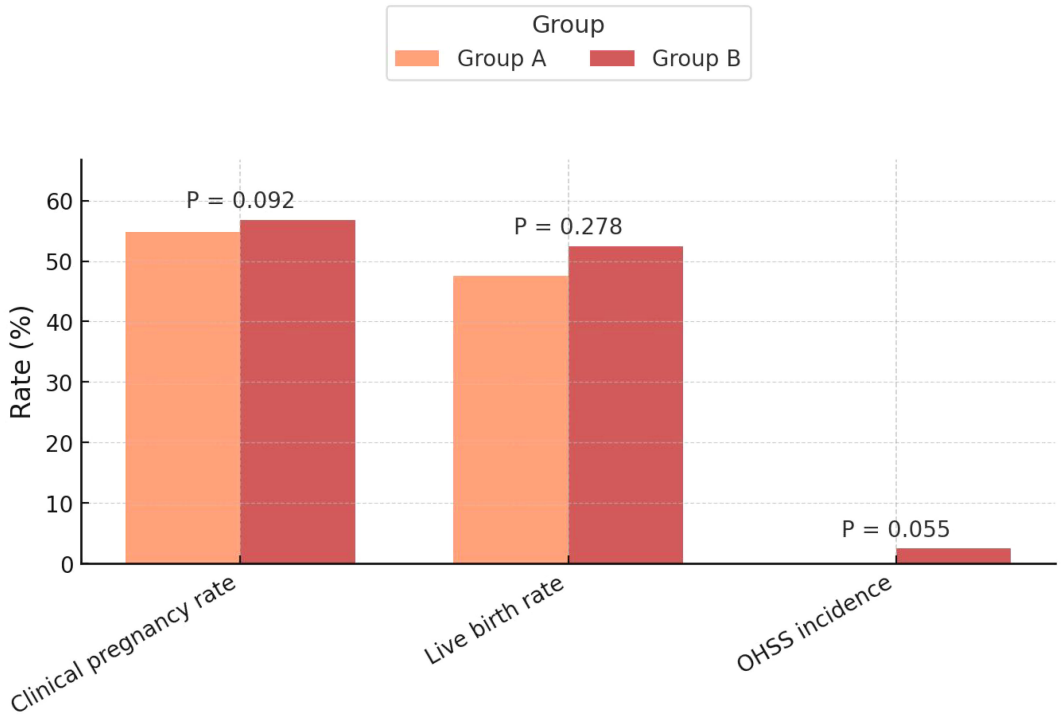

FIGURE 2
Comparison of primary clinical outcomes between the two groups.

showed trends toward significance but did not reach statistical significance ($P > 0.05$).

Subgroup analyses

Table 5 presents the age-related subgroup analysis of clinical pregnancy and live birth rates for Group A and Group B, stratified into >20–25, 30–35, and 35–<40 years subgroups. In the >20–25 years subgroup, clinical pregnancy rates were 66.7% (Group A, n=6) *vs.* 72.2% (Group B, n=18, *P*=0.706), and live birth rates were both 66.7% (*P*=1.000). For the 30–35 years subgroup, clinical pregnancy rates were 57.1% (Group A, n=28) *vs.* 57.7% (Group B, n=78, *P*=0.953), and live birth rates were 50.0% *vs.* 53.8% (*P*=0.717). In the 35–<40 years subgroup, clinical pregnancy rates were 37.5% (Group A, n=8) *vs.* 40.9% (Group B, n=22, P=0.861), and live birth rates were 25.0% *vs.* 36.4% (*P*=0.684). No significant differences were observed between groups, but success rates decreased with increasing age, highlighting age as a key factor influencing IVF/ICSI outcomes.

Discussion

This study compares two commonly used ovarian stimulation protocols— the long luteal phase GnRH agonist protocol and the flexible GnRH antagonist protocol— in patients with normal ovarian reserve undergoing IVF/ICSI. Our findings demonstrate no significant differences between the two protocols in terms of clinical pregnancy rates, live birth rates, or incidence of OHSS. Significant predictors of clinical pregnancy were identified, including AFC, AMH levels, endometrial thickness, number of oocytes retrieved, and embryo quality, while increasing age negatively impacted pregnancy outcomes.

Our study is consistent with the growing body of literature demonstrating that both the GnRH agonist and antagonist protocols are effective for ovarian stimulation in patients with normal ovarian reserve (14–16). However, the flexibility and patient-centered advantages of the antagonist protocol, such as shorter treatment duration and lower risk of OHSS, suggest that it may be preferable for many patients (17–19). Our findings align with several studies that have found no significant differences in pregnancy or live birth rates between the GnRH agonist and antagonist protocols in patients with normal ovarian reserve (20–23). Lamballk et al. (14) and Venetis et al (15) both conducted systematic reviews and meta-analyses, reporting that although antagonist protocols lead to shorter treatment cycles and lower OHSS rates, pregnancy outcomes are comparable between the protocols. Similarly, Kadoura et al. (24) found no differences in live birth rates between the two protocols, though the antagonist protocol reduced patient discomfort and treatment burden. The absence of significant differences in OHSS incidence in our study is supported by earlier findings from studies such as those by Olivennes et al. (25), which suggested that the antagonist protocol is particularly beneficial in preventing severe OHSS in high responders. A systematic review by Engmann et al. (26) reinforced this view, demonstrating the antagonist protocol's role in enhancing safety without compromising efficacy.

Our findings of equivalent clinical outcomes between the GnRH agonist and antagonist protocols in patients with normal ovarian

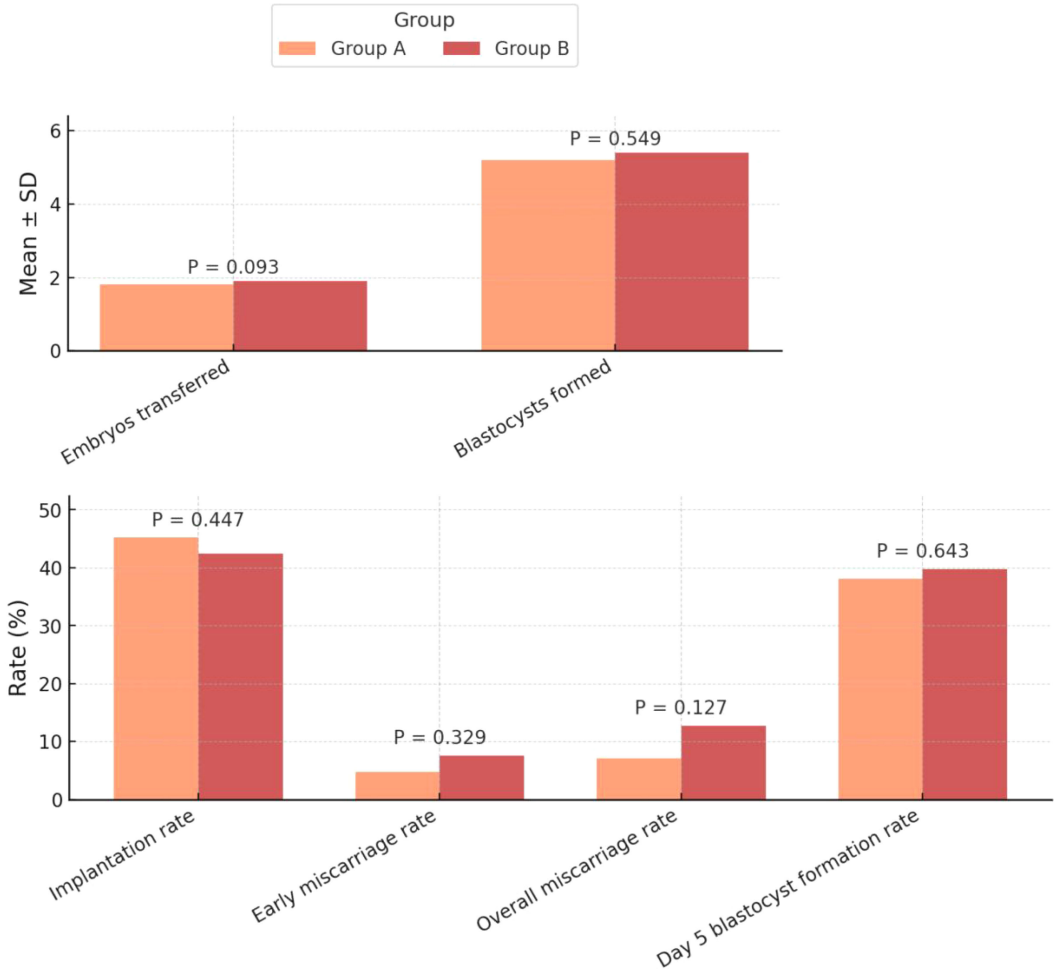

FIGURE 3
Comparison of secondary clinical outcomes between the two groups.

reserve resolve apparent contradictions in the literature. While some studies suggest advantages for the agonist protocol in populations with diminished ovarian reserve or recurrent implantation failure (27, 28), others report comparable efficacy across protocols in broader populations (14, 15). The equivalence observed in our study is attributable to the focus on patients with normal ovarian reserve, who respond well to both protocols, as evidenced by similar clinical pregnancy (54.8% *vs.* 56.8%, *P*=0.092) and live birth rates (47.6% *vs.* 52.5%, *P*=0.278). Systematic reviews by Lambalk et al. (14) and Venetis et al. (15) support this equivalence, demonstrating no significant differences in live birth rates, while Kadoura et al. (24) further confirm comparable outcomes in polycystic ovary syndrome patients, a group with robust ovarian response. The antagonist protocol's shorter treatment duration and lower OHSS incidence, as noted by Nie et al. (20), enhance its appeal without compromising efficacy, providing clinicians with flexibility to tailor treatments based on patient preferences and risk profiles.

The absence of protocol-specific differences in our study contrasts with studies reporting advantages for one protocol over the other, a discrepancy attributable to differences in patient populations and study methodologies (14, 15, 27, 28). Studies observed improved outcomes with the agonist protocol in patients with diminished ovarian reserve, where prolonged downregulation may enhance follicular synchronization (27, 28). In contrast, our study's focus on patients with normal ovarian reserve, who exhibit robust responses to both protocols, likely minimizes differential effects. Additionally, our prospective design with randomization and standardized clinical protocols, such as endometrial thickness ≥8 mm and a freeze-all strategy for high-risk cases, reduced variability that may amplify protocol-specific differences in retrospective or heterogeneous studies. Systematic reviews (14, 15) corroborate that protocol equivalence is more pronounced in normal responders, supporting our findings and highlighting the importance of patient selection in ART outcomes.

The results suggest that both protocols are equally effective for patients with normal ovarian reserve, providing flexibility in treatment options. The flexible antagonist protocol offers distinct advantages, including reduced treatment burden and a lower incidence of severe OHSS. These benefits are particularly relevant in modern clinical practice, where patient comfort and safety are paramount. The role of AFC, AMH, and embryo quality as predictors of success in IVF/ICSI cycles has been well documented in previous studies. AFC and AMH

TABLE 3 Univariable logistic regression for selected predictors of clinical pregnancy.

Variables	β	SE	OR	95% CI	P value
Age (years)	-0.062	0.029	0.940	0.888 - 0.995	0.035
BMI (kg/m²)	-0.051	0.025	0.950	0.904 - 0.998	0.045
Duration of infertility (years)	-0.105	0.062	0.900	0.797 - 1.017	0.090
AFC	0.140	0.057	1.150	1.029 - 1.286	0.015
AMH (ng/mL)	0.336	0.126	1.400	1.094 - 1.792	0.008
FSH (U/L)	-0.083	0.047	0.920	0.839 - 1.009	0.080
Gonadotropin Dose (IU)	0.003	0.002	1.003	1.001 - 1.008	0.044
Estradiol on Trigger Day (pg/mL)	0.002	0.001	1.002	1.000 - 1.004	0.050
Endometrial Thickness (mm)	0.166	0.070	1.180	1.029 - 1.354	0.018
Progesterone on Trigger Day (ng/mL)	-0.198	0.091	0.820	0686 - 0.980	0.031
Number of Oocytes Retrieved	0.223	0.096	1.250	1.035 - 1.509	0.020
Mature Oocyte Count	0.199	0.088	1.220	1.026 - 1.451	0.025
Fertilization Rate (%)	0.029	0.016	1.030	0.998 - 1.063	0.070
Embryo Quality (Grade I-II)	0.470	0.182	1.600	1.120 - 2.286	0.010

have been shown to be reliable markers of ovarian reserve and predictors of ovarian response to stimulation (29). Our findings further emphasize the importance of these markers in guiding treatment decisions, particularly in identifying patients who may benefit from individualized gonadotropin dosing. Age remains a critical factor influencing clinical pregnancy outcomes, as demonstrated by the negative association between increasing age and pregnancy success in our study. This is consistent with the extensive literature highlighting the impact of maternal age on oocyte quality and reproductive outcomes (30, 31). As age increases, the cumulative impact of genetic and cellular changes within oocytes leads to reduced implantation potential and increased miscarriage rates (32). Although ovarian reserve markers like AFC and AMH provide valuable information, they cannot entirely mitigate the age-related decline in fertility.

Additionally, endometrial factors, such as endometrial thickness, and hormonal levels, such as progesterone on trigger day, are critical for pregnancy success, particularly in fresh embryo transfer cycles (33). In our study, endometrial thickness was standardized (≥8 mm) for fresh transfers, and progesterone levels were monitored to defer fresh transfers in cases of elevation (>1.5 ng/mL), employing a freeze-all strategy to optimize outcomes. These factors were not included in the logistic regression model (Table 3) due to their standardization across groups and lack of significant differences in preliminary analyses. However, we acknowledge that variability in endometrial receptivity or subtle progesterone elevations may still influence outcomes, and their exclusion from the regression analysis is a limitation. Future studies should incorporate these factors as variables to further elucidate their impact on pregnancy outcomes in patients with normal ovarian reserve.

Our findings have several important implications for clinical practice. The comparable efficacy of the two protocols means that clinicians have the flexibility to select a protocol based on patient preferences, clinical workflow, and the risk of OHSS. The antagonist protocol, with its shorter duration and lower complication risk, may be more suitable for patients seeking a less invasive treatment approach (33). The importance of embryo quality in predicting

TABLE 4 Multivariable logistic regression for selected predictors of clinical pregnancy.

Variables	β	SE	OR	95% CI	P value
Age (years)	-0.045	0.020	0.956	0.920 - 0.993	0.042
AFC	0.120	0.045	1.127	1.035 - 1.227	0.018
AMH (ng/mL)	0.305	0.090	1.357	1.137 - 1.620	0.005
Gonadotropin Dose (IU)	0.002	0.001	1.002	1.000 - 1.004	0.050
Endometrial Thickness (mm)	0.150	0.065	1.162	1.023 - 1.320	0.021
Number of Oocytes Retrieved	0.210	0.065	1.234	1.084 - 1.408	0.023
Embryo Quality (Grade I-II)	0.395	0.075	1.485	1.265 - 1.742	0.002

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TABLE 5 Age-Related subgroup analysis of clinical pregnancy and live birth rates.

Age Subgroup	Outcome	Group A	Group B	P value
>20–25 years		(n=6)	(n=18)	
	Clinical pregnancy rate (%)	4/6 (66.7%)	13/18 (72.2%)	0.706
	Live birth rate (%)	4/6 (66.7%)	12/18 (66.7%)	1.000
30–35 years		(n=28)	(n=78)	
	Clinical pregnancy rate (%)	16/28 (57.1%)	45/78 (57.7%)	0.953
	Live birth rate (%)	14/28 (50.0%)	42/78 (53.8%)	0.717
35–<40 years		(n=8)	(n=22)	
	Clinical pregnancy rate (%)	3/8 (37.5%)	9/22 (40.9%)	0.861
	Live birth rate (%)	2/8 (25.0%)	8/22 (36.4%)	0.684

Clinical pregnancy rate and live birth rate are calculated as the number of events divided by the number of embryo transfer cycles within each age subgroup (one per patient, including both fresh and first frozen embryo transfer [FET] cycles).

clinical pregnancy further emphasizes the need for careful embryo selection and grading during IVF/ICSI cycles. As demonstrated in several studies, morphologic criteria remain a critical component of embryo assessment, contributing significantly to treatment success. Our study also highlights the value of tailoring ovarian stimulation protocols to individual patient characteristics, including age and ovarian reserve markers. By adjusting stimulation protocols based on these factors, clinicians can optimize outcomes while minimizing the risk of complications such as OHSS.

Strengths and limitations

This study's prospective design and large sample size are key strengths, allowing for rigorous data collection and robust comparisons between the two protocols. The random assignment of patients to each protocol minimized selection bias and enhanced the generalizability of the findings to other patient populations with normal ovarian reserve (34). The randomization methhod ensured balanced baseline characteristics and minimized the selection bias. Additionally, the use of multivariable logistic regression further adjusted for potential confounders, reducing the risk of bias in identifying predictors of clinical pregnancy.

However, there are several limitations to consider. First, the study was conducted at a single center, which may limit the external validity of the findings. Multi-center studies are needed to confirm these results in more diverse populations and clinical settings. Second, while the sample size was sufficient for the primary outcome, it may be considered moderate for detecting smaller differences in secondary outcomes, such as implantation or blastocyst formation rates, potentially limiting the study's power for these endpoints. Nevertheless, the observed non-significant differences in primary outcomes ($P > 0.05$) suggest that the sample size was appropriate for the study's main objectives. Additionally, while the study focused on short-term outcomes such as clinical pregnancy and live birth rates, it did not assess long-term neonatal outcomes or maternal health beyond the postpartum period. These outcomes are crucial for evaluating the overall safety and effectiveness of ovarian stimulation protocols. Another limitation is the potential for unmeasured confounders, such as genetic factors or variations in laboratory techniques, that may have influenced the outcomes. Future studies should aim to control for these variables and include more detailed assessments of patient and embryological characteristics.

Future research should focus on further refining ovarian stimulation protocols to improve patient outcomes and safety (35, 36). Randomized controlled trials with larger, more diverse populations are needed to confirm the equivalence of the GnRH agonist and antagonist protocols in different patient groups, including those with diminished ovarian reserve and those undergoing multiple IVF cycles.

Conclusion

In conclusion, this prospective cohort study found no significant differences in clinical pregnancy or live birth rates between the long luteal phase GnRH agonist protocol and the flexible GnRH antagonist protocol in patients with normal ovarian reserve undergoing IVF/ICSI. While both protocols are effective, the flexible antagonist protocol offers a more patient-friendly approach, with shorter treatment duration and lower risk of OHSS. Clinicians should consider individual patient characteristics, including age, ovarian reserve markers, endometrial factors, and personal preferences, when selecting the most appropriate protocol. Further research is needed to confirm these findings in larger, multi-center studies and to explore the long-term outcomes associated with different ovarian stimulation protocols.

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 humans were approved by Yellow River Sanmenxia Affiliated Hospital of Henan University of Science and Technology. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

YT: Conceptualization, Data curation, Funding acquisition, Writing – original draft. JH: Conceptualization, Data curation, Formal analysis, Investigation, Writing – original draft. XT: Formal analysis, Methodology, Validation, Visualization, Writing – review & editing. SF: Investigation, Methodology, Writing – original draft. ML: Investigation, Methodology, Writing – original draft. YC: Data curation, Investigation, Methodology, Writing – original draft. ZC: Conceptualization, Writing – original draft.

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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.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

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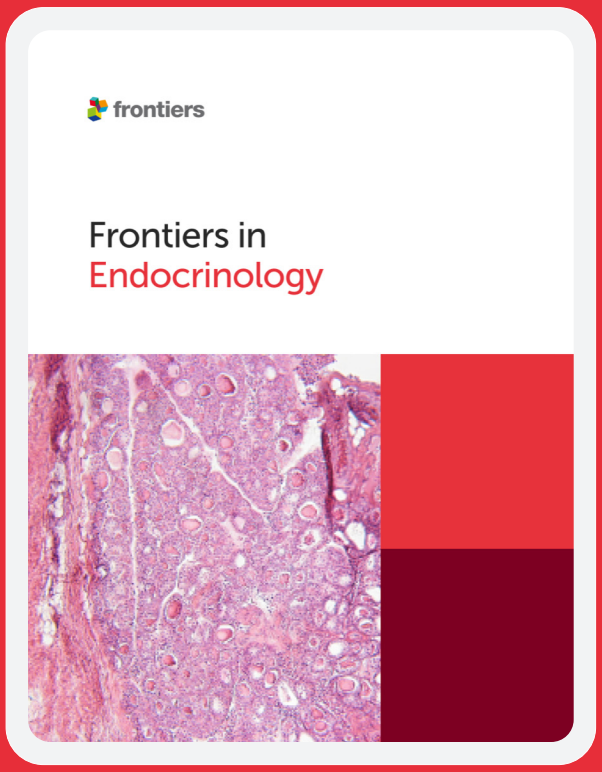

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