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Benefits and risks of agonist triggering strategies

Edited by
Emanuele Garzia, Jan Tesarik and Francesco Maria Fusi

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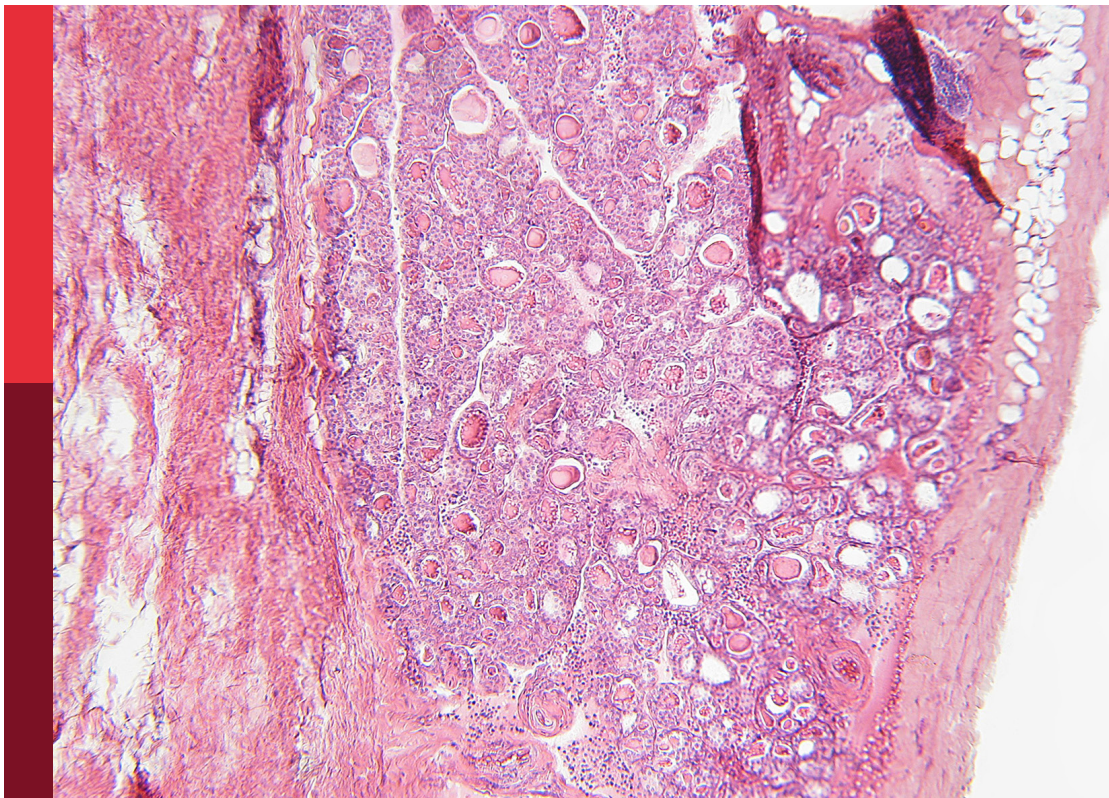

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Benefits and risks of agonist triggering strategies

Topic editors

Emanuele Garzia — Santi Paolo e Carlo Hospital, Italy

Jan Tesarik — MARGen Clinic, Spain

Francesco Maria Fusi — Papa Giovanni XXIII Hospital, Italy

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EDITED AND REVIEWED BY
Richard Ivell,
University of Nottingham, United Kingdom

*CORRESPONDENCE
Jan Tesarik
jantesarik13@gmail.com

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Editorial: Benefits and risks of agonist triggering strategies

Jan Tesarik*

MARGen Clinic, Granada, Spain

KEYWORDS

GnRH agonist, GnRH antagonist, controlled ovarian stimulation, embryo viability, uterine receptivity, early pregnancy

Editorial on the Research Topic
Benefits and risks of agonist triggering strategies

1 Introduction

Since many years, the GnRH analogues GnRHa and GnRHant are used alternatively for preventing premature LH surge and ovulation in controlled ovarian stimulation protocols (1). Recently, GnRHa is also used in GnRHant-controlled cycles as an alternative to human chorionic gonadotropin (hCG) to trigger final oocyte maturation and ovulation (2). The use of these GnRH analogues simplifies the ovarian stimulation protocol and reduces the risk of ovarian hyperstimulation syndrome (1, 2). After initial warning voices, based on animal experiments and suggesting that GnRH and its analogues may interfere with the early pregnancy through their action on the corpus luteum and the uterus (3), these fears were not substantiated in clinical practice (1, 2). However, some doubts may still persist. This Research Topic addresses this question, in addition to bringing together other new data relative to the efficacy and safety of controlled ovarian stimulation protocols.

2 The main points of individual contributions

This series includes 9 original research articles, focusing on GnRHa effects on embryo viability, uterine receptivity and early pregnancy, as well as some other new aspects of controlled ovarian stimulation in general. In this section, they are presented in a chronological order of publication in the Journal. Pang et al. investigated into the relationship between serum luteinizing hormone (LH) concentration on the day of the beginning of GnRHant administration during ovarian stimulation for conventional *in-vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI), on the one hand, and laboratory indicators and clinical outcomes on the other hand. They report a significant positive correlation between LH concentration on the antagonist administration day and the numbers of oocytes retrieved, of two-pronucleated embryos and of blastocysts. In a propensity score-matched study, Zhang et al. explored the cycle characteristics and pregnancy outcomes of progestin-primed ovarian stimulation using fixed versus degressive doses of medroxyprogesterone acetate (MPA) in conjunction with letrozole (LE) in infertile women. They did not find any significant differences in the incidence of premature LH surge, the number of oocytes retrieved, the number of top-quality embryos,

clinical pregnancy rate, cumulative live birth rate or fetal malformation rate between the two groups, while the combination of a degressive MPA dose with LE proved effective in reducing total MPA dose. A parallel, open-label randomized trial by Li et al., including 245 women, examined the usefulness of intramuscular injection of human chorionic gonadotropin (hCG) after embryo transfer as luteal phase support in artificial cycle frozen-thawed embryo transfer attempts failed to find any improvement of clinical outcomes in the hCG group. A retrospective cohort study by Cao et al. compared pregnancy outcomes in fresh IVF/ICSI cycles in 294 women who had recovered from COVID-19 infection with those of 631 women who had not been infected. No substantial evidence was found between the two groups. Li et al. used transcriptome profiling to analyze the impact of using GnRHa as ovulation trigger on embryo implantation and early pregnancy in superovulated mice. Their findings suggest that a combination of ovarian stimulation and GnRHa trigger impair embryo implantation in mice, presumably due to changes in endometrial gene expression, namely concerning the genes responsible for endometrial remodeling, ion transport, and immune response. A retrospective cohort study conducted by Cao et al. compared live birth rates after IVF/ICSI in 924 treatment cycles using GnRHant original reference product Cetrotide with those in 1984 cycles using a generic GnRHant (Ferpront). No differences between the attempts using either of the two preparations were detected. Hao et al. compared retrospectively clinical outcomes of frowen-thawed embryo transfer in patients prepared with the combined use of hormone replacement therapy (HRT) and GnRHa (leuprorelin) with those achieved with HRT (estradiol valerate) alone. Clinical pregnancy and implantation rates achieved with the combined (HRT + GnRHa) protocol were higher as compared with HRT alone. Luo et al. used logistic regression analysis to identify the risk factors for empty follicle syndrome (EFS). They further analyzed IVF cycles of patients with EFS and performed long-term follow-up of those who had got pregnant until live birth was achieved. They identified polycystic ovary syndrome as an independent risk factor for EFS and showed that repeated instances of EFS are associated with poor reproductive prognosis. Finally, Hsu et al. investigated the correlation between the ovarian sensitivity index (OSI) and clinical parameters in GnRHa and GnRHant cycles. Serum anti-Mullerian hormone, cycle 2 follicle stimulating hormone (FSH), LH and estradiol concentrations, numbers of large follicles, fertilization rate, and the incidence of premature LH surge were positively correlated with the OSI. The GnRHa and GnRHant protocols did not differ as to the incidence of premature LH surge and ovulation, but higher numbers of mature oocytes and good-morphology embryos were obtained in the GnRHa cycles.

3 Synthetic view and conclusions

Taken together, the data presented in this Research Topic touch various aspects of GnRHa and GnRHant effects on assisted reproduction outcomes. In addition, data unrelated to these two substances but important for improving controlled ovarian stimulation protocols are also included. Most of data presented support the inclusion of GnRHa and GnRHant in these protocols.

Author contributions

JT: Writing – original draft, Writing – review & editing.

Conflict of interest

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

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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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EDITED BY
Jan Tesarik,
MARGen Clinic, Spain

REVIEWED BY
Firat Tulek,
Acıbadem Ataşehir Hospital, Türkiye
Akmal El-Mazny,
Cairo University, Egypt
Zainab M. Alawad,
University of Baghdad, Iraq

*CORRESPONDENCE
Kehua Wang
sdwangkh@126.com

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LH level on the antagonist administration day as a predictor of the reproductive outcomes in women with normal ovarian function

Conghui Pang[1,2], Kehua Wang[2]*, Ruihan Wang[1], Danyang Guo[1] and Zixi Wen[1]

[1]The First Clinical College, Shandong University of Traditional Chinese Medicine, Jinan, Shandong, China, [2]Department of Reproduction and Genetics, The Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Jinan, Shandong, China

Introduction: The addition of antagonists is mainly based on estrogen level and follicle size, while LH level has not received sufficient attention.In this study, LH Level on the antagonist administration day was used as the main research objective to explore its relationship with laboratory indicators and pregnancy outcomes.

Methods and Analysis: We enrolled 854 patients with normal ovarian function undergoing *in-vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) between May 2021 to May 2022 at the Reproductive Center of Shandong University of Traditional Chinese Medicine.We used the quartile method to group LH levels on the antagonist administration day. There were four groups: Q1 (0.53IU/L≤LH ≤ 1.89IU/L); Q2 (1.89IU/L<LH ≤ 3.01IU/L); Q3 (3.01IU/L<LH≤ 5.29 IU/L); Q4 (5.29IU/L<LH ≤ 8.72IU/L). A total of 452 fresh embryo transplantation cycles and 1726 Frozen embryo transplantation cycles were carried out.

Result: There were significant differences among the four groups in terms of total Gn dosage, E2, P and LH on trigger day, number of retrieved oocytes, number of 2PN embryos, number of blastocysts, Number of ET and fresh ETR.There is a significant correlation between LH on antagonist administration day and Basal LH Level,LH on trigger day,number of oocytes retrieved,number of 2PN embryos,number of blastocysts, number of ET.Using Fresh ETR,Fresh CPR,OHSS and Cumulative CPR as the criterion respectively, the optimal cut-off value for evaluating LH on antagonist administration day was 4.18IU/L,3.99IU/L,4.63IU/L,4.66IU/L.

Conclusion: There was a significant positive correlation between LH on the antagonist administration day and number of oocytes retrieved,number of 2PN embryos,number of blastocysts.LH on the antagonist administration day could predict Fresh CPR,OHSS and Cumulative CPR to some extent.

KEYWORDS

GnRH antagonist protocol, LH level, antagonist administration day, laboratory indicators, pregnancy outcomes

1 Introduction

In recent decades, *in vitro* fertilization-embryo transfer (IVF-ET) has grown rapidly throughout the world, becoming an important method of treating infertility. The treatment process revolves around controlled ovarian hyperstimulation (COH). Gonadotropin-releasing hormone antagonist (GnRH-ant) protocols are widely used due to their advantages of short stimulation time, low costs, and a lower incidence of ovarian hyperstimulation syndrome (OHSS) (1–3). GnRH-ant binds to specific receptors on the pituitary gland and inhibits endogenous Luteinizing hormone (LH). It can prevent the appearance of early follicular LH surge, thereby inhibiting premature follicle production and reducing the cycle cancellation rate, which brings a new choice for clinical ovulation induction programs (4).

LH is a glycoprotein hormone secreted by the pituitary gland, which plays an important role in estrogen synthesis, follicle development, and ovulation induction (5).On the one hand, high LH levels are harmful to pregnancy outcomes in both the natural and ovarian stimulation cycles. Too little LH, on the other hand, is linked to pregnancy loss (6, 7). Scholars generally agree that an adequate level of LH is required for follicular development. According to some studies, the LH window has a range of 1.2–5 IU/L (8, 9). The team led by Professor Li Yuan proposed that LH≥4IU/L be considered the critical value, and antagonists should be considered when the threshold was exceeded (10). However, there is no consensus on the appropriate value of LH during COH with antagonist protocol.

At present, antagonist protocols are mainly divided into fixed and flexible protocols (11). The starting day of GnRH antagonist administration (i.e., both the fixed and flexible protocols) is mainly based on the day of ovarian stimulation, the diameter of the follicles, the estradiol levels, or a combination of these parameters (3). However, LH levels on the antagonist administration day have received less attention.

A retrospective analysis was used in this study. We divided the LH level on the antagonist administration day into four groups according to the quartile method and compared the laboratory indicators and pregnancy outcomes among the four groups.

Statistical methods were used to analyze the effects of LH level on the antagonist administration day on laboratory indicators and estimate the cut-off values of LH on antagonist administration day for predicting various pregnancy outcomes,so as to illustrate that the LH Level on the antagonist administration day could be used as a predictor of the reproductive outcomes in women with normal ovarian function.

Abbreviations: COH, controlled ovarian hyperstimulation; GnRH-ant, gonadotropin-releasing hormone antagonist; OHSS, ovarian hyperstimulation syndrome; LH, Luteinizing hormone; IVF, *in-vitro* fertilization; ICSI, intracytoplasmic sperm injection; FSH, follicle-stimulating hormone; TSH, thyroid stimulating hormone; E2:estradiol, 2PN, 2 pronuclear stage; P, progesterone; T, testosterone; fresh ETR, Fresh embryo transfer rate; fresh CPR, Clinical pregnancy rate of fresh embryo transplantation; Cumulative CPR, Cumulative Clinical pregnancy rate; PCOS, polycystic ovarian syndrome; EMS, Endometriosis.

2 Materials and methods

2.1 Participants

Through a database search, the data of patients who underwent IVF/ICSI cycles in the Affiliated Hospital of Shandong University of Traditional Chinese Medicine from May 2021 to May 2022 were selected. Only those infertile patients who received the GnRH-ant regimen to generate usable embryos and had all embryos transferred were included. All enrolled patients signed informed consent.This study was approved by the Reproductive Medicine Ethics Committee of the Affiliated Hospital of Shandong University of Chinese Medicine(No.20210713).The patients' flow chart detailing the whole process is shown in Figure 1 3mL of fasting elbow venous blood was collected and plasma LH, FSH and E2 levels were detected by luteinizing hormone assay kit, follicle-stimulating hormone assay kit and estradiol assay kit (Beckman Coulter, Inc, USA).

Inclusion criteria were: patients aged 20–40 years; patients with body mass index (BMI) ≤32 kg/m^2; basal follicle-stimulating hormone (FSH) ≤10mIU/mL; normal thyroid stimulating hormone (TSH) and prolactin levels as well as patients with no preconditioning with oral contraceptives.

Exclusion criteria were: recurrent abortion or chromosomal abnormalities; patients with a history of uterine malformations and intrauterine adhesions;woman with PCOS diagnosed according to Rotterdam criteria (12);Poor responders identified according to Bologna criteria (13);Presence of clinically significant systemic diseases or other endocrine diseases.

2.2 Protocol for controlled ovarian stimulation

Ovarian stimulation began on days 2 or 3 of the menstrual cycle with recombinant FSH (150–450 IU) (Gonal-F, Merck Serono, Coinsins, Switzerland) daily with or without 75–300 IU of human menopausal gonadotropin (hMG, Livzon, Shanghai, China). Moreover, hMG was used in patients where a poor response was anticipated because of advanced age, low antral follicle count.The starting dose of Gn (FSH/hMG) was based on the patient's age, BMI, antral follicle count(AFC), and hormonal profile. The doses were adjusted according to serum estradiol (E2) level and ovarian response, which was evaluated by transvaginal ultrasound. The administration of GnRH-ant, Ganirelix, or Cetrotide (0.25 mg daily at10:00 AM) was started either on the 6th day of recombinant FSH stimulation until the hCG injection or when the dominant follicle's diameter was ≥ 12–14 mm or estrogen level >250pg/mL.

After the three follicles reached a mean diameter of 17 mm, or two follicles were over 18 mm, final oocyte maturation was triggered by administering recombinant human chorionic gonadotropin (rhCG, 250 μg, Merck Schlano, Germany) or Decapeptyl (0.2 mg) either alone or in combination with urinary hCG (2000 IU, Livzon, China)When a patient was suspected to be at risk for ovarian

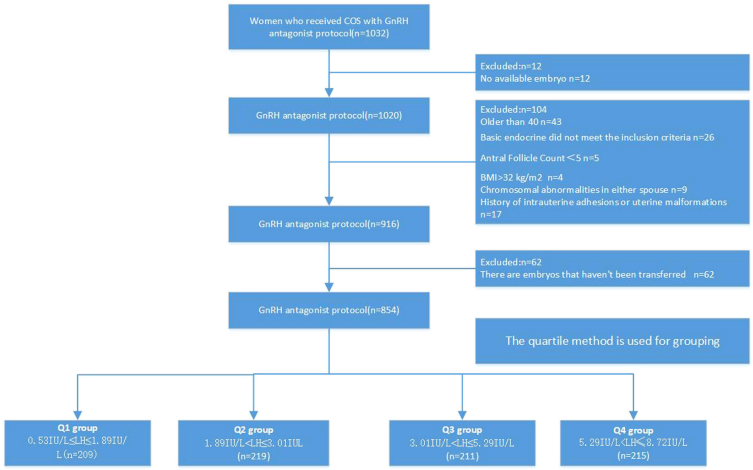

FIGURE 1
A flow chart describing the GnRH-ant protocol in all patients.

hyperstimulation syndrome. After 35 to 37 h, the eggs were harvested by transvaginal ultrasound.

2.3 Embryo transfer and luteal support

On the 3rd to 5th day after fertilization, 1–2 embryos of high-quality were selectively transferred. Embryo grading was done in accordance with the proceedings of the Istanbul consensus (14). High-quality embryos in our center were defined as having 6-10 blastomes on the third day, basically uniform size of blastomes, and fragmentation rate ≤20%.We divided blastocysts into 1-6 stages according to the degree of blastocyst expansion and incubation,the quality of inner cell mass (ICM) and trophoblast cell (TE) was further evaluated for the blastocyst of stage 3-6.Blastocysts with scores ≥ 3BB were defined as high quality blastocysts.

The luteal phase support was started on the day of oocyte retrieval with intramuscular progesterone injections (20 mg, Xian Ju Pharmaceutical Co, China) twice a day. Additionally, dydrogesterone (20 mg, Abbott Laboratories, USA) was taken twice each day.

2.4 FET protocol

At least one of the above three features must be present,the patient underwent frozen-thawed embryo transfer (FET).①E2 on trigger day≥5000pg/ml;②E2 on trigger day was between 4000 and 5000 pg/ml,number of oocytes retrieved was between 15 and 20, but the patient has symptoms such as bloating;③number of oocytes retrieved ≥20.

2.5 Outcome measures

The primary outcome measure was the Clinical pregnancy rate of fresh embryo transfer(fresh CPR) and all embryo transfer cycles (Cumulative CPR). These secondary outcomes included the number of retrieved oocytes, the number of high-quality embryos, the fresh embryo transfer rate (fresh ETR), the rate of Ovarian hyperstimulation syndrome (OHSS rate). Clinical pregnancy was defined as the confirmation of gestational sac and fetal heartbeats by transvaginal ultrasound 28 days after ET. Fresh CPR was the ratio of the number of pregnancy cycles after fresh-ET to the total number of fresh embryo transfer cycles. Cumulative CPR was the ratio of the number of clinical pregnancies following the transfer of all embryos from one ovulation cycle to the total number of ovulation cycles. Fresh ETR was defined as the ratio of fresh embryo transfer cycles to oocyte retrieval cycles. OHSS is defined by Golan et al. Standards (15).

2.6 Statistical analysis

Statistical software SPSS(version 26.0) was used for statistical analysis. Kolmogorov-Smirnov test was used to test whether continuous numerical variables obeyed normal distribution, If the data was distributed normally, it was expressed by mean and standard deviation.If continuous numerical variables do not follow the normal distribution, the data was represented by the median and upper and lower quartiles [M(P25, P75)], and the rank sum test was used for comparison.Counting data was described by n(%) and Chi-square test was used to compare the distribution differences between groups.If sample size > 40 and theoretical frequency > 5, Pearson Chi-square test was used for non-parametric test;If the sample size is less than 40 or the theoretical frequency is less than 5, Fisher's exact probability method is used to test.a=0.05 was used as the test level, $P<0.05$ was considered statistically significant, And the cut-off value of Yoden index was calculated by ROC curve.

3 Results

A total of 1032 patients were included in the initial analysis, and 854 patients were included in the final study after applying the exclusion criteria. No cycle cancellation due to unexpected premature ovulation was reported among patients of groups.

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Age, BMI, infertility type, infertility years, causes of infertility, basal FSH level, basal E2 level, basal T level, started Gn dose, time of antagonist administration, and fertilization method were not significantly different among the four groups. Basal LH levels were significantly different among the four groups. (Table 1).

Laboratory indicators and pregnancy outcomes were compared among the four groups. There were no significant differences among the four groups in terms of duration of Gn, endometrium on trigger day, number of embryos (D3),number of high-quality embryos,fresh CPR,OHSS rate and Cumulative CPR.There were significant differences among the four groups in terms of total Gn dosage, E2 on trigger day, P on trigger day,LH on trigger day, number of retrieved oocytes, number of 2PN embryos, number of blastocysts, Number of ET and fresh ETR. (Table 2).

Pearson chi-square test was used to verify the correlation between LH on antagonist administration day and basal LH level, LH on trigger day,endometrium on trigger day,number of oocytes retrieved,number of 2PN embryos,number of embryos (D3), number of blastocysts,number of ET and number of high-quality embryos.The results showed that LH on antagonist administration day had a significant correlation with basal LH level,LH on trigger day,number of oocytes retrieved,number of 2PN embryos,number of blastocysts,number of ET. (Figures 2, 3).

Linear regression analysis revealed that there is a significant positive correlation between LH on antagonist administration day and basal LH level ($p<0.05$, Figure 4), LH on trigger day ($p<0.05$, Figure 5), number of oocytes retrieved ($p<0.05$, Figure 6), number of 2PN embryos ($p<0.05$, Figure 7), number of blastocysts ($p<0.05$, Figure 8), number of ET ($p<0.05$, Figure 9).

The optimal cut-off value of LH on antagonist administration day of various pregnancy rates was analyzed by ROC curve.The results showed that the optimal cut-off value of LH on antagonist administration day was 4.18IU/L using Fresh ETR as the standard (AUC=0.559; P=0.003; Figure 10). Using Fresh CPR as the criterion, the optimal cut-off value for evaluating LH on antagonist administration day was 3.99IU/L (AUC=0.515; P=0.534; Figure 11). Using OHSS as the criterion, the optimal cut-off value for evaluating LH on antagonist administration day

TABLE 1 Analysis of demographic and clinical characteristics among the four groups.

Variables	Q1 group (n = 209)	Q2 group (n = 219)	Q3 group (n = 211)	Q4 group (n = 215)	F/H/X^2	*P* value
Age (years)	33.07 ± 4.38	32.36 ± 4.26	33.17 ± 4.18	32.31 ± 4.29	2.404	0.066
BMI	24.69 ± 3.54	24.07 ± 3.39	24 ± 3.48	23.93 ± 3.52	2.085	0.101
Infertility type, n (%)					5.713	0.126
Primary infertility	90 (43)	102 (47)	75 (36)	93 (43)		
Secondary infertility	119 (57)	117 (53)	136 (64)	122 (57)		
Infertility years (years)	3 (2, 4)	3 (2, 4)	3 (2, 4.5)	3 (2, 4)	2.607	0.456
Causes of infertility					1.326	0.97
Tubal factor	178 (85)	185 (84)	175 (82)	180 (84)		
Male factor	29 (14)	31 (14)	35 (16)	32 (15)		
Tubal factor and Male factor	2 (1)	3 (2)	4 (2)	3 (1)		
Basal E2 Level (pg/mL)	45.95 ± 11.15	46.15 ± 8.96	46.91 ± 10.04	46.94 ± 9.64	0.566	0.637
Basal FSH Level (IU/L)	7.13 ± 1.76	7.04 ± 1.34	7.07 ± 1.41	6.77 ± 1.29	2.449	0.062
Basal LH Level (IU/L)	4.29 ± 1.43	4.68 ± 1.55	5.18 ± 1.68	5.75 ± 1.9	31.119	< 0.001
Basal T Level (μg/L)	0.39 ± 0.12	0.38 ± 0.1	0.37 ± 0.11	0.39 ± 0.11	1.29	0.276
Started Gn dose(IU)	224.46 ± 41.64	218.88 ± 39.27	217.71 ± 40.21	214.01 ± 38.64	2.481	0.06
Time of antagonist administration (D)	5.77 ± 1.1	5.74 ± 1.09	5.85 ± 1.11	5.84 ± 0.93	0.572	0.633
Fertilization method (n,%)					2.141	0.544
IVF	170 (81)	172 (79)	176 (84)	177 (84)		
ICSI	39 (19)	45 (21)	34 (16)	34 (16)		

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TABLE 2 Comparison of laboratory indicators and pregnancy outcomes among four group.

Variables	Q1 group (n = 209)	Q2 group (n = 219)	Q3 group (n = 211)	Q4 group (n = 215)	F/H/X^2	*P* value
Duration of Gn(d)	9.53 ± 1.58	9.21 ± 1.56	9.34 ± 1.63	9.31 ± 1.68	1.431	0.232
Total dosage of Gn(IU)	2250 (1800, 2700)	2000 (1620, 2475)	2025 (1800, 2400)	1925 (1575, 2400)	22.692	< 0.001
E2 on trigger day (pg/mL)	2056 (1386, 2886)	2194 (1450.5, 3685)	2729 (1727, 3972.5)	3330 (1980, 4924.5)	52.142	< 0.001
P on trigger day (nmol/L)	1.08 (0.73, 1.49)	1.08 (0.78, 1.54)	1.13 (0.8, 1.48)	1.25 (0.84, 1.77)	9.762	0.021
LH on trigger day (IU/L)	1.82 (1.05, 2.83)	1.98 (1.35, 2.81)	2.67 (1.83, 4.54)	3.14 (1.79, 5.44)	74.62	< 0.001
Endometrium on trigger day(cm)	1.18 (1, 1.33)	1.16 (0.97, 1.31)	1.15 (1, 1.29)	1.15 (0.98, 1.28)	3.017	0.389
Number of oocytes retrieved	9 (7, 12)	10 (6.5, 13)	10 (6, 14)	12 (7, 17)	11.888	0.008
Number of 2PN embryos	6.53 ± 2.96	6.92 ± 3.47	7.16 ± 3.69	7.56 ± 4.2	3.012	0.029
Number of embryos(D3)	4 (2, 4)	4 (2, 4)	3 (2, 4)	3 (2, 4)	2.097	0.552
Number of blastocysts	0 (0, 1)	1 (0, 2)	1 (1, 2.5)	1 (1, 3)	147.658	< 0.001
Number of high-quality embryos	1 (0, 2)	1 (0, 2)	1 (0, 2)	1 (0, 2)	0.567	0.904
Number of ET	2 (0, 2)	2 (0, 2)	2 (0, 2)	0 (0, 2)	14.241	0.003
Fresh ETR, n (%)	124 (59)	122 (56)	114 (54)	92 (43)	13.088	0.004
Fresh CPR, n (%)	46(37)	52(43)	46(40)	48(52)	2.042	0.564
OHSS rate, n (%)	1(0)	1(0)	1(0)	2(1)	Fisher	0.94
Cumulative CPR, n (%)	160(77)	170(78)	170(81)	178(83)	3.142	0.37

was 4.63IU/L (AUC=0.605; P=0.36; Figure 12). Using Cumulative CPR as the criterion, the optimal cut-off value for evaluating LH on antagonist administration day was 4.66IU/L(AUC=0.557; P=0.005; Figure 13).

4 Discussion

Total dosage of Gn in Q4 group was the least, but the number of oocytes retrieved was the most.Although there was no difference in the

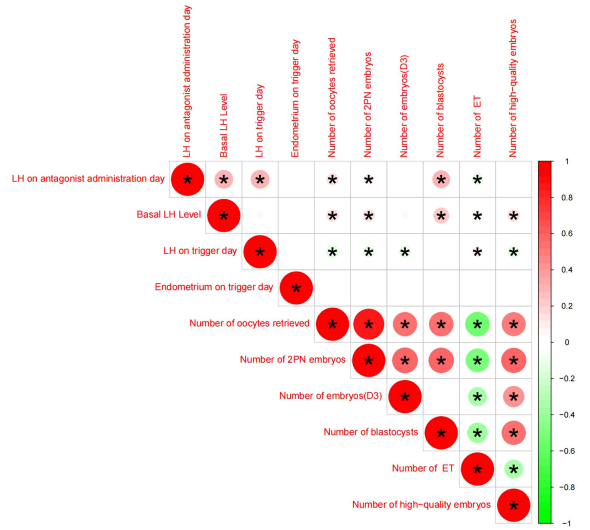

FIGURE 2
Correlation analysis between LH on the antagonist administration day and other indicators, An asterisk between the two indicators indicates statistical significance (there is a significant correlation),Red means positive correlation, green means negative correlation.

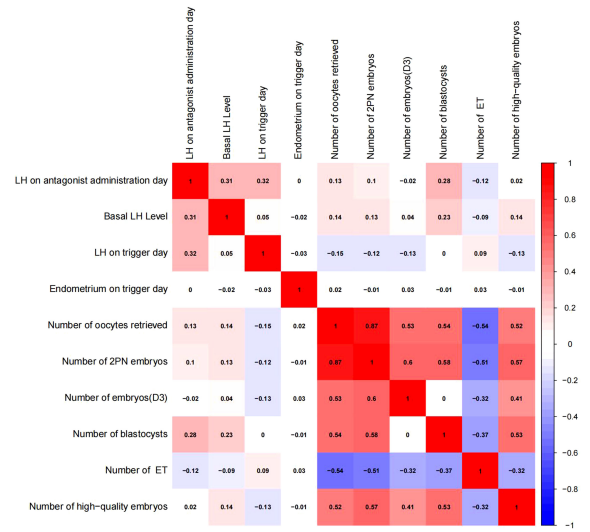

FIGURE 3
Correlation analysis between LH on the antagonist administration day and other indicators.The value between indexes represents the correlation coefficient R,R>0 means positive correlation, R < 0 means negative correlation.

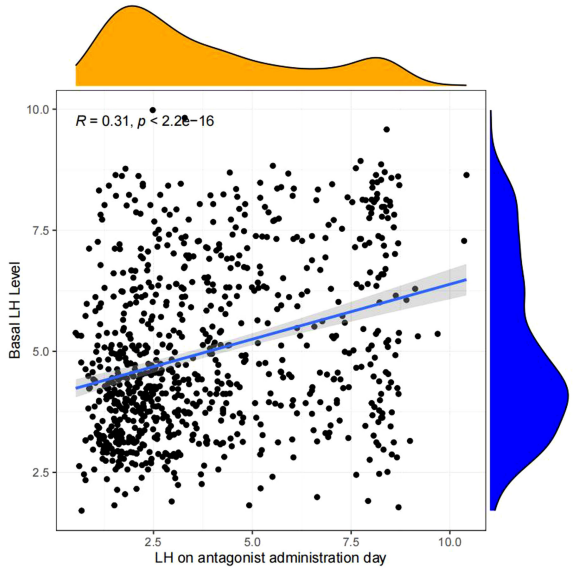

FIGURE 4
Linear correlation between LH on antagonist administration day and basal LH level.

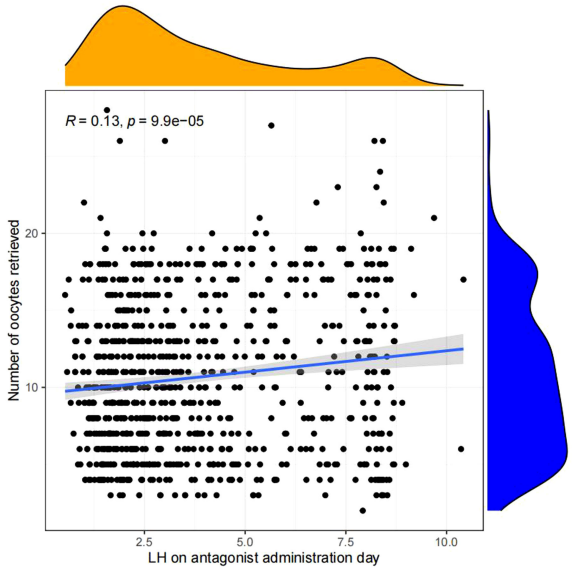

FIGURE 6
Linear correlation between LH on antagonist administration day and number of oocytes retrieved.

number of high-quality embryos among the four groups, both Fresh CPR and Cumulative CPR in group Q4 were higher than those in the other three groups.The results were not statistically significant, but they did give us some insight.In predicting the optimal cut-off value of LH on antagonist administration day in multiple pregnancy rates,Almost all the optimal cut-off value are greater than 4, which is similar to the view of Professor Li Yuan's team (10). Their study concluded that LH≥4IU/L be considered the critical value, and antagonists should be considered when the threshold was exceeded.Our study suggests that fresh ETR would be increased if adding antagonists after LH Level on the antagonist administration day > 4.18IU/L, Cumulative CPR would be increased if adding antagonists after LH Level on the antagonist administration day > 4.66IU/L.

Current research on the relationship between LH levels, ovarian reactivity, and pregnancy outcome during ovulation stimulation has yielded inconclusive results.According to Benmachiche et al. (16) a low LH level on the trigger day was associated with a lower rate of continued pregnancy and live birth and an increased rate of early abortion.Lahoud et al. (6) discovered that mid-follicular LH levels were related to ovarian reactivity but not to live birth rate.Another

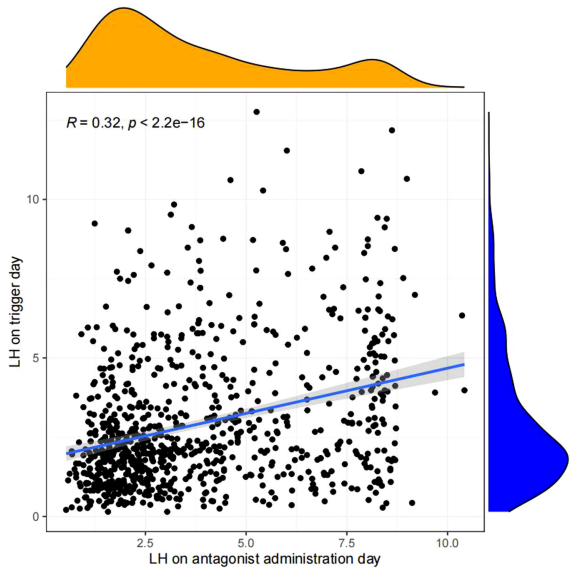

FIGURE 5
Linear correlation between LH on antagonist administration day and LH on trigger day.

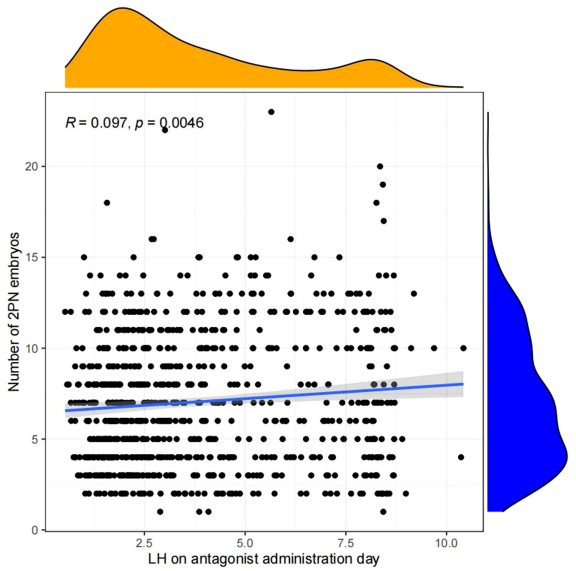

FIGURE 7
Linear correlation between LH on antagonist administration day and number of 2PN embryos.

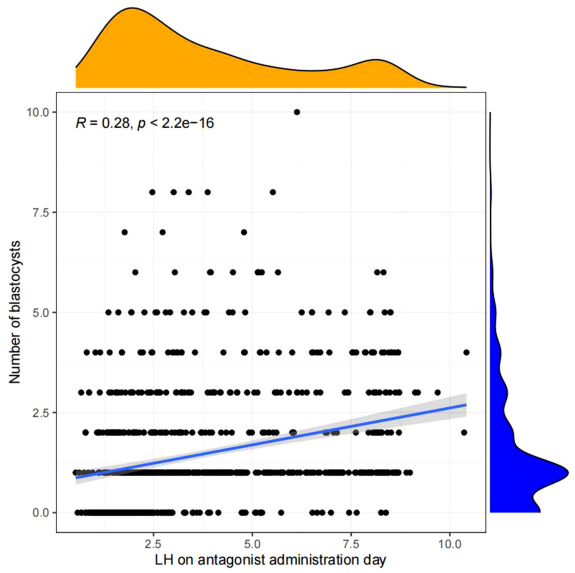

FIGURE 8
Linear correlation between LH on antagonist administration day and number of blastocysts.

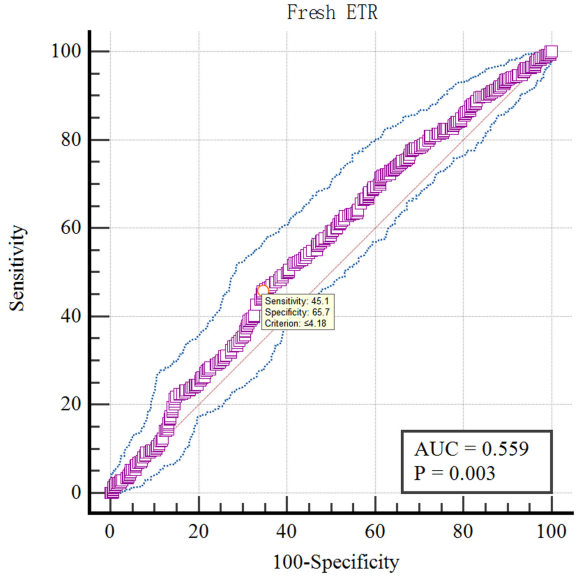

FIGURE 10
ROC curves of LH on antagonist administration day to predict Fresh ETR.

study (17) found that patients with low basal LH levels (≤3U/L) had no special ovarian responsiveness during ovulation induction but had a poorer pregnancy outcome than those with LH≥3U/L.

LH level is of great significance for maintaining a more appropriate follicle development environment and better receptivity of implanted endometrium in the COH regimen (18, 19). The premature addition of GnRH-ant, excessive use of GnRH-ant, or GnRH-ant usage beyond recommended days may lead to excessive ovarian suppression, thereby resulting in low serum LH levels and a relative lack of estrogen that might affect the growth and development of oocytes. Excessive inhibition of LH level was not found in this study. However, if GnRH-ant is added too late or the dose is insufficient, it may lead to high LH levels and an early LH surge, resulting in decreased follicle quality, reduced pregnancy rate, premature ovulation, and cycle cancellation (20). The increase in serum P level induced by an LH surge can also affect the expression of genes related to endometrial receptivity, thus, affecting embryo implantation (21). Another study reported that an increase in serum P level during the late follicular phase affected not only the embryo quality (22, 23) but also reduced implantation and clinical pregnancy rates (21, 24, 25). However, there

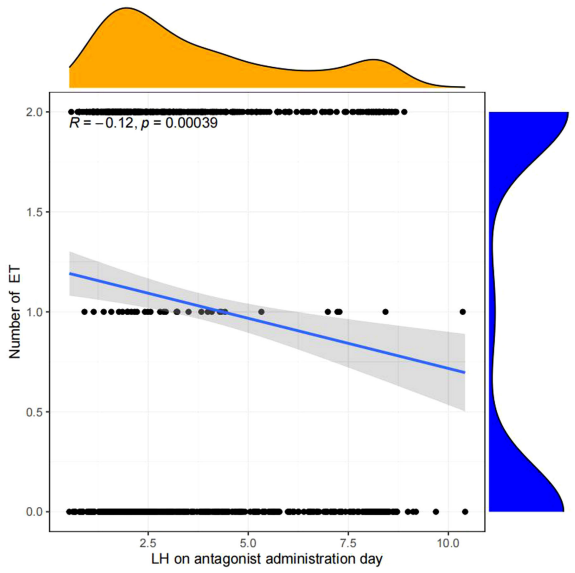

FIGURE 9
Linear correlation between LH on antagonist administration day and number of ET.

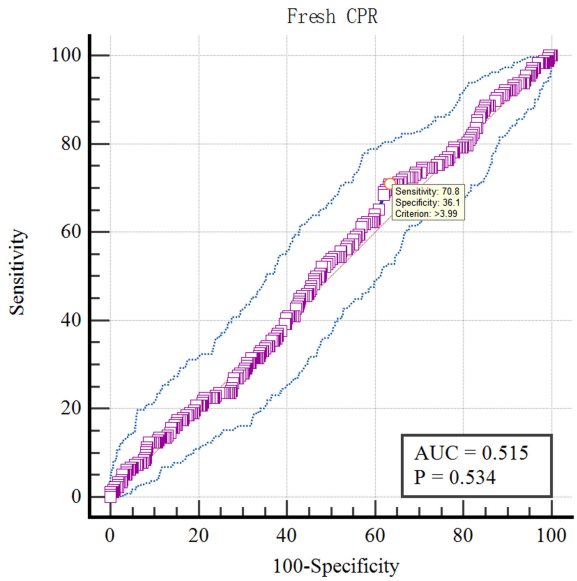

FIGURE 11
ROC curves of LH on antagonist administration day to predict Fresh CPR.

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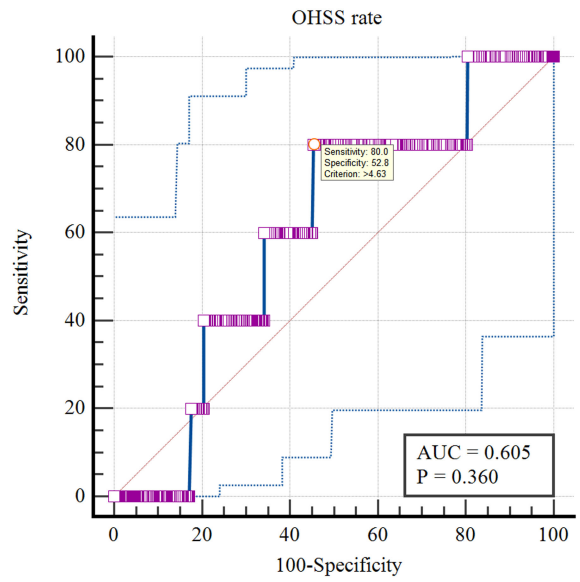

FIGURE 12
ROC curves of LH on antagonist administration day to predict OHSS.

are also views that the increase of serum P level at the late follicular stage does not affect embryo quality and cumulative live birth rate (26). This study found that as LH on the antagonist administration day increased, so did LH on the trigger day and P on the trigger day. However, there were no significant differences between the four groups in terms of the number of high-quality embryos and fresh CPR.

Current antagonist protocols are divided into fixed and flexible regimens and are mainly based on Gn stimulation time, follicle development size, and estrogen levels. However, little attention has been paid to LH levels on the antagonist administration day. A study

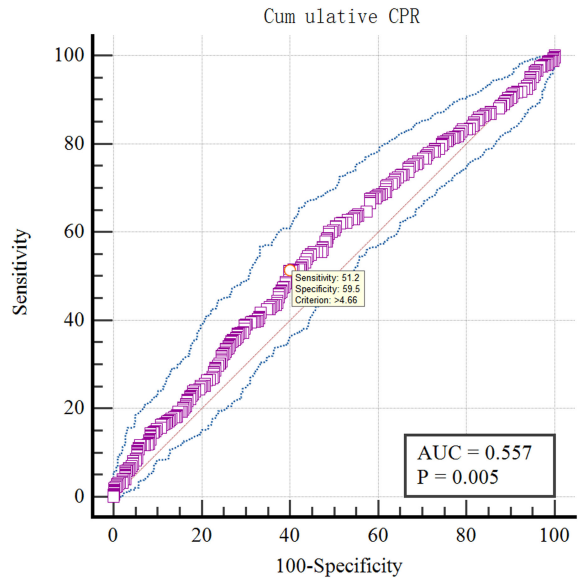

FIGURE 13
ROC curves of LH on antagonist administration day to predict Cumulative CPR.

suggested that LH levels can be used as an indicator for the addition of antagonists during COS. Patients with persistently low LH levels (LH<4.0 IU/L) may not require an antagonist (10). It is suggested that the implantation and pregnancy rates decreased with an increased antagonist dosage (27). A study stated that the number of natural killer cells and the expression level of perforin in endometrium were increased in patients treated with GnRH-Ant, hence suggesting that GnRH-ant may reduce endometrial receptivity (28). However, a randomized controlled trial showed that administration of GnRH-ant during the proliferative phase did not affect endometrial receptivity and embryo implantation; the pregnancy rates were not significantly different when compared with controls (29). Studies on the negative effects caused by elevated LH levels have mostly focused on embryo quality and endometrial receptivity. Therefore, the main observation indicators of this study were fresh ETR, fresh CPR, Cumulative CPR,the number of high-quality embryos, and OHSS rate. Although there was no significant difference in the number of high-quality embryos,fresh CPR and Cumulative CPR among the four groups, However, we give the optimal cut-off value of LH on antagonist administration day affecting different pregnancy rates,This has important guiding significance for clinical work.

This is a manuscript with LH on antagonist administration day as the main object of study, and LH on antagonist administration day 's significance in pregnancy outcomes of GnRH-ant protocols had not received enough attention before.As this was a retrospective study, many confounding factors limit the generalization of the findings to a certain extent. The sample size is not very large, which weakens the credibility of the study. Additionally, we only included patients undergoing IVF-ET due to female tubal factors, but did not include patients with other common clinical diseases such as PCOS and EMS.In the future, we can consider increasing the sample size for corresponding research.In addition, the lack of studies on abortion rate and live birth rate of pregnancy indicators is also a pity, which can be considered to supplement data based on later follow-up.

5 Conclusion

The LH Level on the antagonist administration day could be used as a predictor of the reproductive outcomes in women with normal ovarian function.There was a significant positive correlation between LH on the antagonist administration day and number of oocytes retrieved,number of 2PN embryos, number of blastocysts.LH on the antagonist administration day could predict Fresh CPR, OHSS and Cumulative CPR to some extent.

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 Reproductive Medicine Ethics Committee of the Affiliated Hospital of Shandong

University of Chinese Medicine. 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. 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

CP contributed to study design, data collection, statistical analysis and drafting of the manuscript. KW assisted with data collection and interpretation and reviewed the analyzed results. HW provided ART-related clinical theory and technical support. CP and KW reviewed the analyzed results and revised the manuscript. All authors contributed to the article and approved the submitted version.

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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

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EDITED BY
Francesco Maria Fusi,
Papa Giovanni XXIII Hospital, Italy

REVIEWED BY
Fu-Jen Huang,
Specialist Hospital, Poland
Akmal El-Mazny,
Cairo University, Egypt

*CORRESPONDENCE
Changjun Zhang
changjunzhang@hotmail.com
Hong Cao
33556511@qq.com
Honglu Diao
hldiao1976@hotmail.com

†These authors have contributed equally to this work

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The comparison between fixed versus degressive doses of medroxyprogesterone acetate combined with letrozole in patients of progestin-primed ovarian stimulation protocol: a propensity score-matched study

Ying Zhang[1,2,3,4,5†], Hao Li[1,2†], Shanshan Zhu[1,2,3,4,5], Shengfang Jiang[1,2,3,4,5], Wenxian Zhao[1,2], Xiaoning Wang[1,2,3,4,5], Liu Tian[1,2,3,4,5], Guangming Zhao[1,2,6], Nongqiao He[1,2,6], Honglu Diao[1,2,3,4,5*], Hong Cao[7*] and Changjun Zhang[1,2,3,4,5*]

[1]Reproductive Medicine Center, Renmin Hospital, Hubei University of Medicine, Shiyan, China, [2]Hubei Clinical Research Center for Reproductive Medicine, Shiyan, China, [3]Biomedical Engineering College, Hubei University of Medicine, Shiyan, China, [4]Biomedical Research Institute, Hubei University of Medicine, Shiyan, China, [5]Hubei Key Laboratory of Embryonic Stem Cell Research, Hubei University of Medicine, Shiyan, China, [6]The Third Medical School, Hubei University of Medicine, Shiyan, China, [7]Department of Orthopaedic Surgery, Renmin Hospital, Hubei University of Medicine, Shiyan, China

Objective: To explore the cycle characteristics and pregnancy outcomes of progestin-primed ovarian stimulation (PPOS) using fixed versus degressive doses of medroxyprogesterone acetate (MPA) in conjunction with letrozole (LE) in infertile women by propensity score matching (PSM) analysis.

Design: A retrospective cohort study.

Setting: Tertiary-care academic medical center.

Population: A total of 3173 infertile women undergoing their first *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) treatment within the period from January 2017 to December 2020.

Methods: A total of 1068 and 783 patients who underwent a fixed dose of MPA combined with LE and a degressive dose of MPA combined with LE protocols, respectively, were enrolled in this study. The freeze-all approach and later frozen-thawed embryo transfer (FET) were performed in both groups. Propensity score matching (1:1) was performed.

Main outcome measures: The primary outcomes were the dosage of MPA and the incidence of premature luteinizing hormone (LH) surges. The secondary outcomes were the number of oocytes retrieved, the cumulative live birth rate (CLBR) and the fetal malformation rate.

Results: We created a perfect match of 478 patients in each group. The dosage of MPA, the LH serum level on the eighth day of stimulation, progesterone (P) level and LH level on the hCG trigger day were significantly higher in the LE + fixed MPA group than in the LE + degressive MPA group (52.1 ± 13.1 mg vs. 44.9 ± 12.5 mg; 5.0 ± 2.7 IU/L vs. 3.7 ± 1.7 IU/L; 0.9 ± 0.5 ng/ml vs. 0.8 ± 0.5 ng/ml; 3.3 ± 2.4 IU/L vs. 2.8 ± 1.9 IU/L; $P < 0.01$). The duration of Gn, the number of follicles with diameter more than 16 mm on trigger day, the estradiol (E_2) level on the hCG trigger day were lower in the LE + fixed MPA group than in the LE + degressive MPA group (9.7 ± 1.7 days vs. 10.3 ± 1.5 days; 5.6 ± 3.0 vs. 6.3 ± 3.0; 1752.5 ± 1120.8 pg/ml vs. 1997.2 ± 1108.5 pg/ml; $P < 0.001$). No significant difference was found in the incidence of premature LH surge, the number of oocytes retrieved, the number of top-quality embryos, clinical pregnancy rate (CPR), CLBR or fetal malformation rate between the two groups.

Conclusion: The combination of a degressive MPA dose with LE proved effective in reducing the total MPA dosage with comparable premature LH surge and pregnancy outcomes in women undergoing the PPOS protocol.

KEYWORDS

progestin primed ovarian stimulation, medroxyprogesterone acetate, dose reduction, controlled ovarian stimulation, letrozole

Introduction

The progestin-primed ovarian stimulation (PPOS) protocol has become widely used in *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) treatments as an alternative to gonadotropin-releasing hormone (GnRH) analog protocols for inhibiting premature luteinizing hormone (LH) surges (1, 2). This protocol offers several advantages, making it a favored option in clinical practice. First, it can be administered orally, which is highly convenient for patients. Second, it is more cost-effective than other controlled ovarian hyperstimulation (COH) protocols. Third, the PPOS protocol is associated with shorter treatment durations, saving time for both patients and healthcare providers. Most importantly, it significantly reduces the occurrence of ovarian hyperstimulation syndrome (OHSS), a severe complication associated with other COH protocols. Due to these benefits, the PPOS protocol is considered suitable for women with various ovarian responses, including those with poor ovarian response (3–6), normal responders (7, 8), and even high responders (7, 9) in IVF/ICSI cycles.

Since its introduction in 2015 (10), the PPOS protocol has been subject to various progestin administration investigations, with medroxyprogesterone acetate (MPA) being the most commonly used. MPA is a potent synthetic progestin that effectively suppresses pulsatile GnRH and LH secretion. Previous research has shown that 10 mg of MPA effectively inhibits spontaneous ovulation, whereas 5 mg does not yield the same results (11). However, conflicting findings have been reported regarding the appropriate MPA dosage for preventing untimely LH surges, with some studies suggesting that daily doses of 4 mg (12, 13) or 6 mg (3, 6) are sufficient. In our previous study, we demonstrated that coadministration of letrozole (LE) with MPA during ovarian stimulation for IVF achieved comparable embryo and pregnancy outcomes while reducing the required MPA dosage (14). Nonetheless, it is crucial to address the potential teratogenicity and toxicity associated with MPA, as several human and animal studies have indicated a dose-related relationship (15–20). As a result, we have been exploring avenues to reduce the MPA dose while maintaining its inhibitory effect and ensuring the safety of the PPOS protocol.

Hence, we hypothesized that coadministration of LE with a degressive dose of MPA based on serum LH levels may offer the potential for further reducing the required MPA dosage. The objective of this retrospective cohort study was to investigate the effects of this degressive MPA dose combined with LE on cycle characteristics, endocrinological profiles, and neonatal outcomes in IVF/ICSI cycles.

Materials and methods

Study setting and subjects

We conducted a hospital-based retrospective cohort study, adhering to the principles outlined in the Declaration of Helsinki, and obtained approval from the Ethics Committee of Renmin Hospital, Hubei University of Medicine. The data were collected from the Reproductive Medicine Center, Renmin Hospital, Hubei University of Medicine, covering the period from January 2017 to December 2020. All data collected were anonymized to ensure patient confidentiality and privacy.

Patients who underwent the PPOS protocol were included in the study if they met the following criteria: women with regular menstrual cycles ranging from 25 to 35 days, aged between 20 and 40 years, and had a body mass index (BMI) between 18 and 28 kg/m^2. Additionally, bilateral antral follicle counts (AFCs) were required to be between 3 and 20, and normal basal serum levels of follicle-stimulating hormone (FSH) (<10 IU/L) and anti-Müllerian hormone (AMH) (≥1.1 ng/ml) were determined on day 2 or 3 of the cycle before COH. Study exclusion criteria included patients with metabolic disorders, polycystic ovarian syndrome (PCOS), endometriosis, pelvic tuberculosis, congenital uterine malformations, chromosomal abnormalities, single-gene disorders, and immunological diseases (Figure 1). Pregnancy outcomes were followed through telephone contact with the participants.

Controlled ovarian stimulation

All patients received an ultrasound scan and serum concentration tests on day 2 or 3 of the cycle.

In the LE+ fixed MPA group, oral LE (Jiangsu Hengrui Pharmaceuticals Co.,Ltd, China) 2.5 mg/day was started on day 2 or 3 of menstruation for 3 days, along with gonadotropin (Gn) stimulation of recombinant FSH (Gonal-f, Merck Serono, Germany) 100-150 IU/day intramuscularly, and the doses of urinary human menopausal gonadotropin (HMG, Livzon Pharmaceutical, China) and recombinant FSH were adjusted according to the growth trend of the follicles and serum hormone changes (150-450 IU per day). MPA (Zhejiang Xianju Pharmaceutical Co., China) 10 mg/day was started on day 5 of Gn use and stopped on the trigger day. Triptorelin (Decapeptyl, Ferring Pharmaceuticals, Germany) at a dose of 0.1 mg and urinary hCG (Livzon Pharmaceutical, China) at a dose of 2,000 IU were given to trigger oocyte maturation when two or more follicles reached preovulatory size (18-22 mm). Oocyte retrieval was performed 36 hours after the trigger (Figure 2). According to the standard insemination procedures used in the laboratory, all oocytes were inseminated using IVF or ICSI. Embryo scoring was conducted based on morphologic criteria; 6-8 cells with less than 20% fragmentation were considered good-quality embryos. These embryos were cultured forward when the number equaled or was more than three until they reached the blastocyst stage and were frozen on day 5 or day 6.

In the LE+ degressive MPA group, MPA 10 mg/day was started on day 5 of Gn use, and then, the dosage of MPA was gradually reduced if the serum LH level did not increase (Figure 2). We used 10 mg per day when the LH level increased to more than 10 IU/L in the process of stimulation. The other treatments were the same as above.

Hormonal measurement

Serum FSH, LH, estradiol (E_2), and progesterone (P) were measured on day 3 of the stimulation cycle (first day of stimulation), cycle day 6 (fourth day of stimulation), cycle day 8 (sixth day of stimulation), cycle day 10 (eighth day of stimulation), hCG trigger day, and the day after hCG trigger (approximately 12 hours after the injection of GnRH-a and hCG). Hormone levels were measured with electrochemiluminescence (Beckman Coulter, USA). Skilled technicians carried out all measurements in accordance with the manufacturer's instructions. The detection limits of sensitivity were as follows: FSH, 0.2 IU/L; LH, 0.2 IU/L; E_2, 15 pg/ml; and P, 0.1 ng/ml. The in-house inter and intra-assay coeffients of variation were no more than 10%.

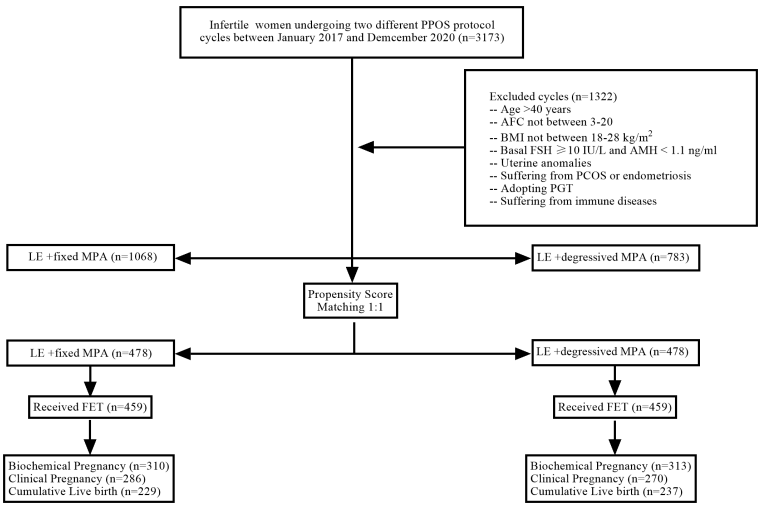

FIGURE 1
Flow chart of patient inclusion/exclusion. PPOS, progestin-primed ovarian stimulation; n, number of participants; AFC, antral follicle count; BMI, body mass index; FSH, follicle-stimulating hormone; AMH, anti-Müllerian hormone; PCOS, polycystic ovarian syndrome; PGT, preimplantation genetic diagnosis/screening; LE, letrozole; MPA, medroxyprogesterone acetate; FET, frozen embryo transfer.

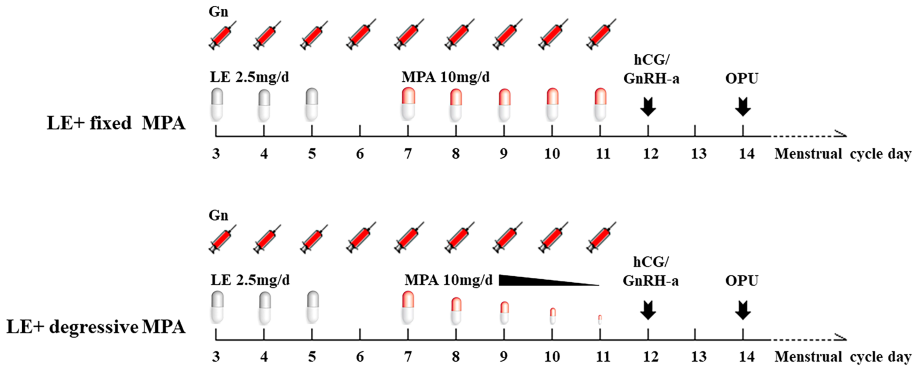

FIGURE 2
The diagram of the two PPOS protocols. LE, letrozole; MPA, medroxyprogesterone acetate; Gn, gonadotropin; hCG, human chorionic gonadotropin; GnRH-a, gonadotropin-releasing hormone agonist; OPU, oocyte pick-up.

Endometrial preparation and frozen-thawed embryo transfer

Endometrial preparation was performed with natural cycle, hormone replacement treatment (HRT) or downregulation combined with HRT for the second cycle after oocyte retrieval. The decision of the therapy was determined according to patient and physician preference.

In the natural cycle, the follicle growth was exanimated by transvaginal ultrasound from day 10 of menstruation per 2 days till ovulation happened, then luteal-phase support was initiated with 10 mg twice oral dydrogesterone (Duphaston, Abbott, USA) and continued daily until 3 months of gestation.

In the HRT cycle, women were administered 2 mg twice oral estradiol valerate tablets (Progynova, Berlin, Germany) on day 3 of spontaneous menses or P-induced withdrawal bleeding. The dosage of Progynova was adjusted according to the endometrial thickness and serum E_2 levels, and the maximum dose was 8 mg per day. After 16 days, when the endometrial thickness reached ≥ 7 mm and the serum concentration of E_2 was ≥ 100 pg/ml, luteal-phase support was initiated with the application of 90 mg vaginal progesterone gel (Crinone; Merck Serono) or 60 mg intramuscular progesterone (Zhejiang Xianju Pharmaceutical Co., China) and 10 mg twice oral dydrogesterone.

In the downregulation combined with HRT cycle, the patients received a single intramuscular injection of 3.75 mg long-acting triptorelin acetate (Decapeptyl; Ferring, SaintPrex, Switzerland) on day 3 of the cycle. After 35 days of downregulation, oral estradiol valerate tablets were added, and the other procedure was the same as above.

Outcome measurements

Primary outcomes

The primary outcomes were the dosage of MPA and the incidence of premature luteinizing hormone (LH) surges. The premature LH surge was defined as serum LH > 10 IU/L during stimulation. Viable embryos were estimated based on embryo morphologic scoring conducted on day 3 after oocyte retrieval.

Secondary outcomes

Secondary efficacy parameters include the number of oocytes retrieved, the cumulative live birth rate (CLBR) and the fetal malformation rate from a single IVF cycle. The endpoint was cumulative live birth or the use of all embryos.

Moderate/severe OHSS was diagnosed in women who fulfilled more than one of the following criteria: clinical ascites, hydrothorax, or dyspnea (exertional or at rest). Biochemical pregnancy was defined as hCG >10 IU/L two weeks after embryo transfer (ET). Clinical pregnancy was defined as an intrauterine gestational sac identified by ultrasonography 30 days after ET. Early pregnancy loss was defined as spontaneous pregnancy loss before 12 weeks. Live birth was considered when a living fetus was born after 28 weeks of pregnancy. CLBR was calculated as the number of live birth cycles/total number of oocyte retrieval cycles.

Statistical analysis

All analyses were performed using the statistical packages R (The R Foundation; http://www.r-project.org; version 3.4.3), EmpowerStats (http://www.empowerstats.com) and SPSS 26.0 (IBM, Armonk, NY, USA). Continuous variables were presented as mean with standard deviation or median with interquartile range, and one-way analysis of variance or Kruskal–Wallis test was used to compare the differences among groups. Categorical variables were described as number with percentage and compared by Pearson's chi-square test or Fisher's exact test. We constructed a multivariable regression model to quantify the related factors of pregnancy outcomes in all participants. Statistical significance was accepted as a two-sided P value < 0.05. Graphs were generated by using Originpro 2018C version 9.5.1.195 (Originlab).

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Results

Patient characteristics

From the initial cohort of 3,173 IVF/ICSI cycles, 1,322 cycles were excluded from the analysis. After the exclusions, the eligible cohort included 1,068 women using the LE+ fixed MPA protocol, 783 women using the LE+ degressive MPA protocol, and 478 patients in each group when propensity score matching (PSM) was performed (Table 1). There were no statistically significant differences in female age, BMI, AFC, AMH, infertility duration, infertility type, or infertility diagnosis between the two groups ($P > 0.05$) (Table 1).

Ovarian stimulation characteristics

The ovarian stimulation characteristics of the two groups are given in Table 2. After PSM, there were significant differences in the dose of MPA, duration of Gn, and number of follicles with diameter > 16 mm on trigger day (P <0.05). However, there were no statistically significant differences between the two groups in terms of total dosage of Gn, premature LH surge, endometrial thickness on the hCG trigger day, number of oocytes retrieved, number of mature oocytes, fertilization rate, nonviable embryo cycles, blastocyst progression rate, number of frozen embryos and moderate/severe OHSS rate ($P > 0.05$).

Hormone profile

For hormone levels during ovarian stimulation, there were no statistically significant differences in LH and E_2 levels in the two cohorts on the first day, the fourth day, and the sixth day of stimulation, as well as E_2 levels on the eighth day of stimulation and LH levels on the day after hCG trigger ($P > 0.05$), but there were significant differences in LH levels on the eighth day of stimulation, and LH, E_2, and P levels on the hCG trigger day ($P < 0.01$) (Table 3; Figures 3, 4).

Pregnancy outcomes in frozen-thawed embryo transfer cycles

Descriptive statistics for the reproductive outcomes of frozen-thawed embryo transfer (FET) are summarized in Table 4. There was no statistically significant difference between the two groups in the number of transferred embryos, endometrial preparation methods, embryo transfer stage, clinical pregnancy rate (CPR), ectopic pregnancy rate, early pregnancy loss rate, mid- and late-

TABLE 1 Baseline characteristics of the two PPOS protocols.

	Before propensity matching			After propensity matching		
	LE+ fixed MPA	LE +degressive MPA	*P*-value	LE+ fixed MPA	LE +degressive MPA	*P*-value
No. of cycles	1068	783	/	478	478	/
Female Age (years)	(1068) 33.0 ± 4.2	(783) 32.6 ± 4.2	0.032	(478) 32.4 ± 4.1	(478) 32.4 ± 4.2	0.957
BMI (kg/m^2)	(1068) 22.4 ± 2.4	(783) 22.9 ± 2.5	<0.001	(478) 22.7 ± 2.5	(478) 22.6 ± 2.4	0.370
AFC	(1068) 7.5 ± 3.3	(783) 7.8 ± 3.3	0.033	(478) 7.8 ± 3.4	(478) 7.7 ± 3.2	0.860
AMH (ng/ml)	(1068) 2.5 ± 1.8	(783) 2.4 ± 1.9	0.734	(478) 2.5 ± 1.8	(478) 2.5 ± 2.1	0.964
Infertility duration (years)	(1068) 4.1 ± 3.3	(783) 3.8 ± 3.1	0.112	(478) 3.8 ± 2.9	(478) 4.0 ± 3.0	0.491
Primary Infertility n (%)	846/1068 (79.2%)	773/783 (98.7%)	<0.001	460/478 (96.2%)	468/478 (97.9%)	0.125
Infertility diagnosis, n (%)			0.094			0.587
Tubal factor	475/1068 (44.5%)	358/783 (45.7%)		226/478 (47.3%)	231/478 (48.3%)	
Male factor	111/1068 (10.4%)	94/783 (12.0%)		56/478 (11.7%)	56/478 (11.7%)	
DOR	358/1068 (33.5%)	229/783 (29.3%)		145/478 (30.4%)	127/478 (26.6%)	
Combined	94/1068 (8.8%)	66/783 (8.4%)		35/478 (7.3%)	42/478 (8.8%)	
Unexplained /other	30/1068 (2.8%)	36/783 (4.6%)		16/478 (3.3%)	22/478 (4.6%)	
Insemination method, n (%)			0.061			0.877
IVF	862/1068 (80.7%)	604/783 (77.1%)		372/478 (77.8%)	370/478 (77.4%)	
ICSI	206/1068 (19.3%)	179/783 (22.9%)		106/478 (22.2%)	108/478 (22.6%)	

Date: mean ± SD or (%) (no./total no.). PPOS, progestin-primed ovarian stimulation; LE, letrozole; MPA, medroxyprogesterone acetate; BMI, body mass index; AFC, antral follicle count; AMH, anti-Mullerian hormone; DOR, diminished ovarian reserve; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection.

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TABLE 2 Ovarian stimulation characteristics of the two PPOS protocols.

	Before propensity matching			After propensity matching		
	LE+ fixed MPA	LE +degressive MPA	*P*-value	LE+ fixed MPA	LE +degressive MPA	*P*-value
Total dosage of MPA (mg)	(1068) 53.6 ± 13.4	(783) 42.6 ± 12.4	<0.001	(478) 52.1 ± 13.1	(478) 44.9 ± 12.5	<0.001
Duration of Gn (days)	(1068) 9.5 ± 1.5	(783) 10.3 ± 1.6	<0.001	(478) 9.7 ± 1.7	(478) 10.3 ± 1.5	<0.001
Total dosage of Gn (IU)	(1068) 1862.2 ± 475.9	(783) 1939.0 ± 604.1	0.177	(478) 1899.9 ± 450.4	(478) 1993.3 ± 597.3	0.260
Premature LH surge (LH > 10mIU/ml)	147/1068 (13.8%)	38/783 (4.9%)	<0.001	30/478 (6.3%)	26/478 (5.4%)	0.582
Endometrial thickness on the hCG trigger day (mm)	(1061) 8.8 ± 2.4	(770) 8.8 ± 2.3	0.441	(475) 9.2 ± 2.5	(470) 8.8 ± 2.3	0.075
No. of follicles with diameter > 16 mm on trigger day	(1063) 5.2 ± 2.9	(776) 6.3 ± 3.1	<0.001	(477) 5.6 ± 3.0	(474) 6.3 ± 3.0	<0.001
No. of oocytes retrieved	(1068) 6.3 ± 2.9	(783) 6.6 ± 2.7	0.031	(478) 6.4 ± 2.9	(478) 6.7 ± 2.7	0.194
No. of mature oocytes	(1068) 5.5 ± 2.8	(783) 5.7 ± 2.6	0.041	(478) 5.5 ± 2.9	(478) 5.8 ± 2.6	0.175
Fertilization rate (2PN) (%)	(1068) 84.0 ± 17.4	(783) 84.3 ± 16.5	0.707	(478) 82.8 ± 17.8	(478) 83.7 ± 16.8	0.428
Cleavage rate (%)	(1068) 98.4 ± 9.3	(783) 98.5 ± 9.4	0.738	(478) 98.2 ± 10.1	(478) 98.3 ± 10.7	0.899
Nonviable embryo cycles	34/1068 (3.2%)	20/783 (2.6%)	0.427	13/478 (2.7%)	8/478 (1.7%)	0.270
No. of viable embryos obtained	(1068) 2.7 ± 1.4	(783) 2.7 ± 1.3	0.332	(478) 2.6 ± 1.4	(478) 2.7 ± 1.3	0.148
No. of top-quality embryos	(1068) 2.0 ± 1.5	(783) 2.0 ± 1.4	0.509	(478) 1.9 ± 1.4	(478) 2.0 ± 1.4	0.126
Blastocyst progression rate (%)	1590/2061 (77.1%)	1482/1814 (81.7%)	<0.001	773/968 (79.9%)	885/1102 (80.3%)	0.797
No. of frozen embryos	(1068) 2.2 ± 1.3	(783) 2.3 ± 1.3	0.133	(478) 2.2 ± 1.3	(478) 2.3 ± 1.3	0.298
Moderate/severe OHSS, n (%)	0	0	/	0	0	/

Date: mean ± SD or (%) (no./total no.). PPOS, progestin-primed ovarian stimulation; LE, letrozole; MPA, medroxyprogesterone acetate; Gn, gonadotropin; LH, luteinizing hormone; PN, pronuclear number; OHSS, ovarian hyperstimulation syndrome.

TABLE 3 Hormone profiles during ovarian stimulation of the two PPOS protocols.

	Before propensity matching			After propensity matching		
	LE+ fixed MPA	LE +degressive MPA	*P*-value	LE+ fixed MPA	LE +degressive MPA	*P*-value
1st day of stimulation						
FSH (IU/L)	(1067) 7.8 ± 1.8	(783) 7.7 ± 1.8	0.158	(478) 7.7 ± 1.8	(478) 7.7 ± 1.8	0.878
LH (IU/L)	(1068) 4.2 ± 1.9	(782) 3.7 ± 1.7	<0.001	(478) 3.9 ± 1.5	(478) 3.9 ± 1.7	0.874
E_2 (pg/ml)	(1068) 41.5 ± 20.8	(783) 41.4 ± 22.1	0.915	(478) 41.2 ± 21.3	(478) 41.7 ± 20.9	0.697
P (ng/ml)	(1062) 0.6 ± 0.5	(777) 0.6 ± 0.5	0.564	(478) 0.7 ± 0.5	(478) 0.7 ± 0.6	0.937
4th day of stimulation						
LH (IU/L)	(1047) 5.1 ± 2.3	(771) 4.5 ± 2.1	<0.001	(478) 4.8 ± 2.3	(478) 4.8 ± 2.2	0.694
E_2 (pg/ml)	(1064) 47.1 ± 29.2	(781) 42.4 ± 27.6	<0.001	(478) 42.8 ± 27.0	(478) 44.5 ± 28.7	0.354
6th day of stimulation						
LH (IU/L)	(1015) 5.2 ± 3.1	(680) 3.5 ± 2.0	<0.001	(478) 3.9 ± 2.3	(478) 3.8 ± 2.1	0.548
E_2 (pg/ml)	(1017) 188.3 ± 140.3	(683) 159.7 ± 124.2	<0.001	(478) 168.6 ± 111.1	(478) 173.4 ± 136.9	0.547

(Continued)

TABLE 3 Continued

	Before propensity matching			After propensity matching		
	LE+ fixed MPA	LE +degressive MPA	*P*-value	LE+ fixed MPA	LE +degressive MPA	*P*-value
8th day of stimulation						
LH (IU/L)	(813) 5.2 ± 2.6	(378) 3.8 ± 1.8	<0.001	(333) 5.0 ± 2.7	(208) 3.7 ± 1.7	<0.001
E_2 (pg/ml)	(814) 592.9 ± 441.9	(378) 488.3 ± 422.5	<0.001	(333) 597.9 ± 397.0	(208) 625.9 ± 438.2	0.444
hCG trigger day						
LH (IU/L)	(1065) 3.6 ± 2.5	(778) 2.8 ± 1.9	<0.001	(477) 3.3 ± 2.4	(475) 2.8 ± 1.9	0.002
E_2 (pg/ml)	(1067) 1708.0 ± 1231.1	(779) 1921.2 ± 1110.4	<0.001	(478) 1752.5 ± 1120.8	(476) 1997.2 ± 1108.5	<0.001
P (ng/ml)	(1019) 0.9 ± 0.5	(777) 0.8 ± 0.4	<0.001	(461) 0.9 ± 0.5	(476) 0.8 ± 0.5	<0.001
Day after hCG trigger						
LH (IU/L)	(864) 65.9 ± 29.3	(695) 56.6 ± 27.0	<0.001	(285) 61.2 ± 29.3	(364) 58.0 ± 27.3	0.150

Date: mean ± SD or (%) (no./total no.). PPOS, progestin-primed ovarian stimulation; LE, letrozole; MPA, medroxyprogesterone acetate; FSH, follicle-stimulating hormone; LH, luteinizing hormone; E_2, estradiol; P, progesterone; hCG, human chorionic gonadotropin.

term pregnancy loss rate, live birth rate, CLBR, fetal birth weights, fetal sex, or malformation rate ($P > 0.05$) (Table 4).

To account for potential confounders, multivariable regression analysis was performed. After controlling for female age, BMI, AFC, AMH, duration of infertility, infertility type, infertility diagnosis, fertilization method, serum FSH, LH, E_2 and P levels on the 1st day of stimulation and serum LH and E_2 levels on the 4th day and 6th day of stimulation, there were significant differences in total dosage of MPA and number of follicles with diameter more than 16 mm on trigger day ($P < 0.001$) (Table 5). Furthermore, there were no

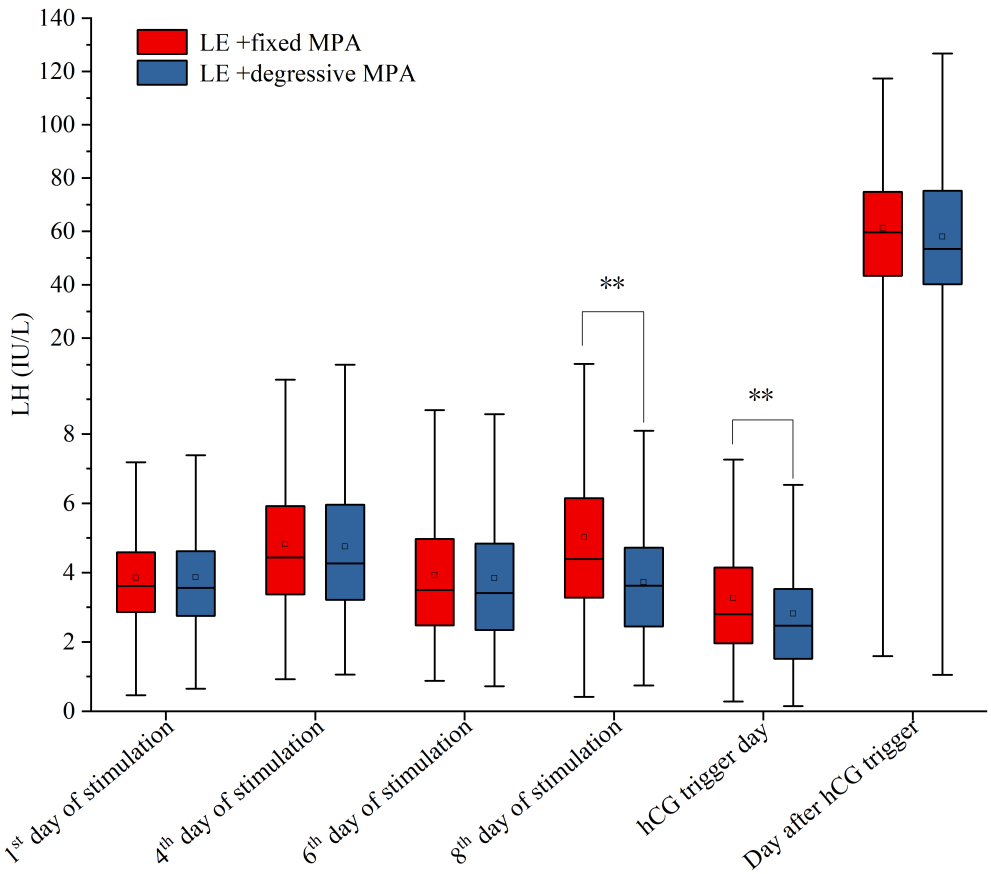

FIGURE 3
The serum levels of LH on the 1st, 4th, 6th, 8th of stimulation, hCG trigger day and the day after hCG trigger between the two PPOS protocols. **p-value < 0.01.

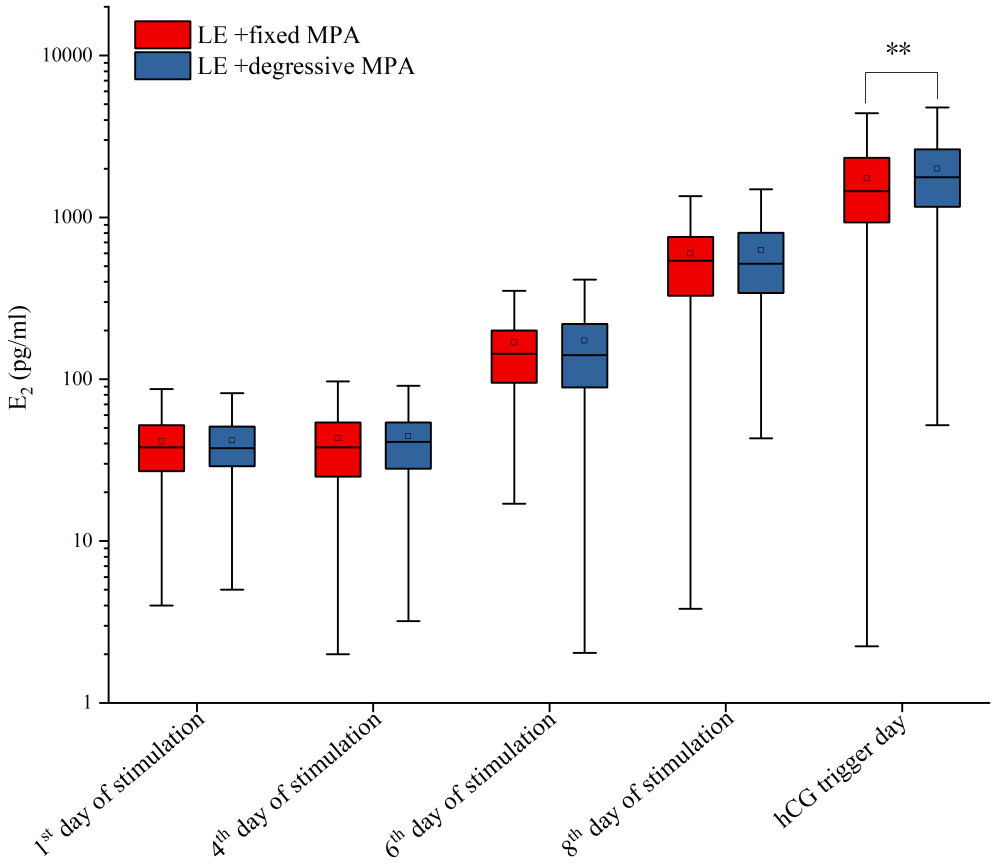

FIGURE 4
The serum levels of E_2 on the 1st, 4th, 6th, 8th of stimulation and hCG trigger day between the two PPOS protocols. **p-value < 0.01.

significant differences in the premature LH surge rate, number of oocytes retrieved, CLBR or fetal malformation rate after multivariable regression analysis ($P > 0.05$) (Table 5).

Discussion

Our study found that the LE + degressive MPA group exhibited lower dosages of MPA, and lower hormone levels (LH and E_2) during the late follicular stage compared to the fixed 10 mg daily MPA group. Additionally, the LE + degressive MPA group showed a higher duration of Gn and greater numbers of follicles with diameter more than 16 mm on trigger day. However, there were no significant differences between the two groups in terms of premature LH surge, number of oocytes retrieved, moderate/severe OHSS rate, CPR, CLBR, or fetal malformation rate. The use of a degressive MPA dose combined with LE proved effective in reducing the total MPA dosage and promoting follicle maturation in women undergoing the PPOS protocol.

TABLE 4 Freeze-thaw transplantation cycle and reproductive outcome between the two PPOS protocols.

	Before propensity matching			-	After propensity matching		
	LE+ fixed MPA	LE +degressive MPA	*P*-value		LE+ fixed MPA	LE +degressive MPA	*P*-value
No. of FET	1027	772			459	459	
No. of transferred embryos (per transfer)	(1027) 1.8 ± 0.5	(772) 1.7 ± 0.5	0.003		(459) 1.7 ± 0.5	(459) 1.7 ± 0.5	0.281
Endometrial preparation, n (%)			0.512				0.446
Natural cycle	4/1027 (0.4%)	3/772 (0.4%)			1/459 (0.2%)	2/459 (0.4%)	
HRT	101/1027 (9.8%)	89/772 (11.5%)			39/459 (8.5%)	49/459 (10.7%)	
Down-regulation + HRT	922/1027 (89.8%)	680/772 (88.1%)			419/459 (91.3%)	408/459 (88.9%)	

(Continued)

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TABLE 4 Continued

	Before propensity matching			-	After propensity matching		
	LE+ fixed MPA	LE +degressive MPA	*P*-value		LE+ fixed MPA	LE +degressive MPA	*P*-value
Embryos transferred n (%)			<0.001				0.084
Cleavage stage	318/1027 (31.0%)	160/772 (20.7%)			116/459 (25.3%)	94/459 (20.5%)	
Blastocyst stage	709/1027 (69.0%)	612/772 (79.3%)			343/459 (74.7%)	365/459 (79.5%)	
Biochemical pregnancy rate, n (%)	677/1027 (65.9%)	521/772 (67.5%)	0.486		310/459 (67.5%)	313/459 (68.2%)	0.832
Clinical pregnancy rate, n (%)	612/1027 (59.5%)	435/772 (56.3%)	0.175		286/459 (62.3%)	270/459 (58.8%)	0.160
Implantation rate, n (%)	807/1808 (44.6%)	559/1305 (42.8%)	0.318		385/797 (48.3%)	356/781 (45.6%)	0.278
Ectopic pregnancy rate, n (%)	8/612 (1.3%)	2/435 (0.5%)	0.286		6/286 (2.1%)	2/270 (0.7%)	0.181
Early pregnancy loss rate, n (%)	89/612 (14.5%)	51/435 (11.7%)	0.283		39/286 (13.6%)	28/270 (10.4%)	0.237
Mid- and late-term pregnancy loss rate, n (%)	17/612 (2.8%)	8/435 (1.8%)	0.327		8/286 (2.8%)	2/270 (0.7%)	0.068
Preterm birth rate, n (%)	118/612 (19.3%)	79/435 (18.2%)	0.648		52/286 (18.8%)	49/270 (18.1%)	0.883
Twin pregnancy rate, n (%)	141/612 (23.0%)	93/435 (21.4%)	0.525		72/286 (25.2%)	62/270 (23.0%)	0.542
Live birth rate, n (%)	490/1027 (47.7%)	373/772 (48.3%)	0.800		229/459 (49.9%)	237/459 (51.6%)	0.403
Cumulative live birth rate, n (%)	490/871 (56.3%)	373/666 (56.0%)	0.922		229/401 (57.1%)	237/413 (57.4%)	0.936
Birth weights (kg)	(627) 2.94 ± 0.67	(466) 2.96 ± 0.66	0.713		(300) 2.92 ± 0.65	(299) 2.94 ± 0.65	0.704
Fetus's sex, n (%)			0.695				0.653
A girl	154/490 (31.4%)	126/373 (33.8%)			73/229 (31.9%)	76/220 (32.1%)	
A boy	198/490 (40.4%)	154/373 (41.3%)			85/229 (37.1%)	99/220 (41.7%)	
Two girls	19/490 (3.9%)	16/373 (4.3%)			11/229 (4.8%)	9/220 (3.8%)	
Two boys	51/490 (10.4%)	37/373 (9.9%)			21/229 (9.2%)	23/220 (9.7%)	
A boy and a girl	68/490 (13.9%)	40/373 (10.7%)			39/229 (17.0%)	30/220 (12.7%)	
Fetal malformation rate (%)	6/490 (1.2%)	6/373 (1.6%)	0.633		4/229 (1.7%)	2/220 (0.9%)	0.440

Date: mean ± SD or (%) (no./total no.). PPOS, progestin-primed ovarian stimulation; LE, letrozole; MPA, medroxyprogesterone acetate; FET, frozen-thawed embryo transfer; HRT, hormone replacement therapy.

In the current study, three steps were taken to reduce the total dose of MPA. First, we used LE instead of MPA from day 1 to day 3 of ovarian stimulation. Then, on day 4 of stimulation, no MPA or LE was administered. The third step involved administering MPA from day 5 of stimulation until the hCG trigger day, with gradual reduction until complete withdrawal.

LE, a third-generation aromatase inhibitor, promotes folliculogenesis by accumulating androgen in the follicle while increasing FSH receptor expression and stimulating insulin-like growth factor-I (IGF-I) (21, 22). Notably, LE treatment in women with PCOS resulted in a trend of monofollicular growth in the late follicular stage (23). Two retrospective studies on PCOS patients using a combination of LE and MPA in IVF cycles reported a higher follicular output rate (24) without compromising mature and fertilized oocyte yields, despite decreased oocyte maturity and fertilization rates (13). These studies used LE for at least five days, similar to the 5-day clomiphene citrate (CC) regimen for ovulation induction (21). However, some research has shown that a single dose of LE (20-25 mg) on day 3 of the cycle or a 5-day LE regimen yields similar reproductive outcomes (25, 26), suggesting possibilities for reducing the LE usage days. To ensure multiple follicular development while preventing monofollicular growth, this study employed a 3-day LE treatment. Adding LE to Gn has been shown to effectively lower Gn requirements in previous reports (21). In our study, we adopted a sequential application of LE and MPA instead of simultaneous use, which our team previously found effective in patients with normal ovarian reserve (14). This approach allows for a reduction in the MPA dose and initial Gn dose, leading to cost savings during ovarian stimulation. Additionally, LE has a mean half-life of approximately 45 hours and is quickly reversible after discontinuation (21). Thus, abstaining from the administration of LE and MPA for approximately 2 days after the 3-day LE treatment provides another feasible strategy for decreasing the MPA dose.

There are two crucial aspects of MPA administration: dosage and timing. The inhibitory effect on an untimely LH increase can be determined by considering both factors. While a previous study by Wikström et al. (27) demonstrated that a 5 mg MPA dose failed to

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TABLE 5 Comparison of the correlation between the two PPOS protocols and pregnancy outcomes using multivariable regression analysis before and after propensity score matching.

Exposure	Before propensity matching		After propensity matching	
	Non-adjusted	Adjust I	Non-adjusted	Adjust II
Total dosage of MPA (mg)				
LE + fixed MPA	0	0	0	0
LE + degressive MPA	-11.1 (-12.3, -9.9) <0.001	-7.9 (-9.3, -6.6) <0.001	-7.4 (-9.0, -5.9) <0.001	-6.9 (-8.5, -5.4) <0.001
Premature LH surge				
LE + fixed MPA	1.0	1.0	1.0	1.0
LE + degressive MPA	0.3 (0.2, 0.5) <0.001	0.7 (0.4, 1.3) 0.264	0.8 (0.5, 1.4) 0.473	0.7 (0.3, 1.5) 0.319
number of oocytes retrieved				
LE + fixed MPA	0	0	0	0
LE + degressive MPA	0.3 (0.0, 0.5) 0.031	0.2 (-0.0, 0.5) 0.058	0.1 (-0.2, 0.5) 0.405	0.1 (-0.1, 0.4) 0.276
No. of follicles with diameter > 16 mm on trigger day				
LE + fixed MPA	0	0	0	0
LE + degressive MPA	1.1 (0.8, 1.4) <0.001	0.7 (0.4, 1.0) <0.001	0.7 (0.3, 1.1) <0.001	0.7 (0.4, 1.0) <0.001
Cumulative live birth rate				
LE + fixed MPA	1.0	1.0	1.0	1.0
LE + degressive MPA	1.1 (0.9, 1.3) 0.454	1.0 (0.8, 1.2) 0.837	1.0 (0.8, 1.3) 0.738	1.0 (0.8, 1.3) 0.813
Fetal malformation rate				
LE + fixed MPA	1.0	1.0	1.0	1.0
LE + degressive MPA	1.4 (0.4, 4.3) 0.590	0.9 (0.2, 4.5) 0.900	0.4 (0.1, 2.1) 0.279	0.2 (0.0, 5.6) 0.362

Data was shown as β (95%CI) P value /OR (95%CI) P value.
Non-adjusted model adjusts for: None. Adjust I model and Adjust II model were adjusted for: female age, BMI, AFC, AMH, duration of infertility, infertility type, infertility diagnosis, insemination method, serum FSH, LH, E_2 and P levels on 1st day of stimulation and serum LH and E_2 levels on 4th and 6th day of stimulation.

suppress ovulation, recent research with varied MPA doses, such as 4 mg, 6 mg, and 10 mg daily, proved effective in preventing premature LH surges (3, 6, 12). Hence, our presumption is that the MPA dosage used in IVF cycles is less critical than the precise timing of its administration. To achieve optimal results, MPA should be applied before the LH surge induced by E_2 (10). As a flexible-start MPA protocol, the initiation of MPA usage could occur on stimulation day 7 or when the leading follicle reaches ≥ 12-14 mm or serum E_2 levels reach > 200 ng/mL (5, 28–33). Notably, the peak plasma MPA concentration is typically reached 1-3 hours after oral administration (34), and the pituitary LH levels decrease after 5 days of MPA administration (10). Furthermore, it takes three weeks or longer for serum LH levels to recover after oral intake of 10 mg MPA per day for 10 days (35). In our study, we administered MPA on stimulation day 5, which is earlier than the timing mentioned in the literature. We also adopt a degressive administration approach for MPA, based on stable serum LH levels, preventing delayed resumption of LH levels. Our findings suggest a promising beneficial effect, as it allows for a reduction in MPA dosage while ensuring effective pituitary suppression.

Emphasis should be placed on the impact of LH on various stages of follicle growth. A study confirmed that elevated basal LH levels in PCOS patients undergoing IVF treatment with the MPA protocol do not impair pregnancy outcomes (36). To ensure optimal follicle development in IVF cycles with suppressed endogenous LH, LH supplementation is recommended when basal LH levels are less than 1.2 IU/L (37). Furthermore, different stages of follicle development are influenced by distinct survival factors for follicle growth. Although during the antral follicle stage, FSH plays a major role as a survival factor, while IGF1 and IL1b act as potent survival factors (38), elevated LH levels after LE treatment could potentially serve as a predictor for improved ovulation induction outcomes and no need for preinhibition of LH secretion (39). In preovulatory follicles of middle and late follicular stages, both FSH and LH play crucial roles as survival factors (38). Therefore, if the serum LH levels of the ovarian stimulation process remain stable, adopting MPA later than early follicular stage and administration degressively is considered safe.

Previous studies have reported varying LH levels on the hCG trigger day in different patient groups using the MPA protocol. In women with PCOS, LH levels ranged from 1.62 to 2.52 IU/L (40–43), while in infertile women with normal ovarian reserve, LH levels were between 1.56 and 3.54 IU/L (12, 14, 44–46). Poor responders showed LH levels in the range of 2.4 to 5.55 IU/L (6, 47–49). Moreover, research indicated that the LH level at the hCG trigger was 3.68 ± 2.69 IU/L for patients younger than 35 years and 4.77 ±

3.10 IU/L for patients older than 35 years (49). Although the suitable values for LH levels on the hCG trigger day require further investigation, it appears that they are positively correlated with age and negatively correlated with ovarian reserve. In this study, the LH level on the hCG trigger day was lower in the LE + degressive MPA group than in the LE + fixed MPA group, suggesting that individualized degression could effectively result in ovarian suppression without affecting ovulation and pregnancy outcomes.

When assessing the efficacy of MPA in pituitary suppression, the incidence of a premature LH surge serves as a crucial indicator for evaluation. In PCOS patients, no cases of premature LH surge were reported (41, 43), while normal responders among infertile women had an incidence of 0-0.7% (10, 12). Studies on poor responders revealed a range of 0.6%-5.6% premature LH increase (3, 48, 50). These findings suggest a negative correlation between the incidence of premature LH surge and ovarian reserve; however, further investigations are required to establish strong and direct evidence. In our study, we observed a comparable occurrence of premature LH increase during the middle to late stage of follicular growth in the LE + fixed MPA group compared to the LE + degressive MPA group (6.3% vs 5.4%), with no cases canceled in either group. Therefore, we presume that the MPA degressive regimen has an efficiency on pituitary suppression.

It is crucial to consider the potential impact of MPA on oocyte quality, and consequently, embryo quality and fetal growth, during the administration process. Despite some case series reporting adverse reproductive development after *in utero* exposure, there are reassuring findings regarding neonatal outcomes following MPA usage in a collection of retrospective studies (51–53). In accordance with these results, our study revealed no significant difference in reproductive outcomes and fetal malformation rates, leading to the conclusion that MPA at a daily dose of 10 mg for approximately 10 days or less appears to be relatively safe.

To our knowledge, this is the first study aimed at evaluating the efficacy of a step-by-step reduction in MPA dosage compared to a daily 10 mg dose in IVF/ICSI patients with PPOS protocols, focusing on endocrinological characteristics and clinical outcomes. This novel approach offers valuable insights to improve the regimen for PPOS ovarian stimulation. Another notable strength of our study is the implementation of PSM analysis, which helps mitigate bias in this retrospective cohort study. Additionally, this study benefits from a relatively large sample size, encompassing a diverse population aged between 20 and 40 years, providing meaningful representation of women facing infertility. Furthermore, recording neonatal outcomes adds to the credibility and reliability of this study.

However, this study has several limitations that should be acknowledged. First, its retrospective nature calls for further validation through randomized controlled trials and multicenter studies to confirm the results. Second, the study population from our reproductive center had a higher average age and lower AFC than other research, potentially limiting the generalizability of the findings to younger women, PCOS patients, or other specific groups of infertility patients. Additionally, the administration of different stimulation drugs (recombinant FSH and HMG) and flexible initial Gn doses may have influenced the hormonal outcomes, adding a degree of complexity to the analysis. While the CLBR was utilized as a recommended measure for evaluating IVF/ICSI treatment outcomes, it is worth noting that 308 and 355 embryos were still awaiting transfer in the LE + fixed MPA and LE + degressive MPA groups, possibly affecting the precision of the conclusion.

Conclusion

This retrospective study demonstrates the effectiveness of degressive MPA combined with LE in reducing the total MPA dose without compromising the stimulation outcomes in IVF patients. This approach offers advantages such as cost-effective stimulation, personalized treatment, and comparable reproductive outcomes. To validate the practicality of this regimen and to determine the optimal LH level and initial Gn dose for IVF, further prospective randomized controlled trials are warranted.

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/s.

Ethics statement

The studies involving humans were approved by This study was performed in accordance with the principles of the Declaration of Helsinki and was approved by the Ethics Committee of Renmin Hospital, Hubei University of Medicine (No: syrmyy2023-051). 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

CZ: Conceptualization, Project administration, Resources, Supervision, Writing – review & editing. YZ: Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing. HL: Data curation, Formal Analysis, Investigation, Software, Visualization, Writing – original draft, Writing – review & editing. SZ: Data curation, Writing – review & editing. SJ: Data curation, Writing – review & editing. WZ: Data curation, Writing – review & editing. XW: Data curation, Writing – review & editing. LT: Data curation, Writing – review & editing. GZ: Data curation, Writing – review & editing. NH: Data curation, Writing – review & editing. HD: Project administration, Supervision, Writing – review & editing. HC: 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.

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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.2023.1295787/full#supplementary-material

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Francesco Maria Fusi,
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REVIEWED BY
Bindu Menon,
University of Toledo, United States
Lixia Zhu,
Huazhong University of Science and Technology, China

*CORRESPONDENCE
Na Li
Drlina_ART@163.com

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Intramuscular injection of human chorionic gonadotropin as luteal phase support in artificial cycle frozen-thawed embryo transfer does not improve clinical outcomes: a parallel, open-label randomized trial

Xiaofang Li[1], Yu Huang[1,2], Zan Shi[1], Juanzi Shi[1] and Na Li[1*]

[1]Assisted Reproduction Center, Northwest Women's and Children's Hospital, Xi'an, Shaanxi, China, [2]Department of Reproductive Medicine, Xian Yang Central Hospital, Xianyang, Shaanxi, China

Background: Human chorionic gonadotropin (hCG) as one of the first signals secreted by the embryo to the mother may have a direct effect on the endometrium at implantation. The current study was aim to compare the clinical outcomes after frozen-thawed embryo transfer (FET) treated with artificial cycles (AC) between women who were administered intramuscular injection of human chorionic gonadotropin (hCG) as luteal phase support and the routine group.

Methods: A randomized controlled trial of 245 women was conducted at the Assisted Reproduction Center, Northwest Women's and Children's Hospital, Xi'an, China from January 2019 to January 2020. Women <40 years of age undergoing their first FET treated with AC were included. Patients were randomly allocated into either: (1) the hCG treatment group, who received intramuscular injection of hCG since the third day of progesterone administration, at a dose of 2000 IU once every two days, for a total of four times, (2) the control group, receiving routine protocol without placebo on these four days. Clinical outcomes of the two groups were analyzed.

Results: The primary outcome ongoing pregnancy rate in the hCG treatment group versus the control group was 73/124 (58.87%) versus 75/121 (61.98%), respectively (odds ratio [OR], 95% confidence interval [CI]:0.88, 0.53-1.47, P = 0.619). Secondary clinical outcomes including biochemical pregnancy, clinical pregnancy, early pregnancy loss, multiple pregnancy, live birth and preterm birth were also comparable between the two groups through the univariate analysis and multivariable regression analysis ($P > 0.05$).

Conclusion: In women undergoing AC-FET, there was no significant difference in the clinical outcomes between the hCG treatment group and the control

group. Clinicians should be cautious about adding IM-hCG as luteal phase support to improve the clinical outcome after AC-FET.

Clinical trial registration: http://www.chictr.org.cn/showprojen.aspx?proj=32511, identifier ChiCTR1800020342.

KEYWORDS

intramuscular injection of hCG, frozen embryo transfer, ongoing pregnancy rate, artificial cycle, randomized controlled trial

Introduction

The proportion of frozen embryo transfer (FET) has increased dramatically in recent years. Artificial cycle (AC) is one of the classic schemes for endometrium preparation, owing to its advantages of less monitoring and lower cancellation rate. AC-FET is suitable for women with ovulation disorder, irregular menstruation or those who do not wish to be monitored frequently. During AC-FET, a gonadotropin releasing hormone agonist (GnRH-a) may be used, followed by sequential supplementation with estrogen and progesterone to promote the proliferation and transformation of endometrium (1, 2). A randomized controlled trial (RCT) suggested that estrogen could be tapered from the day a biochemical pregnancy is established without being detrimental to the clinical pregnancy rate in AC-FET cycles (3). Progesterone should be continued until 10-12 weeks of gestation owing to the absence of corpus luteum (4, 5).

Human chorionic gonadotropin (hCG) is one of the first signals secreted by the embryo to the mother (6). HCG is always used in fresh embryo transfer cycles of *in-vitro* fertilization (IVF) or natural cycles-FET for luteal phase support to maintain progesterone secretion by the corpus luteum (7, 8). HCG has been described as one of the modulators of the implantation site by different molecular pathways and through supporting different immune cells (9). The identification of hCG receptors in the endometrium suggested that hCG may have a direct effect on the endometrium at implantation (10, 11). The direct function of hCG on the endometrium to regulate the implantation process may represent a promising direction apart from its traditional function of stimulating corpus luteum. In embryo culture media, hCG is detected from the stage of fertilization (2PN) (12). However, in ART, since embryos are transferred into the uterus at D3 (cleaved embryo) or D5 (blastocyst), the endometrium lacks stimulation from early embryo-derived hCG. Several studies investigated the function of hCG in AC-FET cycles and showed contradictory results, due to limited power with small sample sizes (13–15). Therefore, whether hCG supplementation before the embryo transfer would be beneficial for the implantation of embryos and ultimately improve the clinical outcomes of women after AC-FET remain unclear.

This prospective RCT aimed to compare the clinical outcomes in AC-FET cycles with and without IM-hCG as luteal phase support at a single center.

Materials and methods

Study design and study population

This single-center RCT was conducted at the Assisted Reproduction Center, Northwest Women's and Children's Hospital, Xi'an, China. The study protocol was approved by the ethics committee of Northwest Women's and Children's Hospital (approval number: 2018027), and was registered as ChiCTR1800020342 at http://www.chictr.org.cn/showprojen.aspx?proj=32511. All women participating in the study provided written informed consent. Participants were able to withdraw from the trial at any time.

Participants' enrollment was scheduled to be completed from January 10, 2019 to January 10, 2020. But the number of planned recruits was not reached by January 10, 2020. We had planned to apply for extending the trial to enroll enough patients, but our center suspended all new IVF treatments due to the COVID-19 pandemic in January 24, 2020. Hence, the trial recruitment was terminated on January 10, 2020.

Women were eligible if they met the following inclusion criteria (1): age < 40 years (2); first FET cycle (3); artificial cycle for endometrium preparation. Women with confirmed endometriosis, uterine malformation, intrauterine adhesion or untreated hydrosalpinx were excluded. Cycles were not eligible if the endometrial thickness was ≤ 8 mm before starting progesterone. Cycles with follicular diameter >14 mm on the day of progesterone administration were also excluded. Women were also excluded if they were participating in other studies.

Randomization

We selected women who met the inclusion criteria and started daily endometrial preparation for FET. The details of the trial were

explained to the women by a member of the project, and eligible women who signed the consent form were randomized on the day of progesterone administration. Women were randomly allocated to the hCG treatment or the control group according to a randomization list generated by a computer. The specific process was as follows: 300 random numbers were produced by a computer and divided into A and B groups, with 150 in each group. Then a random group table was made and blinded on computer. The 300 random numbers obtained above were randomly distributed to 300 sequence numbers. For every patient included, a random number was obtained according to the order of inclusion. Then the number was unblinded by the computer.

The randomization process was completed by a member of the project. Therefore, the staff who conduct the randomization process and the participants were not blinded. The physician who performed the endometrial preparation protocol and determined the number and grade of embryos transferred was blinded to the grouping. Laboratory staff and staff who conducted the data analysis and follow-up were blinded to the allocation.

Preparation of endometrium and luteal phase support

Women received a transvaginal ultrasound on the fifth day of menstruation if their urine hCG examination was negative. Estrogen was administered at starting doses of 4-6 mg/d for five days (oral estradiol valerate tablets, Bayer, Germany), and then adjusted after evaluating the endometrial growth by transvaginal ultrasound. Intramuscular (IM) progesterone (Zhejiang Xianju Pharmaceutical Co., Ltd.) was commenced if the endometrium was ≥ 8 mm and serum progesterone value was < 1.5 ng/ml. The transfer of cleaved embryo or blastocyst was performed after 4 days (20mg per day for one day, then 40mg per day for 2 days, then 60mg per day for 1 day) or 6 days (20mg per day for one day, then 40mg per day for 2 days, then 60mg per day for 3 days) of progesterone administration. For the gonadotropin-releasing hormone (GnRH-a)-AC cycles, GnRH-a (3.75 mg, Beaufort, France) was administered on the 2-4 day of menstruation. Estrogen was started approximately 30 days later as described above for the AC-FET scheme.

In the hCG treatment group, women received IM injection of hCG since the third day of starting progesterone, at a dose of 2000 IU once every two days, for a total of four times. The control group received routine protocol without placebo on these four days (Figure 1).

In addition to continuing progesterone (60mg daily) and estrogen, 20 mg of dydrogesterone (Duphaston, Abbott Biologicals B.V.) was added daily until 10 weeks of gestation. The dose of estrogen was tapered on the 12th or 14th day after cleaved embryo or blastocyst transfer if biochemical pregnancy was confirmed. The dose of progesterone was tapered every three days from the 10th gestational week.

Outcomes

The primary outcome of the present study was ongoing pregnancy, defined as the process of pregnancy beyond 12th week of gestation. The secondary outcomes included biochemical pregnancy: hCG test was positive after 12 (blastocyst) or 14 (cleaved embryo) days of transfer, clinical pregnancy (CP): the presence of intrauterine sac on ultrasound at six weeks of gestation, early pregnancy loss: spontaneous miscarriage before 12 weeks of pregnancy or no gestational sac was confirmed after biochemical pregnancy, multiple pregnancy: more than one gestational sac or embryo bud detected on ultrasound at 6-8 weeks of gestation and preterm birth: a baby born alive at 24-37 weeks of gestational age. At the present stage, we have already obtained the live birth data (defined as the delivery of a live baby at more than 24 weeks of gestation). As the live birth rate is the most concerned outcome for both patients and physicians, we also reported the live birth outcome as one of the secondary outcomes.

All participants received allocated intervention and their follow-up data were all acquired in the present study.

Sample size calculation

A superiority design was performed as hCG may have a direct function on the endometrium at implantation and result in a higher

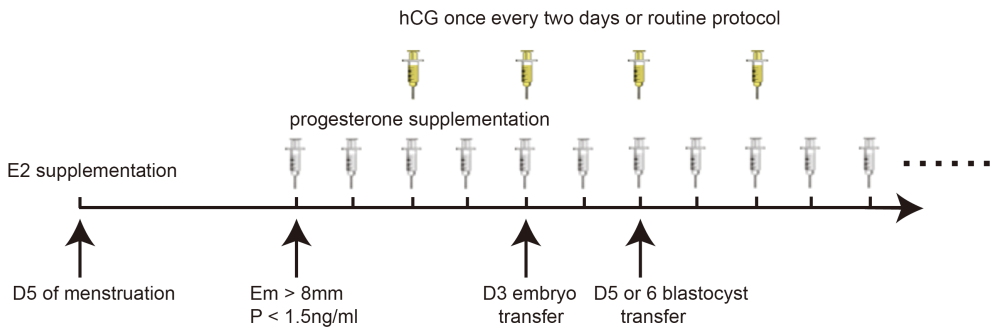

FIGURE 1
Specific programmes for the study.

ongoing pregnancy rate than the routine group. According to Maryam Eftekhar et al., clinical pregnancy rate was 16% in the control group, and 28% in women who received three doses of hCG after embryo transfer (13). To demonstrate 12% increase of ongoing pregnancy rate with a one-sided test, 80% power, and 0.05 alpha error, 147 women at least were required in each group.

Statistical analysis

The analyses in the current study were conducted according to the intention-to-treat principle. The normality of continuous variables was tested by Kolmogorov–Smirnov test. For non-normally distributed continuous variables, the means (25th-75th percentiles) were displayed. Categorical parameters were presented as frequencies (percentages). Kruskal-Wallis test for continuous variables and Pearson's $\chi 2$ test or Fisher's exact test for qualitative data were performed, where appropriate. Univariate analysis was performed to examine the relationship between hCG treatment and clinical outcomes. Multivariate logistic regression analysis was conducted to adjust potential confounders and to further identify the association of hCG treatment with clinical outcomes in AC-FET cycles. Interaction and stratified analyses were performed according to protocol in the FET cycle (AC and GnRH-a+AC), type of embryo transferred (cleavage stage and blastocyst stage), number of embryos transferred (1 and 2) and number of good quality embryos transferred (0,1 and 2). A P value < 0.05 was considered statistically significant. All analyses were performed using IBM® SPSS® software (version: 22.0, SPSS Inc. Headquarters, USA), the statistical packages R (The R Foundation; http://www.r-project.org; version 3.4.3) and EmpowerStats (www.empowerstats.com; version: 3.0, X&Y Solutions Inc.).

Results

Demographic and clinical data of the fresh IVF cycles

Between January 2019 and January 2020, a total of 245 women were eligible and randomly allocated to the hCG treatment group (n = 124) or the control group (n = 121) (Figure 2). Although women in the hCG treatment group had less tubal factors causing infertility when compared with women in the control group (35.48% versus 43.80%, $P > 0.05$), there were no significant differences between the two groups in the baseline characteristics and clinical data of the fresh IVF cycles (Table 1).

In terms of the characteristics of FET cycles, there were more blastocyst stage embryos transferred and more single embryo transfer cycles in the control group as compared to the treatment group (88.43% versus 76.61% and 86.78% versus 74.19%, $P = 0.015$ and 0.013, respectively). Women in the control group were younger at embryo transfer when compared with women in the hCG treatment group ($P = 0.046$). Other parameters including the protocol used in FET cycles (pretreatment with GnRH-a or not), endometrial thickness on the day of progesterone administration, triple-line endometrial pattern and number of good quality embryos transferred were all similar between the two groups ($P > 0.05$) (Table 2).

Clinical outcomes

The primary outcome, ongoing pregnancy rate in the treatment group versus the control group was 58.87% versus 61.98% (OR: 0.88, 95% CI: 0.53-1.47). No differences were found between the two

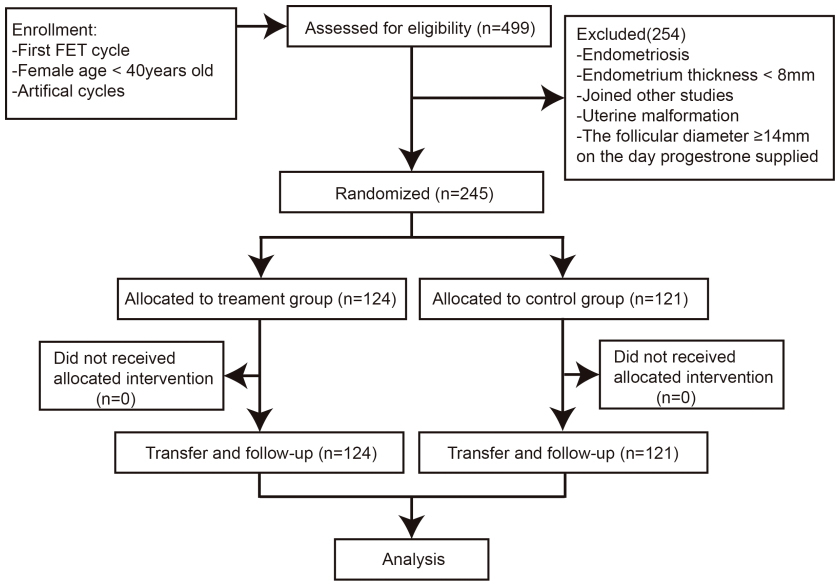

FIGURE 2
Flowchart of the study cohort.

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TABLE 1 Demographic and clinical data of the fresh IVF cycles.

Variables	Control group	Treatment group	*P*-value
n	121	124	
Women's age at oocyte retrieval (years)	29.17 (26–31)	29.94 (28–32)	0.063^{KW}
BMI (kg/m2)	23.02 (19.83-25.39)	23.05 (20.16-25.39)	0.824^{KW}
Infertile years	3.60 (2–5)	3.86 (2–5)	0.503^{KW}
Ovarian reserve function			
bFSH (mIU/mL)	6.92 (5.66-7.71)	6.49 (5.36-7.54)	0.189^{KW}
AFC	15.23 (10–22)	14.21 (9–20)	0.201^{KW}
Cause of infertility			0.757^{K}
Tubal factor	53 (43.80%)	44 (35.48%)	
Ovulation disorder	9 (7.44%)	8 (6.45%)	
Diminished ovarian reserve	2 (1.65%)	2 (1.61%)	
Male factor	20 (16.53%)	21 (16.94%)	
Unexplained	10 (8.26%)	12 (9.68%)	
More than one etiology	27 (22.31%)	37 (29.84%)	
First IVF cycle	116 (95.87%)	112 (90.32%)	0.088^{K}
Protocol in fresh cycle			0.385^{K}
Agonist	94 (77.69%)	92 (74.19%)	
Antagonist	24 (19.83%)	31 (25.00%)	
Others	3 (2.48%)	1 (0.81%)	
Gonadotropin dosage (IU)	2101.24 (1500–2525)	2299.80 (1500–3000)	0.577^{KW}
Duration of stimulations (days)	10.96 (9–12)	11.55 (9–13)	0.249^{KW}
Oestradiol level on the hCG day (pg/mL)	5500.62 (2803.00-7206.00)	5213.74 (3109.25-6615.75)	0.796^{KW}
Progesterone level on the hCG day (ng/mL)	1.60 ± 0.81	1.42 ± 0.66	0.116^{KW}
Insemination			0.195^{K}
IVF	99 (81.82%)	93 (75.00%)	
ICSI	22 (18.18%)	31 (25.00%)	
Number of oocytes retrieved	13.92 (10–18)	13.83 (9–17)	0.807^{KW}
Total number of good quality embryo available	5.24 (3–7)	4.98 (2–7)	0.483^{KW}

KW, Kruskal–Wallis test; K, Pearson's chi-square test; BMI, body mass index; AFC, antral follicle count; FSH, follicle-stimulating hormone; IVF, in-vitro fertilization; hCG, human chorionic gonadotropin; ICSI, Intracytoplasmic sperm injection; Data presented as number (percentage) or median (25th–75th centiles). Both groups were found no statistical significance.

groups in terms of biochemical pregnancy rate (75.81% versus 76.03%, OR: 0.99, 95% CI: 0.55-1.77), implantation rate (57.69% versus 68.61%, OR: 0.62, 95% CI: 0.39-1.01), clinical pregnancy rate (66.13% versus 73.55%, OR: 0.70, 95% CI: 0.41-1.22), early pregnancy loss rate (22.34% versus 18.48%, OR: 1.12, 95% CI: 0.82-1.56), multiple pregnancy rate (7.23% versus 6.61%, OR: 1.05, 95% CI: 0.66-1.67), live birth rate (55.65% versus 57.85%, OR: 0.91, 95% CI: 0.55-1.52) and preterm birth rate (20.29% versus 14.29%, OR: 1.22, 95% CI: 0.83-1.80) (treatment group versus control group, respectively). No ectopic pregnancy occurred in the two groups (Table 3).

TABLE 2 Characteristics of FET cycles.

Variables	Control group	Treatment group	*P*-value
n	121	124	
Women's age at embryo transfer (years)	29.46 (27–32)	30.4 (28–33)	**0.046^{KW}**
FET protocol			0.406^{K}
AC	84 (69.42%)	92 (74.19%)	
GnRH-a+AC	37 (30.58%)	32 (25.81%)	
Em (mm)	10.6 (9.5-11.6)	10.4 (9.5-11.0)	0.322^{KW}
Triple-line endometrial pattern			0.775^{K}
A	34 (28.10%)	40 (32.26%)	
B	75 (61.98%)	72 (58.06%)	
C	12 (9.92%)	12 (9.68%)	
Type of embryo transferred			**0.015^{K}**
Cleavage stage	14 (11.57%)	29 (23.39%)	
Blastocyst stage	107 (88.43%)	95 (76.61%)	
Number of embryos transferred			**0.013^{K}**
1	105 (86.78%)	92 (74.19%)	
2	16 (13.22%)	32 (25.81%)	
Number of good quality embryos transferred			0.082^{KW}
0	24 (19.83%)	40 (32.26%)	
1	88 (72.73%)	75 (60.48%)	
2	9 (7.44%)	9 (7.26%)	

Significant difference values are in bold.
AC, artificial cycle; GnRH-a, gonadotropin-releasing hormone-agonist; Em, endometrial thickness on the day progesterone commenced.

Association of hCG treatment with clinical outcomes in AC-FET cycles

The multivariate logistic regression models were conducted to assess the association between hCG treatment in AC-FET cycles and clinical outcomes, while adjusting for potential confounding factors. In the adjusted model 1 and adjusted model 2, hCG

TABLE 3 Clinical outcomes.

Variables	Control group	Treatment group	OR (95% CI)	*P*-value
n	121	124		
Primary outcome				
Ongoing pregnancy rate (%)	75/121 (61.98%)	73/124 (58.87%)	0.88 (0.53,1.47)	0.619
Secondary outcomes				
Biochemical pregnancy rate (%)	92/121 (76.03%)	94/124 (75.81%)	0.99 (0.55, 1.77)	0.967
Implantation rate (%)	94/137 (68.61%)	90/156 (57.69%)	0.62 (0.39, 1.01)	0.054
Clinical pregnancy rate (%)	89/121 (73.55%)	82/124 (66.13%)	0.70 (0.41, 1.22)	0.207
Early pregnancy loss rate (%)	17/92 (18.48%)	21/94 (22.34%)	1.12 (0.81, 1.56)	0.514
Multiple pregnancy a rate (%)	8/121 (6.61%)	9/124 (7.23%)	1.05 (0.66, 1.67)	0.842
Ectopic pregnancy	0	0		
Live birth rate (%)	70/121 (57.85%)	69/124 (55.65%)	0.91 (0.55, 1.52)	0.728
Preterm birth rate (%)	10/70 (14.29%)	14/69 (20.29%)	1.22 (0.83, 1.80)	0.349

[a]All multiple pregnancies in both groups were twin pregnancies.
OR, odds ratio; CI, confidence interval.

treatment was not a significant factor for ongoing pregnancy (model1:OR: 0.84, 95% CI: 0.48-1.47 and model2:OR: 1.01, 95% CI: 0.53-1.94), live birth (model1:OR: 0.85, 95% CI: 0.49-1.49 and model2:OR: 1.04, 95% CI: 0.54-2.00), biochemical pregnancy (model1:OR: 0.99, 95% CI: 0.52-1.86 and model2:OR: 1.12, 95% CI: 0.54-2.33), clinical pregnancy (model1:OR: 0.67, 95% CI: 0.37-1.22 and model2:OR: 0.70, 95% CI: 0.35-1.40), implantation (model1:OR: 0.61, 95% CI: 0.36-1.05 and model2:OR: 0.84, 95% CI: 0.47-1.52) and early pregnancy loss (model1:OR: 1.61, 95% CI: 0.71-3.63 and model2:OR: 1.36, 95% CI: 0.54-3.40) (Table 4).

Subgroup analyses

Further subgroup analyses were conducted based on FET protocol, type of embryo transferred (cleavage and blastocyst stage), number of embryos transferred (1 and 2) and number of good quality embryos transferred (0,1 and 2) to assess the stability of association of hCG administration in AC-FET cycles and clinical outcomes. The results demonstrated that no significant differences were observed on ongoing pregnancy, live birth, clinical pregnancy and early pregnancy loss between hCG treatment and control group, in all subgroups and no significant interactions were found in any of the subgroups ($P > 0.05$ for all comparisons) (Supplementary Figures 1–4).

Discussion

The present study found no significant differences in ongoing pregnancy and live birth rates between additional hCG supplementation as luteal phase support before the embryo transfer compared with the routine protocol in women undergoing AC-FET.

Traditionally, hCG plays an essential role in maintaining progesterone secretion by corpus luteum in the early stage of pregnancy and it is also used in natural or stimulated cycles in FET to induce ovulation by mimicking the LH surge. However, the transcription of hCG gene by embryo begins very early due the micro-amount of hCG has been detected from the stage of 2PN in embryo culture medium. With the identification of endometrial hCG receptors, it has been thus proposed that hCG may have direct effects on embryo-endometrial communication during implantation of human embryos (11). The application of hCG improves endometrial receptivity by inhibiting the expression of endometrial insulin-like growth factor binding protein-1 (16, 17), while increasing the expression of homeobox A10 (18). Moreover, hCG may stimulate angiogenesis during implantation by targeting VEGF/MEK/ERK or VEGF/NF-κB signaling pathway (19–21). HCG also plays a paracrine role by stimulating the leukemia inhibitory factor or inhibiting macrophage colony stimulating factor, which are important cytokines during implantation (22). On the basis of foregoing, in AC-FET cycles, hCG is likely an adjuvant therapy to enhance the clinical outcome in addition to the essential estrogen and progesterone.

Intrauterine administration of hCG has been suggested to improve clinical outcome in IVF patients. Many studies have investigated the effect of intrauterine hCG infusion on clinical outcomes but the conclusions of these studies were inconsistent due to the heterogeneity of study design (23–25). Mansour et al. reported that pregnancy rate was significantly increased by intrauterine hCG infusion which was in line with Zarei et al's conclusion (26, 27). Two recent RCTs conducted by Barbara Wirleitner et al. and Karim S. Abdallah et al. suggested that intrauterine hCG supplementation does not increases pregnancy rates in IVF patients (23, 24).

The endometrial cells were exposed to exogenous hCG for only a short time when infusion of hCG. However, in experiments

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TABLE 4 Logistic regression analysis of the clinical outcomes.

	Control group	Treatment group
n	121	124
Primary outcome		
Ongoing pregnancy rate		
Adjusted model 1 OR (95%CI)	Reference	0.84 (0.48, 1.47)
Adjusted model 2 OR (95%CI)	Reference	1.01 (0.53, 1.94)
Secondary outcomes		
Biochemical pregnancy		
Adjusted model 1 OR (95%CI)	Reference	0.99 (0.52, 1.86)
Adjusted model 2 OR (95%CI)	Reference	1.12 (0.54, 2.33)
Clinical pregnancy		
Adjusted model 1 OR (95%CI)	Reference	0.67 (0.37, 1.22)
Adjusted model 2 OR (95%CI)	Reference	0.70 (0.35, 1.40)
Implantation rate		
Adjusted model 1 OR (95%CI)	Reference	0.61 (0.36, 1.05)
Adjusted model 2 OR (95%CI)	Reference	0.84 (0.47, 1.52)
Early pregnancy loss		
Adjusted model 1 OR (95%CI)	Reference	1.61 (0.71, 3.63)
Adjusted model 2 OR (95%CI)	Reference	1.36 (0.54, 3.40)
Live birth		
Adjusted model 1 OR (95%CI)	Reference	0.85 (0.49, 1.49)
Adjusted model 2 OR (95%CI)	Reference	1.04 (0.54, 2.00)

OR, odds ratio; CI, confidence interval.
Adjusted Model I: we adjusted for women's age at retrieval; BMI and infertile years.
Adjusted Model II: we adjusted for women's age at retrieval and embryo transfer; BMI; infertile years; bFSH; AFC; protocol in fresh cycle; number of oocytes retrieved; fertilization type; Em; triple-line endometrial pattern; type of embryo transferred; number of transferred embryos and number of good-quality embryos transferred.

conducted by Sherwin et al. showed that prolonged exposure of hCG may be down regulate the pro-implantation factors and have deleterious effects on endometrial receptivity (28). Therefore, moderate hCG supplementation may be more beneficial to clinical outcomes. Asgerally T. Fazleabas et al. suggested that continuous presence of hCG was needed to sustain the impact of the initial single-dose intrauterine hCG (29), indicating that multiple hCG administrations may provide benefits. Intrauterine perfusion after the embryo transfer is obviously not feasible. However, IM-HCG can be given both before and after the embryo transfer. Therefore, the effects of IM-hCG for women undergoing AC-FET were investigated.

There is no consensus on the optimal schedule, such as dose, time point and frequency of supplementation for the introduction of hCG. Some schemes have been reported, including 3000 IU every three days since the third day of starting progesterone for three doses (14), 5000 IU every three days for three doses after embryo transfer (13), 250 μg of recombinant hCG every three days from the day of progesterone initiation for three doses (15). As the half time of hCG is 24-36 hours (30), we used 2000 IU once every two days since the third day of progesterone initiation, for a total of four times, which was somewhat center-selective. However, there was no significant difference between the hCG treatment group and the control group in terms of clinical pregnancy rate, which was in accordance with some previous studies (14, 15), but in contrast to the study of Afsar et al. (13). However, there was a marginal difference with a small sample size (Chemical pregnancy rate: P = 0.048 between the two groups) in the study of Afsar et al. and they did not conduct multivariate regression analysis to control confounding factors (13–15). In addition, ongoing pregnancy and live birth outcomes were not reported in these studies. In the present study, all live births were followed-up and no difference was found between the two groups. Larger sample size and multi-center studies are needed to confirm these findings.

The main strength of the current study is the sufficient follow-up data to report ongoing pregnancy and live birth rate, which is the most important outcome for patients undergoing IVF. The complete follow-up for all women was another strength of the present study. Besides, we conducted logistic regression analysis and subgroup analysis to ensure the stability of the conclusion. On the other hand, our study only included the first FET cycle, which may minimize some potential bias from patients and clinicians (such as clinic variability or patients' psychological factor). Furthermore, in order to reduce bias, the physician who conducted endometrial preparation protocol and staff who conducted the follow-up were blinded to the group assignment.

This study had some limitations. First, some characteristics of FET cycles, such as type and number of embryos transferred, differed between the groups. However, multivariable logistic analysis and subgroup analysis were performed to minimize the potential impact. Second, as this was a clinical trial, it could not explain the mechanism of the ineffectiveness of IM-hCG on clinical outcome. It may be due to the insufficient concentration of hCG in the endometrium. However, at least, the schedule we used had no significant difference when compared with the control group in terms of clinical outcomes. Third, the COVID-19 pandemic resulted in lockdown from January 24, 2020, and all IVF treatments were halted at our hospital. More than 80% (245/294) of the calculated sample size was reached at that time. In addition, it is difficult to require women to visit the clinic or hospital for an HCG injection every day. We stopped our trial early even though the lockdown was lifted two months later. Nevertheless, all the follow-up data were complete. The current study provided the ongoing pregnancy rates of the two groups based on the

admittance standards during the study period. Lastly, no significant difference was found in terms of ongoing pregnancy and live birth rate in women undergoing AC-FET with or without additional hCG administration as luteal phase support through the multivariate analysis and subgroup analysis. The data could be a reference for the multi-center with larger sample sizes analyses and molecular mechanism research. And the trial could also be useful to include in a meta-analysis with other available evidence in the future.

In conclusion, clinicians should be cautious in recommending IM-hCG as an adjuvant therapy to improve clinical outcomes, and the addition of hCG may impose unnecessary financial burden on patients. More multi-center, larger sample sizes analyses are needed to validate the results of this study. In addition, further studies on the physiological level are also needed to analyze the molecular mechanisms of the effect of hCG on embryonic-maternal cross-talk.

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 the Northwest Women's and Children's Hospital (number: 2018027). 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: Data curation, Formal Analysis, Investigation, Methodology, Project administration, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. YH: Data curation, Investigation, Methodology, Resources, Writing – review & editing. ZS: Data curation, Investigation, Methodology, Resources, Writing – review & editing. JS: Conceptualization, Project administration, Writing – review & editing. NL: Conceptualization, Formal Analysis, Funding acquisition, Investigation, Methodology, 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1283197/full#supplementary-material

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EDITED BY
Emanuele Garzia,
Santi Paolo e Carlo Hospital, Italy

REVIEWED BY
Arup Acharjee,
Allahabad University, India
Federico Cirillo,
Humanitas Research Hospital, Italy

*CORRESPONDENCE
Zhiming Zhao
doctor_zhaozhao@sina.com
Wensen Pan
deeprespiration@163.com

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

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Impact of COVID-19 convalescence on pregnancy outcomes in patients undergoing IVF/ICSI during fresh ART cycles: a retrospective cohort study

Mingya Cao[1†], Yan Han[1†], Tengfei Feng[1†], Peiyang Lu[1], Yue Wang[1], Qingyun Sun[1], Zhiming Zhao[1*] and Wensen Pan[2*]

[1]Department of Reproductive Medicine, The Second Hospital of Hebei Medical University, Shijiazhuang, China, [2]Second Department of Respiratory and Critical Care Medicine, The Second Hospital of Hebei Medical University, Shijiazhuang, China

Objective: The aim was to study the impact of coronavirus disease 2019 (COVID-19) convalescence on female fertility and laboratory and clinical outcomes in fresh assisted reproductive technology (ART) cycles.

Methods: In this retrospective cohort study, we analyzed data from 294 patients who had recovered from COVID-19 and who underwent fresh ART cycles between January and March 2023 (COVID-19 group). This group was compared with 631 patients who underwent similar ART cycles in the same period in 2022 but without having been infected with COVID-19 (non-COVID-19 group). The analysis focused on comparison of basic demographic characteristics and laboratory parameters of patients in each group. The primary outcome measure was the clinical pregnancy rate, which was examined to assess the impact of COVID-19 infection on the efficacy of ART treatment.

Results: Basal follicle-stimulating hormone (FSH) levels were significantly lower and antral follicle count (AFC) was markedly higher in the COVID-19 group compared to the non-COVID-19 group (P<0.001 and P=0.004, respectively). The predominant ovarian stimulation protocol in the COVID-19 group was GnRH antagonists (64.85%, P<0.001), with a reduced gonadotropin (Gn) dosage and duration in comparison to the non-COVID-19 group (P<0.05). Although the number of blastocysts formed was lower in the COVID-19 group (P=0.017), this group also exhibited a higher blastocyst freezing rate and a higher rate of high-quality embryos per retrieved oocyte (P<0.001 and P=0.023, respectively). Binary logistic regression analysis indicated that COVID-19 convalescence did not significantly impact clinical pregnancy rates in fresh transfer cycles (odds ratio [OR] = 1.16, 95% confidence interval [CI] = 0.68-1.96, P=0.5874). However, smooth curve-fitting and threshold effect analysis revealed an age-related decline in clinical pregnancy rates in both groups, more pronounced in the COVID-19 group, for women aged over 38 years, with the likelihood of clinical pregnancy decreasing by 53% with each additional year of age (odds ratio [OR] = 0.81, 95% confidence interval [CI] = 0.61–1.08, P=0.1460; odds ratio [OR] = 0.47, 95% CI = 0.21–1.05, P=0.0647).

Conclusions: Our findings present no substantial evidence of adverse effects on clinical pregnancy outcomes in fresh ART cycles in patients undergoing in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) during the period of convalescence from COVID-19. However, age emerges as a significant factor influencing these outcomes. Notably, for women above 38 years of age, the likelihood of clinical pregnancy in patients with a prior COVID-19 infection decreased by 53% with each additional year. This highlights the importance of considering maternal age, especially in the context of COVID-19, when evaluating the likelihood of successful pregnancy following ART treatments.

KEYWORDS

SARS-CoV-2, COVID-19, fertility, IVF, clinical outcomes

Introduction

The coronavirus disease COVID-19 is an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Not only can this virus induce severe respiratory disease, but it can also induce multiple histopathological changes in multiple systems and organs, including the kidney (1), brain (2), and liver (3). It utilizes angiotensin-converting enzyme 2 (ACE2) for cell entry (4). ACE2 receptor expression has been identified in the genitourinary organs and the testis (5–8), so the testis (9) and ovary (10) may also be potential target organs for virus infection. In the initial stages of the pandemic, the American Society for Reproductive Medicine (ASRM) issued guidance recommending the suspension of most assisted reproductive technology (ART) treatments, except in the most urgent cases. This recommendation was in line with the guidance provided by the European Society for Human Reproduction and Embryology (11). As a consequence, there has been a significant decline in the number of patients attending infertility clinics over the past three years. Additionally, the majority of patients undergoing *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) have opted to either cancel their cycles or freeze oocytes or embryos (12, 13). With the easing of nationwide restrictions relating to coronavirus disease 2019 (COVID-19) since December 2022, reproductive centers are likely to encounter an increasing number of infected patients. The region where our center is situated experienced a concentrated outbreak of COVID-19 between December 2022 and January 2023, providing a reliable opportunity for us to gather pertinent data.

Most previous studies have primarily concentrated on the impact of COVID-19 infection on human reproductive function, particularly focusing on analysis of male semen and the potential detection of COVID-19 mRNA or antibodies in semen (14, 15), follicular fluid, oocytes, endometrial tissue (12, 16, 17), and cervicovaginal secretions (18) of infected patients. In contrast, there is a paucity of literature addressing the specific effects of COVID-19 infection on the pregnancy outcomes of IVF/ICSI procedures. Additionally, two studies have reached opposite conclusions regarding the impact of COVID-19 infection on embryos. Chen et al. report that COVID-19 does not adversely affect oocyte quality or embryo development (19). In contrast, another study posits that previous SARS-CoV-2 infection might influence the developmental potential of embryos (20). Although recent research (21–23) has reported no negative impact of COVID-19 infection on the clinical outcomes of ART treatments, these studies may have limitations due to their small case group sample sizes and the lack of consideration for the impact of the woman's age on clinical outcomes.

In light of the above, despite the effective control of COVID-19, the pandemic has not been completely eradicated. Sporadic cases continue to occur, and instances of reinfection have been reported. Consequently, studies investigating the impact of COVID-19 on the clinical outcomes of women undergoing ART cycles remain of critical importance.

Materials and methods

Study population and design

This retrospective cohort study encompassed all couples infected with COVID-19 who underwent fresh IVF/ICSI treatment cycles at the Reproductive Center of the Second Hospital of Hebei Medical University between January 2023 and March 2023. Women who opted for thawing of frozen oocytes, used donated oocytes or sperm, or were not followed up for clinical outcomes were excluded from the study. Patients were allocated to the COVID-19 group if either member of the couple had been infected with SARS-CoV-2 before oocyte retrieval. It is important to emphasize that all patients included in the study were diagnosed with mild cases of COVID-19. No individuals with moderate or

severe symptoms underwent IVF treatment as part of this research. Patients who were not infected with COVID-19 during the same period in 2022 were allocated to the non-COVID-19 group. The diagnosis of COVID-19 infection was confirmed through nucleic acid or antigen testing. Additionally, the interval between recovery time and egg retrieval time was defined as the time from the point at which a patient's serum SARS-CoV-2 antibody or antigen test was negative to egg retrieval. Patients with COVID-19 infection were followed up until the end of June.

In this study, we recorded demographic characteristics including age, partner's age, BMI, type and duration of infertility, baseline hormone levels, IVF treatments, and causes of infertility. Additionally, cycle characteristics such as treatment protocol, total gonadotropins (GT) administered, and fertilization method were documented. The primary outcome measure was clinical pregnancy rate, while secondary outcomes included rates of available and high-quality embryos. Given that the varying time intervals between recovery and retrieval and infection status (whether both members or one member of the couple were infected) may influence cycle outcomes differently, we also conducted further subgroup analyses. These analyses assessed the impact of time interval from SARS-CoV-2 recovery to oocyte retrieval and infection status on clinical pregnancy rates. Informed consent was obtained from all subjects prior to their participation in the study. This research was conducted in accordance with the Declaration of Helsinki and received approval from the Ethics Committee of the Second Hospital of Hebei Medical University (2022-R453).

IVF/ICSI protocols and embryo culture

The controlled ovarian stimulation (COS) protocols carried out at our center were categorized as the gonadotropin-releasing hormone (GnRH) agonist protocol, the GnRH antagonist protocol, the GnRH-a prolonged protocol, or other protocols, including mild stimulation and luteal phase stimulation protocols. The details of COS protocols have been previously presented and thoroughly described (24). For all COS protocols, blood tests and ultrasound were used to monitor hormone levels and follicle growth. When the diameter of the leading follicle reached 18 mm or more than two follicles reached 17 mm, human chorionic gonadotropin (hCG) or GnRH agonists were administered as a trigger. Oocyte retrieval was then performed 36–38 h later.

Oocytes were fertilized through either conventional IVF or ICSI. Pronuclei (PN) were evaluated 16–18 h after insemination. Fertilized oocytes were cultured in G1-plus medium (Gothenburg, Sweden) until day 3, when one or two good-quality embryos were selected for fresh transfer, or cleavage embryos were continued in G2-plus medium until day 5 or day 6; single blastocysts were then transplanted or cryopreserved.

A high-quality embryo (HQE), as evaluated on day 3, was defined as follows: (a) normally fertilized embryo with 4–5 cells on day 2 or 8–10 cells on day 3; (b) <15% fragmentation; (c) uniform blastomeres; (d) absence of multinucleation; (e) absence of zona pellucida defects; (f) absence of perivitelline space granularity; and (g) no inclusions in cytoplasm (25). Blastocyst morphology evaluation was based on the Gardner scoring system (26).

Outcome assessments

The basic characteristics of the patients were collected, including age, body mass index (BMI), type of infertility, infertility duration, causes of infertility, basal hormone levels, COS protocols used, Gn dosage, Gn duration, and so on; among these, basal FSH, AMH, and AFC were taken to reflect ovarian reserve.

Laboratory outcomes included the number of oocytes retrieved, number and rate of normal fertilizations (2PN), number of cleavages and 2PN cleavages, number and rate of available embryos, number and rate of HQEs on day 3, rate of available embryos per egg, rate of HQEs per egg, number and rate of blastocysts formed, blastocyst freezing rate, number of transferred embryos, and clinical pregnancy rate. The normal fertilization rate was the number of 2PN oocytes divided by the number of oocytes retrieved; the rate of available embryos was the number of available embryos divided by the number of 2PN cleavages; the HQE rate was the number of HQEs at the cleavage stage divided by the number of 2PN cleavages; the blastocyst formation rate was the number of blastocysts divided by the number of day 3 embryos for extended culture; the blastocyst freezing rate was number of frozen blastocysts divided by the number of blastocysts formed; and the whole embryo freezing rate was the number of whole-embryo freezing cycles divided by the number of oocyte retrieval cycles.

For clinical outcomes, the clinical pregnancy rate was the primary outcome measure. The criterion for clinical pregnancy was that 28–30 days after embryo transfer, a gestational sac with heartbeat could be seen in the uterine cavity by transvaginal ultrasound examination.

Statistical analyses

All statistical analyses were conducted using the SPSS 26.0 software package or EmpowerStats (X&Y solutions, Inc., Boston, MA). Continuous variables are presented as the mean ± SD or median (Q1–Q3). Categorical variables are presented as percentages. For normally distributed variables, analyses of variance and two-independent-sample tests were conducted for group comparisons. For continuous variables following a non-normal distribution, non-parametric Mann–Whitney U tests were employed for group comparisons. Fisher's exact test or the Chi-square test was performed when comparing categorical variables. Univariate analyses were conducted to identify the possible variables that may affect clinical pregnancy rate. A binary logistic regression analysis was carried out to assess whether COVID-19 infection affects pregnancy outcome in patients undergoing IVF/ICSI. Curve-fitting and threshold effect analyses were conducted to identify non-linear relationships. A p-value <0.05 was considered to indicate statistical significance.

Results

Baseline characteristics

After application of the exclusion criteria, our study included 925 couples. Based on their pre-oocyte-retrieval SARS-CoV-2 infection status, couples were categorized into the COVID-19 group (n=294) or the non-COVID-19 group (n=631), as depicted in Figure 1. Among the former group, both partners were infected in the case of 86.05% of the couples, only the female partner was infected in 7.48% of couples, and only the male partner was infected in 6.46% of couples. The baseline characteristics of the patients are presented in Table 1. There were no significant differences between the groups in terms of female age, male age, BMI for either sex, basal E2, basal LH, AMH, number of cycles, type or duration of infertility, causes of infertility, fertilization method, semen density, or sperm forward motility rate. However, the basal FSH levels were lower (P<0.001) and the antral follicle count (AFC) was higher (P=0.004) in the COVID-19 group. The predominant controlled ovarian stimulation (COS) protocol in the COVID-19 group was GnRH antagonist (64.85%, P<0.001), and both the gonadotropin (Gn) dosage and duration were significantly lower than those in the non-COVID-19 group (P<0.05).

Laboratory indicators and clinical outcomes

Table 2 presents the laboratory indicators and clinical outcomes of the study participants. Several parameters were comparable between the two groups, including the number of oocytes retrieved, 2PN zygotes, normal fertilization rate, cleavage and 2PN cleavage numbers, available embryos, high-quality embryos (HQEs) on day 3, and blastocyst formation rate. However, a notable difference was observed in the whole-embryo freezing rate, which was significantly higher in the COVID-19 group compared to the non-COVID-19 group (P<0.001). In contrast, the number of blastocyst formations was lower in the COVID-19 group (P=0.017), but the rates of blastocyst freezing and high-quality embryo formation per egg were higher than in the non-COVID-19 group (P<0.001 and P=0.023, respectively). There was no significant difference in clinical pregnancy rate between the groups (51.58% in the COVID-19 group vs. 49.10% in the non-COVID-19 group, P=0.677).

The effect of previous COVID-19 infection on clinical pregnancy rate

The results of the univariate analyses are detailed in Supplementary Table 1. We conducted a binary logistic regression analysis, adjusting for factors such as couple ages, AMH level, number of cycles, causes of infertility, COS protocol used, fertilization methods, and type and duration of infertility. The analysis revealed that prior COVID-19 infection did not significantly influence the rate of clinical pregnancy in patients undergoing IVF/ICSI treatment (odds ratio [OR] = 1.16, 95% confidence interval [CI] = 0.68–1.96, P=0.5874) (Table 3). Similarly, the rates of available embryos and high-quality embryos were also not impacted by COVID-19 infection (results are presented in Supplementary Table 2). Furthermore, a sub-analysis of the COVID-19 group under the logistic regression model was

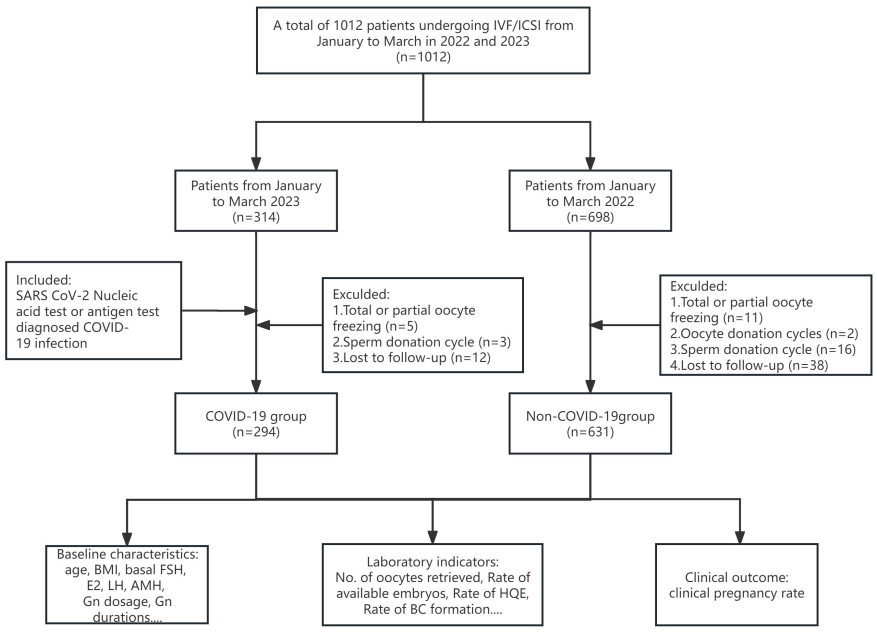

FIGURE 1
Flowchart of the study. A total of 1,012 couples undergoing IVF/ICSI cycles were enrolled from January to March in 2022 and 2023. After application of the inclusion and exclusion criteria, 925 patients were included in the study. HQE, high quality embryo; BC, blastocyst.

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TABLE 1 Baseline characteristics of patients in the COVID-19 and non-COVID-19 groups.

	COVID-19 (n=294)	Non-COVID-19 (n=631)	Standardized diff.	P-value	P-value*
Age (years)	32.93 ± 5.02	33.09 ± 4.96	0.03 (-0.11, 0.17)	0.636	0.865
Age of male partner (years)	33.64 ± 5.56	33.45 ± 5.35	0.03 (-0.10, 0.17)	0.622	0.477
BMI (kg/m^2)	24.12 ± 3.97	23.64 ± 3.65	0.13 (-0.01, 0.27)	0.067	0.076
BMI of male partner (kg/m^2)	25.93 ± 4.22	26.44 ± 4.48	0.12 (-0.02, 0.26)	0.103	0.103
FSH (IU/ml)	7.12 ± 3.57	8.49 ± 4.76	0.33 (0.18, 0.47)	<0.001	<0.001
E2 (pg/ml)	40.59 ± 25.72	45.99 ± 66.42	0.11 (-0.03, 0.25)	0.187	0.58
LH (IU/ml)	5.31 ± 4.25	4.98 ± 5.12	0.07 (-0.07, 0.21)	0.337	0.229
AMH (ng/ml)	3.22 ± 3.07	3.11 ± 3.10	0.04 (-0.10, 0.18)	0.62	0.416
AFC	12.25 ± 8.18	10.68 ± 7.47	0.20 (0.06, 0.34)	0.004	0.006
Cycles			0.10 (-0.04, 0.24)	0.373	–
1	203 (69.05%)	406 (64.34%)			
2	51 (17.35%)	126 (19.97%)			
≥ 3	40 (13.61%)	99 (15.69%)			
Type of infertility			0.07 (-0.07, 0.21)	0.312	–
Primary (%)	142 (48.80%)	284 (45.22%)			
Secondary (%)	149 (51.20%)	344 (54.78%)			
Duration of infertility (years)			0.11 (-0.04, 0.25)	0.349	–
< 1	49 (18.15%)	110 (18.87%)			
1–3	62 (22.96%)	109 (18.70%)			
> 3	159 (58.89%)	364 (62.44%)			
Causes of infertility, n (%)			0.21 (0.08, 0.35)	0.129	–
Tubal factors	117 (39.80%)	236 (37.40%)			
Ovulation disorder	41 (13.95%)	67 (10.62%)			
POR	42 (14.29%)	95 (15.06%)			
EM	14 (4.76%)	60 (9.51%)			
Male factors	31 (10.54%)	76 (12.04%)			
Others	49 (16.67%)	97 (15.37%)			
COS protocols, n (%)			0.34 (0.20, 0.48)	<0.001	–
Antagonist protocol	190 (64.85%)	306 (48.57%)			
Agonist protocol	59 (20.14%)	166 (26.35%)			
GnRH-a prolonged protocol	30 (10.24%)	104 (16.51%)			
Others	14 (4.78%)	54 (8.57%)			
Gn dosage (IU)	2277.45± 903.91	2403.52± 847.32	0.14 (0.00, 0.28)	0.041	0.019
Gn duration (days)	9.22 ± 2.35	9.49 ± 2.59	0.11 (-0.03, 0.25)	0.129	0.002
Fertilization mode, n (%)			0.07 (-0.07, 0.21)	0.336	–
IVF	208 (70.99%)	467 (74.01%)			
ICSI	85 (29.01%)	164 (25.99%)			
Sperm concentration after recovery (10^6/ml)	56.81 ± 56.15	60.17 ± 58.72	0.06 (-0.08, 0.20)	0.413	0.19
Sperm PR after recovery (%)	33.14 ± 16.39	30.38 ± 16.56	0.17 (0.03, 0.31)	0.022	0.048

TABLE 2 Laboratory outcomes in the COVID-19 and non-COVID-19 groups.

	COVID-19 (n=294)	non-COVID-19 (n=631)	Standardize diff.	P-value	P-value*
No. of oocytes retrieved	11.19 ± 8.92	10.76 ± 8.57	0.05 (-0.09, 0.19)	0.487	0.681
No. of 2PN zygotes	6.34 ± 5.26	6.51 ± 5.78	0.03 (-0.11, 0.17)	0.671	0.988
Normal fertilization rate (%)	0.62 ± 0.25	0.62 ± 0.25	0.01 (-0.13, 0.15)	0.903	0.772
No. of cleavages	8.15 ± 6.68	8.35 ± 7.02	0.03 (-0.11, 0.17)	0.679	0.732
No. of 2PN cleavages	6.32 ± 5.26	6.46 ± 5.77	0.03 (-0.11, 0.17)	0.709	0.968
No. of available embryos	3.57 ± 3.06	3.35 ± 2.72	0.08 (-0.06, 0.21)	0.272	0.667
Rate of available embryos (%)	0.67 ± 0.42	0.65 ± 0.44	0.05 (-0.09, 0.20)	0.462	0.256
Available embryos per egg (%)	0.40 ± 0.28	0.37 ± 0.26	0.11 (-0.03, 0.25)	0.11	0.185
No. of high-quality embryos (D3)	2.01 ± 2.57	2.13 ± 2.58	0.05 (-0.09, 0.19)	0.504	0.38
High quality embryo rate (D3) (%)	0.31 ± 0.31	0.33 ± 0.29	0.06 (-0.08, 0.20)	0.405	0.156
Quality embryos per egg (%)	0.19 ± 0.21	0.15 ± 0.19	0.21 (0.07, 0.35)	0.003	0.023
No. of blastocysts formed	2.24 ± 3.51	2.62 ± 3.69	0.10 (-0.04, 0.24)	0.148	0.017
Blastocyst formation rate (%)	0.55 ± 0.29	0.57 ± 0.28	0.08 (-0.11, 0.27)	0.419	0.54
Blastocyst freezing rate (%)	0.82 ± 0.31	0.72 ± 0.28	0.32 (0.12, 0.53)	0.002	<0.001
Sperm concentration on OPU day (10^6/ml)	32.37 ± 12.33	33.17 ± 12.66	0.06 (-0.08, 0.20)	0.37	0.172
Sperm PR on OPU day (%)	23.34 ± 10.64	22.01 ± 10.52	0.13 (-0.01, 0.26)	0.079	0.028
Transferred embryos			0.17 (-0.06, 0.40)	0.169	–
1	14 (14.74%)	59 (21.22%)			
2	81 (85.26%)	219 (78.78%)			
Outcomes			0.28 (0.14, 0.42)	<0.001	–
Whole embryo freezing rate (%)	172 (58.50%)	281 (44.53%)			
Transfer cycle rate (%)	95 (32.31%)	278 (44.06%)			
Cancellation rate (%)	27 (9.18%)	72 (11.41%)			
Clinical pregnancy rate (%)	49 (51.58%)	137 (49.10%)	0.05 (-0.18, 0.28)	0.677	

TABLE 3 The effect of COVID-19 infection on clinical pregnancy rates.

Exposure	Non-adjusted	Adjusted I	Adjusted II
Group (recoded)			
Non-COVID-19	1	1	1
COVID-19	1.10 (0.69, 1.76) 0.6769	1.17 (0.71, 1.94) 0.5333	1.16 (0.68, 1.96) 0.5874

Data presented in the table: β (95% CI) P value/OR (95% CI) P value.
Outcome variable: clinical pregnancy.
Exposure variable: group (recoded).
Non-adjusted: model with no variables adjusted for
Adjusted I: model adjusted for age, age of male partner, FSH, AMH, cycles, and causes of infertility.
Adjusted II: model adjusted for age, age of male partner, AMH, cycles, causes of infertility, COS protocols, fertilization mode, type of infertility, and duration of infertility.

conducted based on the time elapsed between COVID-19 recovery and oocyte retrieval, as well as whether one or both members of the couple were infected. In this sub-analysis, after adjusting for factors such as the ages of both partners, BMI, number of cycles, infertility factors, type of infertility, duration of infertility, and fertilization method, we did not find any impact on pregnancy outcomes of the time interval from recovery to oocyte retrieval or whether one or both members of the couple were infected with COVID-19. The detailed results of this sub-analysis are presented in Supplementary Table 3.

Curve-fitting analysis indicated a curvilinear relationship between female age and clinical pregnancy rate in both groups, even after adjusting for male age, AMH level, number of cycles, causes of infertility, and COS protocol used (Figure 2). Smooth curve-fitting and threshold effect analysis revealed an age-related decline in clinical pregnancy rate in both groups, which was more pronounced in the COVID-19 group for women aged over 38 years,

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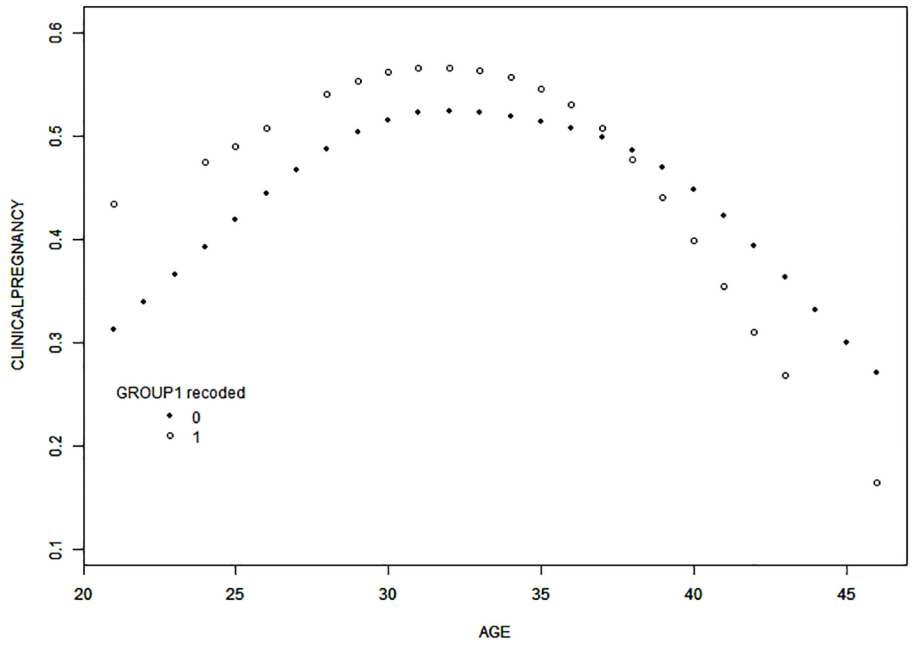

FIGURE 2
Curve-fitting for the relationship between female age and clinical pregnancy rate. After adjustment for male age, AMH, the number of cycles, causes of infertility, and COS protocols employed, the results of curve-fitting revealed a curvilinear relationship between female age and clinical pregnancy rate in a fresh transplant cycle in both groups. Specifically, the clinical pregnancy rate decreased with increasing age in both groups when female age was > 38 years, and the decrease was more significant in the COVID-19 group. Group 0, non-COVID-19 group; Group 1, COVID-19 group.

with the likelihood of clinical pregnancy decreasing by 53% with each additional year of age (odds ratio [OR] = 0.81, 95% confidence interval [CI]= 0.61–1.08, P=0.1460; odds ratio [OR] = 0.47; 95% confidence interval [CI]= 0.21–1.05, P=0.0647) (Table 4).

Discussion

For the general female population, a history of COVID-19 infection may not adversely affect pregnancy outcomes. However, when focusing on different age groups, the study found that for women over the age of 38, the likelihood of clinical pregnancy significantly decreases with each additional year of age.

Serum levels of FSH, AMH, and basal AFC on days 2–3 of the menstrual period are the three most frequently used and effective markers for ovarian reserve. Kolanska et al. found that mild COVID-19 infection does not alter the ovarian reserve in women treated with ART (27), and similar conclusions were reached in a study by Kahyaoglu et al. (28). In our study, we observed that basal follicle-stimulating hormone (FSH) levels were lower and AFC was higher in the COVID-19 group compared to the non-COVID-19 group, while AMH levels were similar between both groups. This suggests that the data from the population examined during this period do not support the conclusion that COVID-19 infection impacts ovarian reserve function. Furthermore, a higher proportion of patients in the COVID-19 group underwent antagonist protocols; this group was also associated with lower Gn dosage and shorter Gn durations than the non-COVID-19 group. This was likely due to the preference for fast entry cycles and short treatment courses during the pandemic to minimize hospital visits, reduce the risk of nosocomial infection, and improve treatment efficiency. Additionally, a significant decrease in the number of blastocyst formations was observed in the COVID-19 group compared to the non-COVID-19 group; this is in alignment with the findings of Jin Lei et al. (22), who also reported a decrease in blastocyst formation rates following COVID-19 infection. This may be related to the significant co-expression of ACE 2 and TMPRSS2 in the trophoblast ectoderm of late blastocysts, which are more sensitive to SARS-CoV-2 (29). Due to the indeterminate nature of the impact on pregnancy outcome after COVID-19 infection, patients in the COVID-19 group were more likely to be selected for whole-embryo freezing. After adjustment for confounding factors, including the ages of the couple, type and durations of infertility, causes of infertility, and controlled ovarian stimulation (COS) protocol, logistic regression analysis revealed that prior COVID-19 infection did not significantly affect clinical pregnancy rate. These findings are in line with several previous studies that have drawn similar conclusions (30–32).

Age of the woman has a significant impact on embryo quality and pregnancy outcome among patients undergoing assisted conception. In our study, the results of curve-fitting indicated that clinical pregnancy rates were lower among women over the age of 38 in both groups, with the difference being more significant in the COVID-19 group. Under a threshold effect model, the results

TABLE 4 Threshold effect analysis for age in both groups in terms of impact on clinical pregnancy rate.

Group (recoded)	Non-COVID-19	COVID-19	Overall
Model I			P-interaction: 0.534
One-line effect	1.01 (0.91, 1.12) 0.8631	0.92(0.75, 1.13) 0.4492	1.00 (0.92, 1.09) 0.9576
model II			P-interaction: 0.380
Turning point (K)	38	38	38
< K effect 1	1.04 (0.93, 1.16) 0.4778	1.01 (0.81, 1.26) 0.9168	1.04 (0.95, 1.14) 0.3731
> K effect 2	0.81 (0.61, 1.08) 0.1460	0.47 (0.21, 1.05) 0.0647	0.72 (0.56, 0.94) 0.0160
effect2-1	0.78 (0.58, 1.05) 0.0995	0.46 (0.20, 1.07) 0.0711	0.69 (0.53, 0.91) 0.0094
Model fit value at K	0.10 (-0.37, 0.57)	0.40 (-0.39, 1.20)	0.18 (-0.22, 0.58)
LRT test	0.085	0.029	0.005

Data in the table: β (95% CI) P value/OR (95% CI) P value.
Outcome variable: clinical pregnancy.
Exposure variable: age.
Variables adjusted for: age of male partner, AMH, cycles, causes of infertility, COS protocols.

showed that for women aged over 38 years, the likelihood of clinical pregnancy declined by 53% for every additional year in the COVID-19 group. In order to explore why the clinical outcomes were poorer among older women infected with COVID-19, we compared the data from couples in which the woman was over the age of 38 between the two groups, and found that the available embryo rate, the rate of available embryos per egg, the high-quality embryo rate, the rate of high-quality embryos per egg, the number of blastocysts formed, the blastocyst formation rate, semen parameters after recovery, and sperm concentration on the day of oocyte retrieval were all lower in the COVID-19 group (the results of analysis of these variables are given in Supplementary Table 4). Some other studies also have explored potential reasons for the decline in fertility caused by COVID-19. Several studies have reported that oxidative stress plays an important role in COVID-19 infection at the molecular level (33, 34), and the antioxidant system and the accumulation of reactive oxygen species are among the possible reasons for poor pregnancy outcomes. Increased oxidative stress activates the pathogenic mechanism of female fertility (35), alters oocyte epigenetics (36), and ultimately has a negative impact on oocyte quality (37). These two factors may constitute one possible explanation for the more significant decrease in clinical pregnancy rate with advanced age after COVID-19 infection. The challenge of poor pregnancy outcomes in the population of older couples undergoing ART is well-recognized among clinicians, and our study suggests that COVID-19 infection exacerbates these outcomes in this demographic. Consequently, individualized treatment approaches, tailored to the specific needs of this population, are warranted.

Does COVID-19 actually affect gametes? Various studies have provided differing answers. Youngster (19) suggests that COVID-19 infection might have a long-term negative effect on oocyte yield when retrieval occurs more than 180 days after infection. However, a study by Dolgushina presents an opposing view, finding that the parameters of oogenesis and embryogenesis, as well as pregnancy and childbirth rates, did not differ between groups with time intervals of ≤180 days or >180 days (38). In our study, we analyzed the impact of the time interval from recovery to oocyte retrieval on clinical pregnancy rate and found no significant effect. This might be attributed to our study's time interval range of 24–167 days, which did not extend to 180 days. However, our result was consistent with that of a study by Huang, Jialyu et al. (32), which indicated that prior SARS-CoV-2 infection in females did not adversely affect subsequent IVF treatment, regardless of the time interval following infection. Regarding sperm, another study (39) involving 120 COVID-19 infected subjects found that sperm parameters gradually improved, during convalescence after documented COVID-19 infection from testing an average 53 days after a positive SARS-CoV-2 nasopharyngeal PCR test, suggesting recovery over time following the viral infection.

This study did not include patients undergoing FET, but there are related studies offering insights. Aizer, Adva et al. found that COVID-19 infection did not affect implantation rates or clinical or ongoing pregnancy rates in subsequent FET cycles (31). However, research by Youngster et al. (40) indicated that the clinical pregnancy rate after FET was significantly lower in women infected less than 60 days prior compared to non-infected patients, although there was no significant difference for patients infected more than 60 days prior. This raises the question of whether appropriately delaying pregnancy to allow for normalization of semen parameters, or opting for egg freezing after retrieval or transfer after resuscitation in whole-embryo freezing cycles, could be effective strategies. Additionally, the impact of increased age on clinical outcomes due to delayed assisted reproduction must be considered. Therefore, the optimal time interval before pregnancy and the effectiveness of these methods in improving pregnancy outcomes require further research.

Although larger in terms of sample size than previous research on COVID-19 and pregnancy outcomes, this study has several limitations. Firstly, the potential for infinite statistical differences suggested by the threshold effect analysis indicates the need for an even larger sample size to achieve significant results. Secondly, as this was a single-center retrospective study, the generalizability of our findings is limited, necessitating validation from multi-center global studies. Additionally, only patients in recovery from mild cases of COVID-19 were included, with moderate and severe cases unaccounted for. The short follow-up period also means that any long-term effects on abortion, live birth, and perinatal outcomes remain unknown. Lastly, the lack of data on vaccination status due to the historical nature of the control group is a notable limitation. However, according to the epidemic prevention policies at the time, the majority of the population had been vaccinated, and the current

literature (41) suggests that vaccination status does not significantly impact clinical outcomes. In future, we will further trace long-term pregnancy outcomes as well as the health of the offspring, and further conduct multiple subgroup analyses of COVID-19-infected patients, considering variables such as the degree of fever, as well as comparing clinical outcomes between reinfected patients and those infected for the first time, so as to draw more comprehensive and reliable conclusions.

In conclusion, our findings suggest that a history of COVID-19 infection does not have a negative effect on clinical pregnancy rates; however, for women aged over 38 years, the clinical pregnancy rate in fresh transplant cycles was lower in both groups, but especially in the COVID-19 group. Specifically, the likelihood of clinical pregnancy declined by 53% for every additional year of age, indicating that COVID-19 further increases the burden of older age in women undergoing assisted reproductive therapy. In order to solve this problem and to offer reasoned and scientific suggestions or measures, we still need to complete more in-depth research.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The studies involving humans were approved by Ethics Committee of the Second Hospital of Hebei Medical University (2022-R453). 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

MC: Data curation, Investigation, Methodology, Writing – original draft. YH: Data curation, Formal analysis, Investigation, Writing – review & editing. TF: Data curation, Software, Writing – review & editing. PL: Investigation, Writing – review & editing, Software. QS: Investigation, Writing – review & editing. YW: Supervision, Writing – review & editing. ZZ: Conceptualization, Writing – review & editing. WP: 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1298995/full#supplementary-material

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EDITED BY
Emanuele Garzia,
Santi Paolo e Carlo Hospital, Italy

REVIEWED BY
Dagan Mao,
Nanjing Agricultural University, China
Ripla Arora,
Michigan State University, United States

*CORRESPONDENCE
Xinglong Wu
wuxl32@hebau.edu.cn

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Transcriptome profiling reveals superovulation with the gonadotropin-releasing hormone agonist trigger impaired embryo implantation in mice

Meng Li[1], Jingmei Han[2], Nana Yang[1], Xiangyun Li[1] and Xinglong Wu[1*]

[1]College of Animal Science and Technology, Hebei Technology Innovation Center of Cattle and Sheep Embryo, Hebei Agricultural University, Baoding, China, [2]College of Veterinary Medicine, Hebei Agricultural University, Baoding, China

Introduction: Superovulation is a critical step in assisted reproductive technology, but the use of human chorionic gonadotropin (hCG) as a trigger for superovulation can result in ovarian hyperstimulation. Thus, the use of Gonadotropin-releasing hormone agonist (GnRHa) trigger has been increasingly adopted, although it has been associated with a higher rate of pregnancy failure compared to natural cycles. This study aimed to investigate the effect of GnRHa trigger on embryo implantation in a mouse model.

Methods: Mice in the superovulation (PG) group were administered 7.5 IU of PMSG, followed by the injection of 3.5 μg of GnRHa (Leuprorelin) 48 h later, while mice in the control group (CTR) mated naturally. We compared the number of oocytes, blastocysts, and corpus luteum between the two groups and the implantation sites after the transfer of natural blastocysts. Ovaries, uterus, and serum 2 and 4 days after mating were collected for qRT-PCR, transcriptome sequencing, and hormone assays.

Results: The PG group had more oocytes, blastocysts, and corpus luteum after superovulation than the CTR group. However, the mRNA expression of leukemia inhibitory factor (*Lif*) and the number of implantation sites were reduced in the PG group. The ELISA assay revealed that superovulation increased ovarian estrogen secretion. The transcriptome analysis showed that superphysiological estrogen led to a response of the uterus to a high estrogen signal, resulting in abnormal endometrium and extracellular matrix remodeling and up-regulation of ion transport and inflammation-related genes.

Conclusion: Our findings suggest that a combination of PMSG and GnRHa trigger impaired embryo implantation in mice, as the excessive uterine response to superphysiological estrogen levels can lead to the change of gene expression related to endometrial remodeling, abnormal expression of uterine ion transport genes and excessive immune-related genes.

KEYWORDS

superovulation, gonadotropin-releasing hormone agonist, uterine receptivity, ovary, transcriptome

1 Introduction

Embryo implantation is a highly coordinated maternal-embryonic communication process (1). Successful embryo implantation requires an implantable blastocyst and a receptive uterus. The uterus undergoes dramatic changes to receive a mature blastocyst, including significant molecular changes and tissue remodeling, such as the proliferation inhibition of endometrial epithelial cells and the decidualization of stromal cells (2). These changes are mainly driven by estrogen and progesterone (2, 3). Subtle hormonal changes during implantation can significantly impact the environment of the uterus. Superovulation technology is widely used in human-assisted and large-scale animal reproduction. In humans, superovulation is an essential step within *in vitro* fertilization-embryo transfer (IVF-ET) cycles and is a popular technology to obtain many oocytes (4, 5). In superovulation protocols, human chorionic gonadotrophin (hCG) is commonly used to trigger ovulation (6). However, hCG has been shown to cause ovarian hyperstimulation and produce stunted embryos (7, 8). The half-life of hCG is as long as 24 h, which can lead to significant changes in the hormonal environment (9). This nonphysiological maternal hormonal environment continues through embryo implantation and early placenta formation (10, 11). Gonadotropin-releasing hormone agonists (GnRHa), which have a shorter half-life as an alternative to hCG, reduce the occurrence of ovarian hyperstimulation (7, 9, 12). At present, GnRHa has been gradually used to replace hCG to trigger ovulation in human-assisted reproductive technology. However, the stimulation of the ovaries with GnRHa still results in more pregnancy failures compared to the natural cycle (13, 14). Such results can be related to abnormal hormone levels caused by impaired ovarian function after superovulation.

The classic superovulation protocol of the mouse model administers hCG to induce ovulation after the intraperitoneal injection of pregnant mare serum gonadotropin (PMSG) for 48 h (15). In the mouse model, the GnRHa trigger results in ovulation like the hCG trigger (16, 17). Decreased VEGF expression in the ovaries was observed with the GnRHa trigger compared to the hCG trigger, which could explain the reduced ovarian hyperstimulation. Numerous research works have evaluated the ovarian, uterine, and fetal abnormalities caused by superovulation (18–20). Previous studies have shown that superovulation with the hCG trigger alters the expression of genes about tissue remodeling and placenta formation during implantation, resulting in abnormal placental and fetal growth (10, 21). The study found that GnRHa triggers altered the expression of angiogenic factors in the mouse uterus (17). Another study found that after the GnRHa trigger, the mouse uterus showed abnormal leukocyte distribution and higher inflammatory response (22). There are several studies showed that the use of HCG to trigger ovulation leads to impaired uterine receptivity (20, 23). However, the effect of GnRHa-triggered protocols on embryo implantation has not been clarified, especially the ovarian and uterine changes in pre-implantation.

In this study, we used natural blastocyst transfer into the uterus of mice, which used the GnRHa trigger to explore the effect of the GnRHa trigger on uterine receptivity. In mice, the maximal receptive period of the uterus for the embryo (the implantation window) typically occurs 4.5-5 days post coitus (dpc) (2). Therefore, the uterus and ovaries were collected on 2 dpc (before implantation) and 4 dpc (close to implantation), and then transcriptome sequencing was performed, respectively. The results revealed the impact of the superovulation protocol of PMSG combined with GnRHa on the transcriptome expression profile of the ovary and uterus before implantation. This study provides valuable insights for optimizing the outcome of assisted reproduction.

2 Materials and methods

2.1 Animals

All mice (CD-1, 6-8 weeks old, 20-25 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All mice were housed at 20-25°C with 12 h/12 h light-dark cycles and received food and water ad libitum during the experiment. Male mice that received a vasectomy were used to create pseudopregnant mice. The Ethical Committee of Hebei Agricultural University approved this study.

2.2 Superovulation, embryo transfer, and samples collection

102 female mice were split randomly into two groups of 51 mice each. For the superovulation (PMSG combined with GnRHa, PG) group, mice were administered 7.5 IU of PMSG (Ningbo Sansheng Biological Technology Co., Ltd) by an Intra-peritoneal (i.p) injection, followed by an i.p injection of 3.5 μg of GnRHa (Leuprorelin, Selleckchem) 48 h later. At the same time, the female mice mated to males. The control (CTR) group mice were not treated with hormones and mated with males during estrus. Vaginal plugs confirmed successfully mating the following day when it was designated 1-day post coitum (dpc). Cumulus–oocyte complexes were collected in the oviduct on 1 dpc morning (no mating was required in the PG group), and cumulus cells were then removed in the 0.1% hyaluronidase. Blastocysts were collected at the uterine horn on 4 dpc mornings. The number and quality of oocytes and blastocysts were counted. In embryo transfer experiments, recipients mated with vasectomy males. Natural blastocysts (7 or 8) were transferred into a single uterine horn of the recipients on 3 dpc. Embryo implantation was examined on 8 dpc. Furthermore, we collected the ovary, uterine horn, and serum of pseudopregnant mice on 2 dpc mornings and 4 dpc nights. One side of the ovary of three mice was stored in 4% paraformaldehyde, and the other was stored in an RNAlater™ solution (Invitrogen) at -20°C until RNA extraction. The uterine was stored in an RNAlater™ solution (Invitrogen) at -20°C until RNA extraction. Mice blood was collected from a retro-orbital vein and centrifuged at 3000 rpm for 10 min at 4°C to extract serum. The serum was stored at -20°C until the enzyme-linked immunosorbent assay (ELISA) was used. In the reproduction experiment, eight mice in the CTR and PG groups were selected to give birth, and the number of pups in each group was counted.

2.3 Haematoxylin-eosin staining of the ovaries

The ovaries were fixed in 4% paraformaldehyde for more than 48 h. Ovaries were placed in embedding boxes and rinsed under running water for 12 h. After dehydration with an alcohol gradient, the ovaries were immersed in xylene for 15 min, soft wax for 60 min, and hard wax for 60 min, and each step was repeated twice. The tissue was embedded and cut into 5 μm sections in an embedding machine, and the slices were dried at 50°C for more than 30 min before being deparaffinized and rehydrated. HE staining was performed on these sections. The ovarian tissue sections were observed under a microscope, and the number of corpus luteum was counted.

2.4 Enzyme-linked immunosorbent assay

Serum samples were analyzed using an enzyme-linked immunosorbent assay (ELISA) kit (Shanghai Jianglai Biotechnology Co., Ltd., China). The E_2 ELISA kit on the intra-assay CV was less than 9%, the inter-assay CV was less than 11%, and the assay sensitivity was 0.1 pg/mL. The P_4 ELISA kit on the intra-assay CV was less than 9%, the inter-assay CV was less than 11%, and the assay sensitivity was 0.1 ng/mL.

2.5 RNA extraction, library construction, and sequencing

The total RNA was extracted using a Trizol reagent kit (Invitrogen) according to the manufacturer's protocol. RNA quality was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and RNase-free agarose gel electrophoresis. After the total RNA was extracted, mRNA was enriched by Oligo(dT) beads. Then, the enriched mRNA was fragmented into short fragments using a fragmentation buffer and reversely transcribed into cDNA using the NEBNext Ultra RNA Library Prep Kit for Illumina (NEB). The purified cDNA fragments were end-repaired, and the A base was added and ligated to Illumina sequencing adapters. The ligation reaction was purified with AMPure XP Beads (1.0X). Ligated fragments were subjected to size selection by agarose gel electrophoresis and a polymerase chain reaction (PCR), which was amplified to obtain the final cDNA library. The cDNA library was sequenced using Illumina No-vaseq6000 by Gene Denovo Biotechnology Co. (Guangzhou, China).

2.6 Quality control of sequencing data

The reads were further filtered by using fastp (version 0.18.0). The parameters were as follows: 1) removing reads containing adapters; 2) removing reads containing more than 10% of the unknown nucleotides(N); 3) removing low-quality reads containing more than 50% of low-quality (Q-value ≤ 20) bases. The short read alignment tool Bowtie2 (version 2.2.8) was used for mapping reads to the ribosome RNA (rRNA) database. The rRNA-mapped reads were then removed. The remaining clean reads were further used in an assembly and gene abundance calculation.

2.7 Quantification of gene expression level

The clean reads were mapped to the reference genome using HISAT2, and then mapped reads were assembled using StringTie v1.3.1. The FPKM (fragment per kilobase of transcript per million mapped reads) value was calculated for each transcription region to quantify its expression abundance and variations using RSEM software. RNA differential expression analysis was performed by DESeq2 software between the two groups (and by edgeR between two samples). The genes with the parameter of a false discovery rate (FDR) < 0.05 and absolute fold change > 2 were considered differentially expressed genes (DEGs).

2.8 Gene ontology and pathway enrichment analyses

All DEGs were mapped to GO terms in the Gene Ontology database (http://www.geneontology.org/), gene numbers were calculated for every term, and significantly enriched GO terms in DEGs compared to the genome background were defined by a hypergeometric test ($p < 0.05$). The Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway enrichment analysis identified significantly enriched metabolic pathways or signal transduction pathways in DEGs compared with the whole genome background ($p < 0.05$).

2.9 Quantitative real-time PCR

The total RNA was extracted from the tissues using the RNeasy Plus Mini Kit (QIAGEN, Germany), and the total RNA was reverse transcribed into cDNA as required by the PrimeScript RT reagent kit (TaKaRa, Japan). SYBR fluorescent dye (Biotium, USA) was used for qRT-PCR. The reaction system was 2× qPCR Master Mix 10 μL, the forward primer was 0.4 μL, the reverse primer was 0.4 μL, and cDNA 1 μL, rox 3 μL, and ddH2O were supplemented to 20 μL. The reaction conditions were as follows: predenaturation at 95°C for 120 s, denaturation at 95°C for 15 s, 60°C annealing for 30 s, and amplification for 40 cycles. The $2^{-\triangle\triangle Ct}$ method was used to analyze data. Primers were synthesized by Sangon Biotech (Shanghai, China). The primer information for qRT-PCR is provided in Table 1.

2.10 Statistical analysis

The data were analyzed for significance using unpaired T-test except for sequencing data, and the results are expressed as the data mean ± SEM. $p < 0.05$ was considered statistically significant. Sequencing data are expressed as FPKM mean ± SEM. Images

were produced using GraphPad Prism 8.0 software and Omicsmart (www.omicsmart.com).

3 Results

3.1 Superovulation impaired uterine receptivity in mice

After administering PMSG combined with GnRHa for superovulation, we found that the number of recovered oocytes in the PG group was significantly higher than in the CTR group (Table 2, Figure 1A). Although there were a few poorly developed embryos in the PG group, there were more blastocysts in the PG group than in the CTR group (Table 2, Figure 1B). At the same time, there was a higher number of corpus luteum in the ovary of the PG group compared to the CTR group on 4 dpc (Table 2, Figure 1C). The reproductive test results indicated that although the PG group had more pups than the CTR group, there was a discrepancy in the number of blastocysts of the PG group (Table 3). We used embryo transfer on mice to eliminate the effect of embryo number and quality on the implantation rate. 7-8 natural embryos were transferred to one side of the uterine horn of each recipient mouse. Compared with the CTR group, the PG group exhibited a significant decrease in the implantation rate (70.09% vs. 24.47%, Table 4) and fewer implantation sites (5.66 vs. 2.03, Table 4, Figure 1D). These findings indicate that most pre-implantation embryos appear capable of developing into blastocysts following superovulation, and the reduction in implantation sites is mainly affected by the uterine environment.

TABLE 1 Primer sequences used for qRT–PCR.

Gene	Primer sequences (5′-3′)	Accession No.
Gapdh	Forward: AGGTCGGTGTGAACGGATTTG Reverse: TGTAGACCATGTAGTTGAGGTCA	NM_001289726.2
Lif	Forward: ATTGTGCCCTTACTGCTGCTG Reverse: GCCAGTTGATTCTTGATCTGGT	NM_001039537.3
Hoxa10	Forward: CCTGCCGCGAACTCCTTTT Reverse: GGCGCTTCATTACGCTTGC	NM_008263.4
Itgb3	Forward: CCACACGAGGCGTGAACTC Reverse: CTTCAGGTTACATCGGGGTGA	NM_016780.2
Inhba	Forward: AAATCAGAACGCCTCCGCTA Reverse: TCCCGAGTGTAGAGTTCGGT	NM_008380.2
Cyp17a1	Forward: TGGAGGCCACTATCCGAGAA Reverse: CACATGTGTGTCCTTCGGGA	NM_007809.3
Hsd17b7	Forward: ATAATGTGGCTCGTGTGGCT Reverse: ATGTCCATCTTTTGGCCCGT	NM_001420237.1
Prap1	Forward: AGAAGGTCTGGGATACTAGAGCC Reverse: GCATCTGGACGCTTTTCCTC	NM_009475.2
H2-Ea	Forward: CGTCTGAGGCTACCCCTTTC Reverse: GAGAACCCCAGCCAGACATT	NM_010381.3

On 4 dpc, we used qRT-PCR to evaluate the uterine receptivity-related genes, including the leukemia inhibitory factor (*Lif*), homeobox A10 (*Hoxa10*), and integrin beta 3 (*Itgb3*). The results showed that *Lif* expression was significantly decreased, and the *Hoxa10* and *Itgb3* expression showed no significant difference (Figure 1E). These results suggested that superovulation affects uterine receptivity and leads to embryo implantation failure.

3.2 Transcriptome sequencing data quality control and differentially expressed genes identification

We performed quality control on the raw data before analysis to reduce the analysis interference caused by invalid data. We acquired between 10.9 and 30.9 million clean reads per sample after filtering the fastq files. We used bowtie2 to map the clean reads to the ribosome database, removed the mapped reads, and used unmapped reads for transcriptome analysis. After mapping the unmapped reads to the reference genome, we used Stringtie to reconstruct the transcripts, obtained the expression levels of all genes in each sample, and corrected them to FPKM values for subsequent analysis.

We assessed the differences in gene expression caused by superovulation in the ovaries and uterus. The genes with FDR < 0.05 and a fold change > 2 were selected as differential genes. On 2 dpc, superovulation resulted in 260 (133 up-regulated, 127 down-regulated) differential expression genes (DEGs) in the ovary and 891 DEGs (417 up-regulated, 474 down-regulated) in the uterus compared with the CTR group. On 4 dpc, compared with the CTR group, superovulation resulted in 192 DEGs in the ovary (75 up-regulated, 117 down-regulated) and 101 DEGs (85 up-regulated, 16 down-regulated) in the uterus. From 2 dpc to 4 dpc, In the CTR group, there were 65 DEGs (47 up-regulated, 18 down-regulated) and 1932 genes (836 up-regulated, 1096 down-regulated) that showed differential expression in the ovary and uterus, respectively. In the PG group, 199 (54 up-regulated, 145 down-regulated) and 319 genes (145 up-regulated, 174 down-regulated) were differentially expressed in the ovary and uterus, respectively. (Figures 2A–D).

3.3 Superovulation perturbed ovarian hormone secretion and uterus respond to high estrogen signals

To further evaluate the effect of superovulation on embryo implantation, we performed the GO and KEGG enrichment analysis of DEGs in the ovary. On 2 dpc, DEGs were enriched in biological process terms in relation to ovarian steroid hormones, such as the steroid biological process, cholesterol biological process, and steroid metabolic process (Figure 3A). The KEGG pathway analysis showed that DEGs were significantly enriched in terpenoid backbone biosynthesis (Figure 3B). The analysis of DEGs enriched in this term showed that all the genes abundance were significantly increased (Figure 3C). These genes were also enriched in steroid

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TABLE 2 Statistics of the corpus luteum, oocyte, and embryo number in mice.

Group	Females (No.)	Oocytes (No.)	Oocytes per female (No.)	Blastocysts (No.)	Blastocysts per female (No.)	Corpus luteum (No.)
CTR	7	83	11.86 ± 2.23	79	11.29 ± 0.70	5.57 ± 0.34
PG	7	215	30.71 ± 8.55**	201	28.71 ± 8.71**	18.57 ± 0.78**

Values are mean ± SEM. CTR, control group. PG, superovulation (PMSG combined with GnRHa, PG) group. **p < 0.01.

biosynthesis. On 4 dpc, DEGs are enriched in the FSH secretion process about inhibin in the cell components, molecular functions, and biological processes (Figure 3D). Among the DEGs, the abundance of *Fshr* was down-regulated, and the abundance of *Lhcgr* was up-regulated in the ovary on 2 dpc (Figure 3F). In contrast, the abundance of inhibin-related genes (*Inha*, *Inhba*) was down-regulated (Figure 3H). On 4 dpc, *Fshr* (FDR = 0.164, p = 0.012) and *Lhcgr* (FDR = 0.164, p = 0.017) had the same expression trend as on 2 dpc (Figure 3G), but the difference was not significant. Similarly, the abundance of inhibin-related genes (*Inha*, *Inhba*, *Inhbb*) was down-regulated on 4 dpc (Figure 3I). We speculated that the continuous stimulation of the pituitary gland by GnRHa could reduce the expression of inhibin in the ovary. These led to the excessive secretion of FSH and the induced LHCGR expression in granulosa cells, affecting ovarian function. DEGs were significantly enriched in Ovarian Steroidogenesis, the Ras signaling pathway, and the MAPK signaling pathway by KEGG analysis (Figure 3E). The enriched Ras signaling pathway and the MAPK signaling pathway were related to cell proliferation, differentiation, and apoptosis.

The cooperative changes in steroid hormones in the ovary affect the estrus cycle and pregnancy. On 2 dpc and 4 dpc, we detected the contents of estrogen (E_2) and progesterone (P_4) in the circulating blood of mice, respectively. We found that E_2 concentration was significantly increased and P_4 concentration was significantly decreased after superovulation compared to the CTR group in the

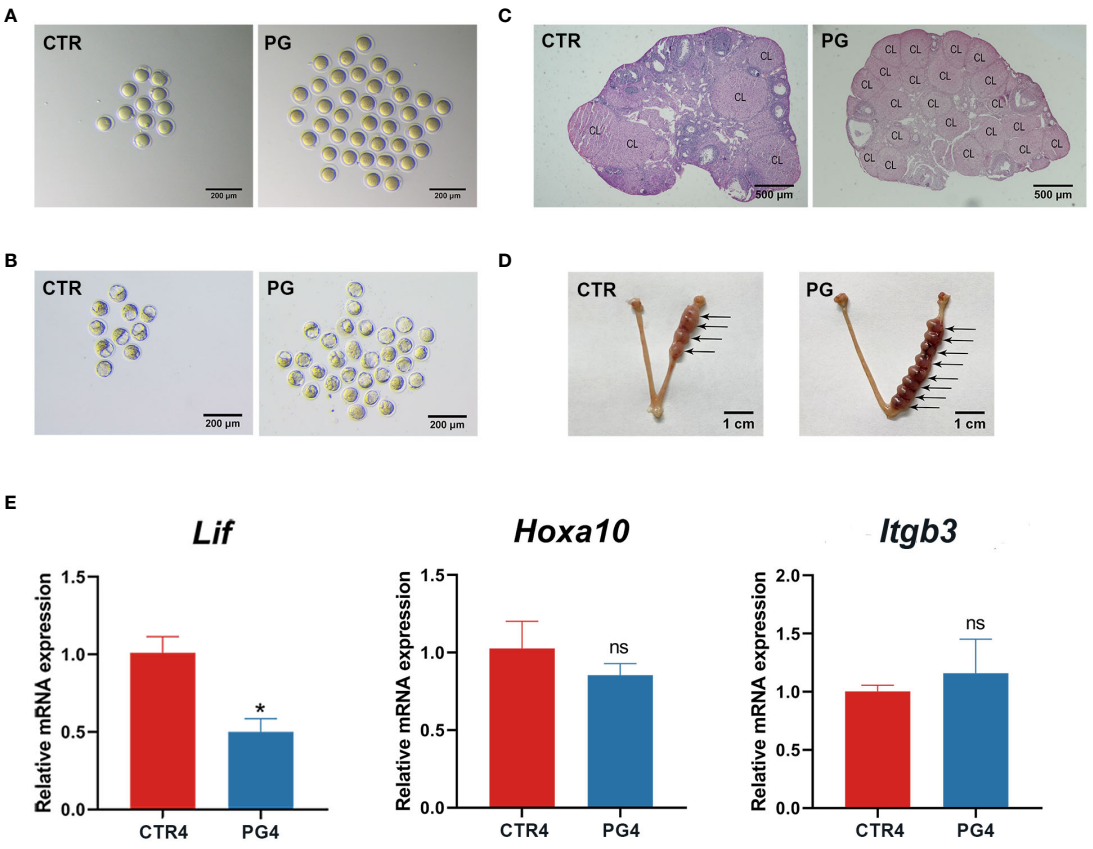

FIGURE 1
Superovulation led to impaired implantation in mice. **(A)** The difference of ovulations in control (CTR) and superovulation (PG) groups on 1 dpc. (n = 7). **(B)** The difference of blastocysts in CTR and PG groups on 4 dpc. (n = 7). **(C)** The difference of corpus luteum (CL) in CTR and PG groups on 4 dpc. (n = 3). **(D)** Implantation sites in CTR and PG groups on 8 dpc. (n = 29). **(E)** Uterine receptivity related gene mRNA expression in CTR and PG groups on 4 dpc. (n = 3). *Lif*, leukemia inhibitory factor. *Hoxa10*, homeobox A10. *Itgb3*, integrin beta 3. CTR4, control group on 4 dpc. PG4, superovulation group on 4 dpc. *p < 0.05. ns, no significance. Scale bar: **(A, B)** is 200 μm; **(C)** is 500 μm; **(D)** is 1 cm.

TABLE 3 Statistics of pups number.

Group	Females (No.)	Pups (No.)	Pups per female (No.)
CTR	8	88	11 ± 1.05
PG	8	118	14.75 ± 2.48^{ns}

Values are mean ± SEM. CTR, control group. PG, superovulation (PMSG combined with GnRHa, PG) group. ns, no significance.

circulating blood at both time points (Figures 4A, B). We analyzed whether the genes with the estrogen and progesterone synthesis of 2 dpc and 4 dpc were DEGs. The key enzyme gene abundance in progesterone synthesis (*Cyp11a1*, *Star*, *Hsd3b1*) was increased. However, the key enzyme gene abundance of the progesterone conversion to estrogen (*Cyp17a1*, *Cyp19a1*, *Hsb17b1*) decreased (Figures 4C, D). The available evidence shows that the synthesis and secretion of ovarian hormones are abnormal in mice after superovulation, affecting embryo implantation.

Given that uterine changes during pregnancy are primarily regulated by estrogen and progesterone signaling, our study focused on examining the expression of the estrogen receptor (*Esr1*) and progesterone receptor (*Pgr*) expression in the uterus on 2 dpc and 4 dpc. Our results revealed an up-regulation of *Esr1* by superovulation only on 2 dpc, with no difference in *Pgr* abundance (Figures 4E, F). However, we observed increased circulating E_2 concentration and decreased P_4 on both 2 dpc and 4 dpc after superovulation (Figures 4A, B). Furthermore, we identified several significantly up-regulated genes on 4 dpc in the uterus, including the estrogen-responsive genes (Figure 4G). These findings suggest that superovulation may lead to increased exposure of the uterus to higher levels of estrogen, potentially interfering with the transition of the uterus to a receptive state.

3.4 Superovulation induced the abnormal gene expression related to endometrial remodeling

An essential event during the change in uterine receptivity is endometrial remodeling. On 2 dpc, extracellular matrix-related GO terms were enriched, including the extracellular matrix (Figure 5A). Additionally, DEGs were significantly enriched in the cell cycle, ECM–receptor interaction, p53 signaling pathway, and pancreatic secretion (Figure 5B). when we screened all genes related to intercellular junctions in DEGs, we found that tight junction-related genes (*Cldn2*, *Cldn4*, *Tjp3*) and gap junction-related genes (*Gjb2*, *Gjb3*, *Gja3*) were significantly down-regulated on 2 dpc (Figures 5C, D), while *Cdh4*, *Cdh16*, *Ajap1*, *Jhy* were significantly up-regulated (Figures 5E, G). On 4 dpc, only *Cdh16* and *Gbj1* were significantly up-regulated (Figures 5D, E). Furthermore, four mucin family members (*Muc1*, *Muc4*, *Muc13*, *Muc20*) were found to be down-regulated on 2 dpc, with *Muc1*, which is known to be absent at implantation (Figure 5F). However, *Muc1* abundance increased (FDR = 0.217, $p = 0.005$) on 4 dpc, although there was no significant difference (Figure 5F). We also analyzed *Cdh1*, despite not being DEGs, as CDH1 deletion in endometrial epithelial cells is critical for embryo implantation. The results showed that *Cdh1* decreased (FDR = 0.183, $p = 0.035$) on 2 dpc, with no difference observed on 4 dpc (FDR = 0.999, $p = 0.524$) (Figure 5E). Additionally, we screened all genes about the extracellular matrix among DEGs. The results showed that the abundance of matrix structural protein genes (*Ecm1*, *Efemp1*, *Spon1*) was up-regulated on 4 dpc (Figure 5H), while several matrix metalloproteinase family members (*Mmp11*, *Mmp13*, *Mmp14*, *Mmp25*) were down-regulated on 4 dpc (Figure 5I). These results indicated that superovulation may have an impact on endometrial remodeling.

3.5 Superovulation led to the imbalance of the microenvironment in the uterus

We performed GO and KEGG enrichment analysis for DEGs in the uterus on 4 dpc. The GO analysis results showed enrichment of ion transport-related GO terms, including chloride transmembrane transporter activity, ion transmembrane transporter activity, chloride transport, and ion transport. (Figure 6A). The top 20 pathways enriched in the KEGG were found to be associated with mineral absorption and immune response, although no pathway showed significant enrichment (Figure 6B). The abnormal expression of ion channels and membrane transporters could disrupt the fluid environment in the uterus, thereby affecting endometrial function and embryo implantation. DEGs related to ion transport were significantly up-regulated on 4 dpc (Figure 6C), suggesting their potential role in altering the uterine fluid environment and leading to embryo implantation failure.

During pregnancy, either semen or embryos induce a wide range of immune responses. On 4 dpc, we observed that immune-related genes accounted for 21.78% of the DEGs (Figure 6D), and immune-related genes accounted for 50% of the top 20 DEGs (Figure 6E). Further evaluation of the expression of these genes revealed that the uterus had a more robust immune response after superovulation than the normal uterus during pregnancy (Figure 6F). These changes may contribute to the inability of the uterus to implant the embryo successfully.

TABLE 4 Statistics of embryo implantation rates by embryo transfer in mice.

Group	Recipients (No.)	Embryos (No.)	Implantation sites (No.)	Implantation sites per female (No.)	Implantation rate (%)
CTR	29	234	164	5.66 ± 1.89	70.09 ± 4.53
PG	29	282	69	2.03 ± 2.08**	24.47 ± 4.65**

Values are mean ± SEM. CTR, control group. PG, superovulation (PMSG combined with GnRHa, PG) group. **p < 0.01.

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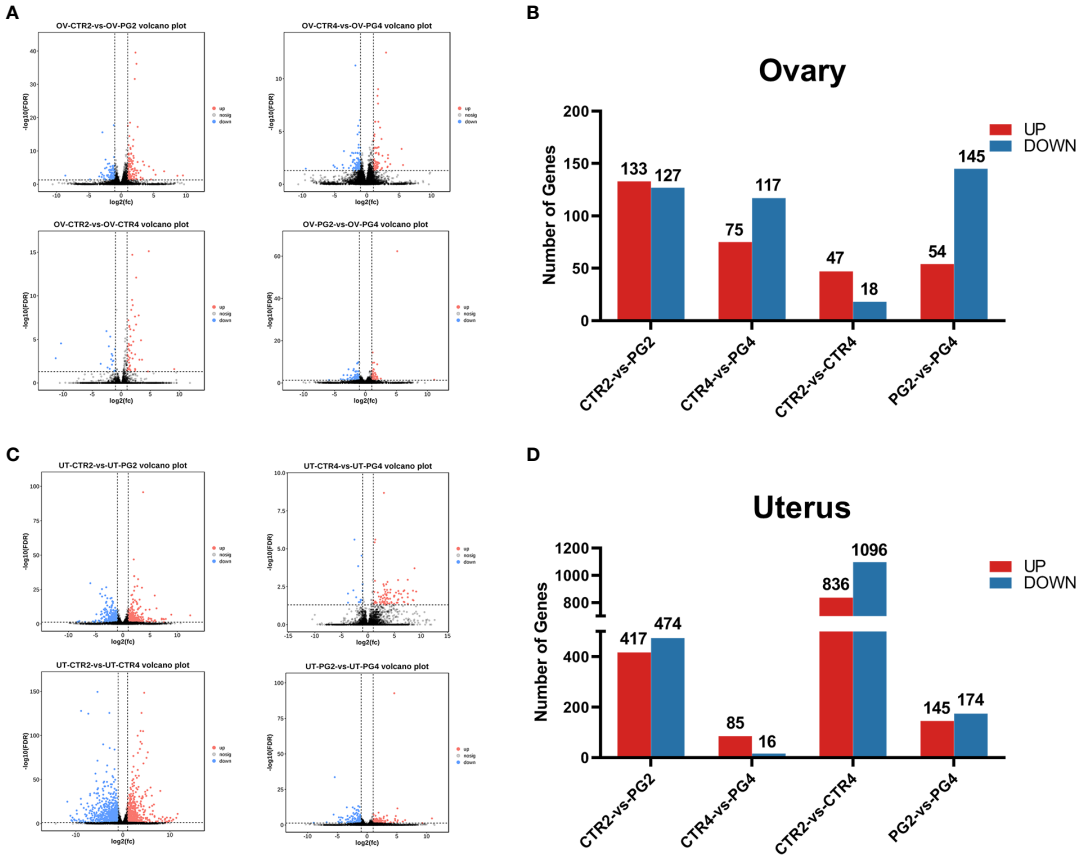

FIGURE 2
Transcriptome differences in mice ovary and uterus by superovulation and different time points after mating. **(A, B)** Volcano plots and bar plots of differentially expressed genes (DEGs) in ovaries between different groups. **(C, D)** Volcano plots and bar plots of differentially expressed genes in the uterus between different groups. CTR2, control group on 2 dpc. PG2, superovulation group on 2 dpc. CTR4, control group on 4 dpc. PG4, superovulation group on 4 dpc. (n = 3).

3.6 Candidate genes identification and RNA sequencing data verification

We screened three candidate genes primarily associated with steroid hormone biosynthesis and signaling pathways in the ovary. Additionally, we focused on three candidate genes close to the implantation stage in the uterus, mainly related to immune response and signal transduction. The expression levels of these candidate genes were detected using qRT-PCR. Notably, all the qRT-PCR results exhibited the same expression pattern as the RNA-Seq results, confirming the reliability of the sequencing data (Figures 6G, H).

4 Discussion

Superovulation is a widely used technique in human reproductive medicine and animal production, but it often leads to complications such as ovarian hyperstimulation syndrome, which can impact ovarian function and embryo implantation (9, 12). Miller et al. demonstrated that GnRHa had a superovulation effect like hCG and relieved ovarian overstimulation using PMSG combined with GnRHa in a mouse model (16). Similarly, our results showed that PMSG combined with GnRHa led to an average of 30 oocytes per mouse and increased corpus luteum in the ovaries. Numerous studies have indicated that superovulation can affect the quality of oocytes and early embryos (18, 24, 25). Our study observed that mice had more blastocysts and stunted or dead embryos after superovulation than normal pregnant mice on 4 dpc, although the litter size was smaller than the number of blastocysts. Previous studies have also demonstrated that embryos implanted after superovulation were smaller than those in naturally pregnant mice, and there was a significantly higher rate of embryo reabsorption (17). However, these studies have not considered the impact of embryo quality or quantity on implantation failure after superovulation (17). In our study, we used embryo transfer to eliminate the effects of embryo number and quality and investigated the effect of superovulation on uterine receptivity. Our results indicated a decrease in embryo implantation sites after superovulation.

In mice, embryo implantation occurred between 4.5 to 5 dpc (2). To explore the effect of superovulation on embryo implantation, samples collected on the morning of the second day (2 dpc) and in the evening of the fourth day (4 dpc) after mating were selected to represent early pregnancy and the period close to implantation, respectively. We evaluated the expression of uterine

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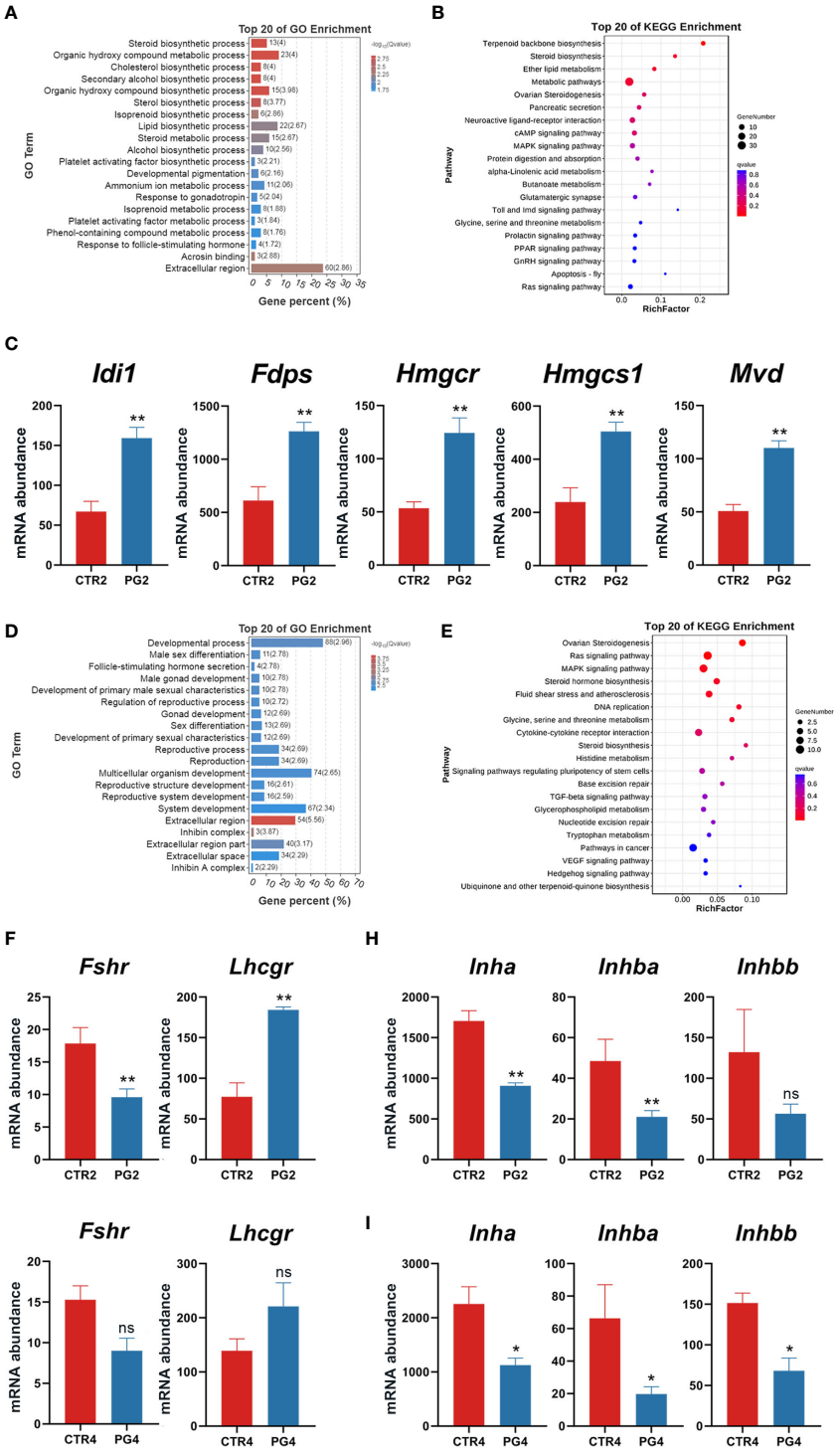

FIGURE 3
Superovulation caused the abnormal ovarian response to gonadotropins. **(A)** The top 20 of Gene Ontology (GO) analysis of differentially expressed genes (DEGs) between CTR2 and PG2 groups. **(B)** The top 20 of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of DEGs between CTR2 and PG2 groups. **(C)** DEGs enriched in terpenoid backbone biosynthesis between CTR2 and PG2 groups. **(D)** The top 20 of GO analysis of DEGs between CTR4 and PG4 groups. **(E)** The top 20 of KEGG pathway analysis of DEGs between CTR4 and PG4 groups. **(F)** Follicle-stimulating hormone receptor (*Fshr*) and luteinizing hormone/choriogonadotropin receptor (*Lhcgr*) mRNA expression between CTR2 and PG2 groups. **(G)** *Fshr* and *Lhcgr* mRNA expression between CTR4 and PG4 groups. **(H)** Inhibin-related gene mRNA expression between CTR2 and PG2 groups. **(I)** Inhibin-related gene mRNA expression between CTR4 and PG4 groups. *Idi1*, isopentenyl-diphosphate delta isomerase. *Mvd*, mevalonate (diphospho) decarboxylase. *Hmgcs1*, 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1. *Hmgcr*, 3-hydroxy-3-methylglutaryl-Coenzyme A reductase. *Fdps*, farnesyl diphosphate synthetase. *Inha*, inhibin alpha. *Inhba*, inhibin beta-A. *Inhbb*, inhibin beta-B. CTR2, control group on 2 dpc. PG2, superovulation group on 2 dpc. CTR4, control group on 4 dpc. PG4, superovulation group on 4 dpc. *FDR < 0.05. **FDR < 0.01. ns, no significance. (n = 3).

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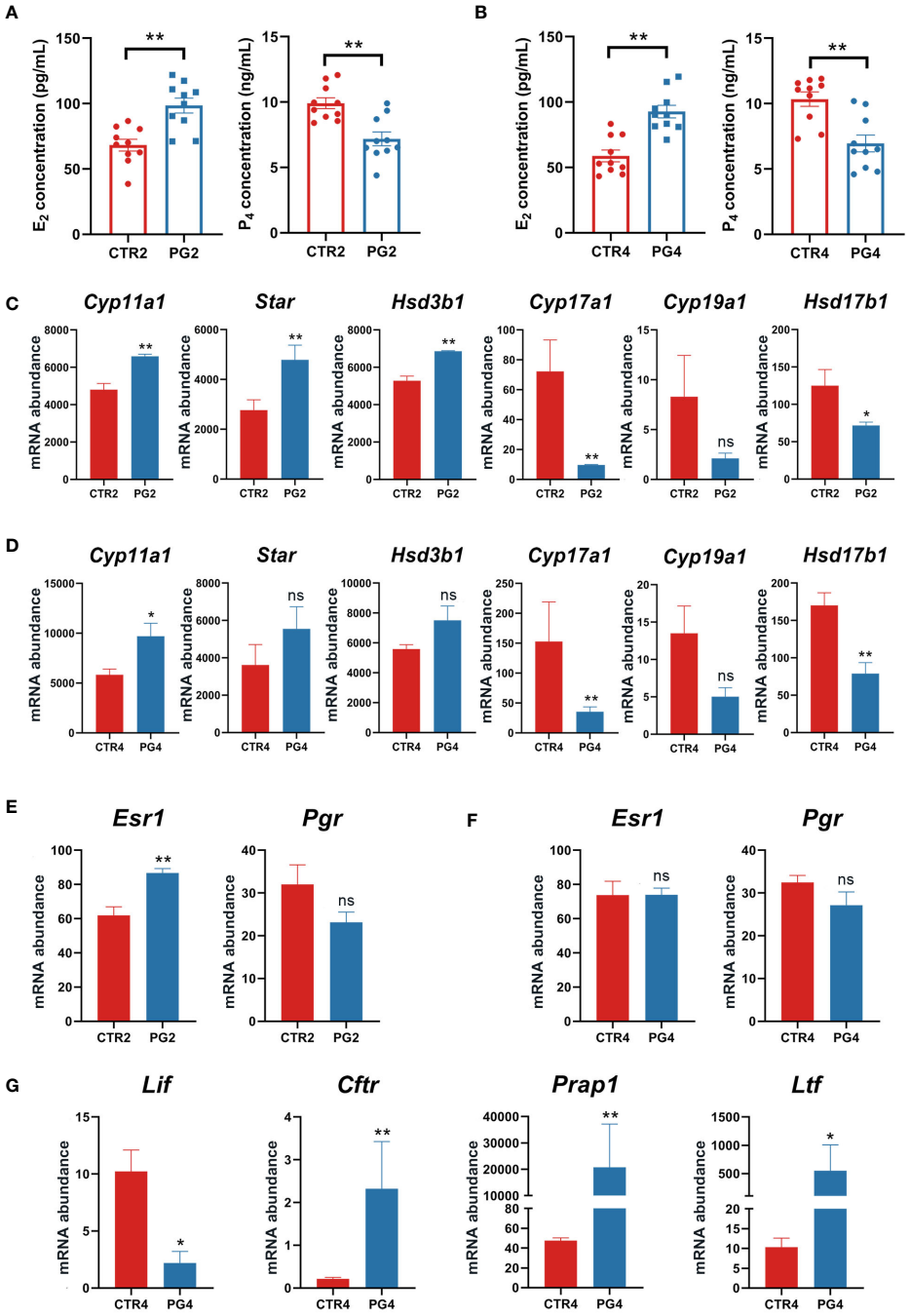

FIGURE 4
Superovulation led to abnormal ovarian steroid hormone synthesis and secretion. **(A)** Estrogen (E_2) and progesterone (P_4) concentrations between CTR2 and PG2 groups. **(B)** Estrogen (E_2) and progesterone (P_4) concentrations between CTR4 and PG4 groups. **(C)** The mRNA expression of key enzymes for steroid hormone synthesis between CTR2 and PG2 groups in the ovary. **(D)** The mRNA expression of key enzymes for steroid hormone synthesis between CTR4 and PG4 groups in the ovary. **(E)** Estrogen receptor 1 (*Esr1*) and progesterone receptor (*Pgr*) mRNA expression between CTR2 and PG2 groups in the uterus. **(F)** *Esr1* and *Pgr* mRNA expression between CTR4 and PG4 groups in the uterus. **(G)** DEGs of E_2-responsive gene mRNA expression between CTR4 and PG4 groups in the uterus. *Cyp11a1*, cytochrome P450, family 11, subfamily a, polypeptide 1. *Star*, steroidogenic acute regulatory protein. *Hsd3b1*, hydroxy-delta-5-steroid dehydrogenase, 3 beta and steroid deltaisomerase 1. *Cyp17a1*, cytochrome P450, family 17, subfamily a, polypeptide 1. *Cyp19a1*, cytochrome P450, family 19, subfamily a, polypeptide 1. *Hsd17b1*, hydroxysteroid (17-beta) dehydrogenase 1. *Lif*, leukemia inhibitory factor. *Cftr*, cystic fibrosis transmembrane conductance regulator. *Prap1*, proline-rich acidic protein 1. *Ltf*, lactotransferrin. CTR2, control group on 2 dpc. PG2, superovulation group on 2 dpc. CTR4, control group on 4 dpc. PG4, superovulation group on 4 dpc. **(A, B)** $^{**}p < 0.01$. **(C-G)** *FDR < 0.05. **FDR < 0.01. ns, no significance. (n = 3).

receptivity-related genes (*Lif*, *Hoxa10*, and *Itgb3*) on 4 dpc. LIF is a crucial cytokine secreted by the uterine glands, playing a vital role in embryo implantation. Successful embryo implantation relies on LIF to activate the JAK-STAT pathway, leading to the phosphorylation of STAT3, which is essential for implantation (26, 27). LIF-null mice exhibit pregnancy failure (28). HOXA10 is a transcription factor that regulates the expression of factors related to embryo implantation (29). It promotes stromal cell proliferation and is

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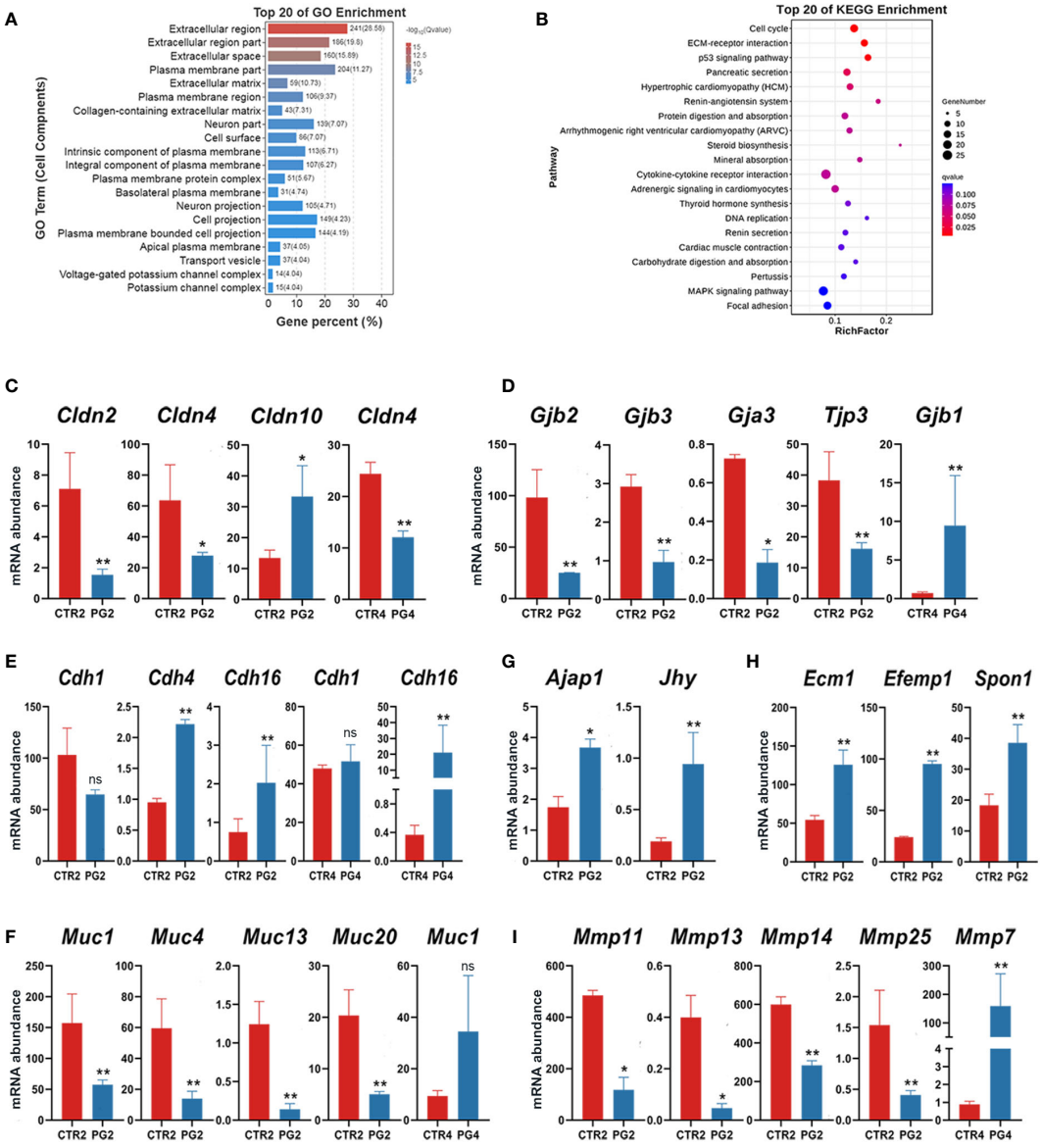

FIGURE 5
Abnormal remodeling of extracellular matrix and epithelial remodeling in the uterus after superovulation. **(A)** The top 20 of Gene Ontology (GO) analysis of differentially expressed genes (DEGs) in cell components between CTR2 and PG2 groups. **(B)** The top 20 of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of DEGs between CTR2 and PG2 groups. **(C)** DEGs of claudin (*Clnd*) family mRNA expression in CTR2 vs. PG2 groups and CTR4 vs. PG4 groups. **(D)** DEGs of gap junction protein family and tight junction protein 3 (*Tjp3*) mRNA expression in CTR2 vs. PG2 groups and CTR4 vs. PG4 groups. **(E)** DEGs of cadherin (*Cdh*) family mRNA expression in CTR2 vs. PG2 groups and CTR4 vs. PG4 groups. **(F)** DEGs of mucin (*Muc*) family mRNA expression in CTR2 vs. PG2 groups and CTR4 vs. PG4 groups. **(G)** Adherens junction-associated protein 1 (*Ajap1*) and junctional cadherin complex regulator (*Jhy*) mRNA expression in CTR2 vs. PG2 groups and CTR4 vs. PG4 groups. **(H)** DEGs of extracellular matrix component proteins mRNA expression between CTR2 and PG2 groups. **(I)** DEGs of matrix metallopeptidase (*Mmp*) family mRNA expression in CTR2 vs. PG2 groups and CTR4 vs. PG4 groups. *Gjb*, gap junction protein, beta. *Gja3*, gap junction protein, alpha 3. *Ecm1*, extracellular matrix protein 1. *Efemp1*, epidermal growth factor-containing fibulin-like extracellular matrix protein 1. *Spon1*, spondin 1, (f-spondin) extracellular matrix protein. CTR2, control group on 2 dpc. PG2, superovulation group on 2 dpc. CTR4, control group on 4 dpc. PG4, superovulation group on 4 dpc. *FDR < 0.05. **FDR < 0.01. ns, no significance. (n = 3).

involved in decidualization (29). Reduced HOXA10 contributes to implantation failure after human embryo transfer (30). Integrin is a transmembrane glycoprotein on the plasma membrane essential to embryo implantation. In pregnant mice, reduced uterine integrin expression is associated with embryo implantation failure (31). Our results indicated a significant reduction in Lif mRNA expression; however, the expression of *Hoxa10* and *Itgb3* was no significant difference. In summary, our findings suggest that the impact of superovulation on embryo implantation primarily involves affecting the uterus rather than the embryo.

To further evaluate the effect of superovulation on mouse embryo implantation, we conducted transcriptome analysis on the pre-

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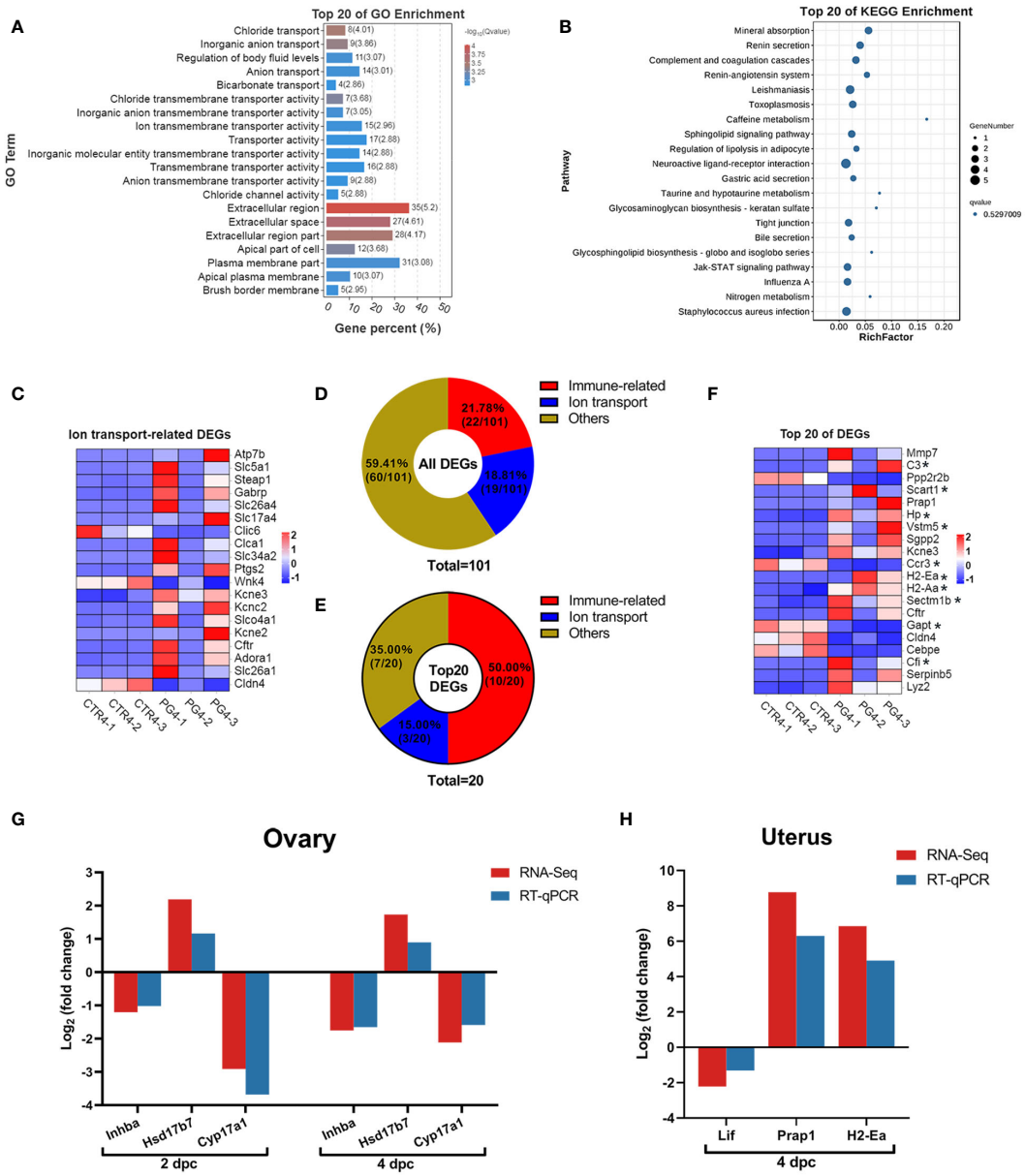

FIGURE 6
Superovulation results in abnormal ion transport and an excessive immune environment in the uterus. **(A)** The top 20 of Gene Ontology (GO) analysis of differentially expressed genes (DEGs) between CTR4 and PG4 groups. **(B)** The top 20 of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of DEGs between CTR4 and PG4 groups. **(C)** DEGs of ion transport-related gene for the heatmap between CTR4 and PG4 groups in the uterus. **(D)** All DEGs were donut-mapped between CTR4 and PG4 groups in the uterus. **(E)** The top 20 DEGs donut map between CTR4 and PG4 groups in the uterus. **(F)** The top 20 of DEGs heatmap between CTR4 and PG4 groups in the uterus. **(G, H)** The mRNA expression level of differentially expressed genes (DEGs) in the ovary and uterus was ascertained by RNA sequencing (RNA-Seq) and quantitative real-time PCR (qRT-PCR). *Immune-related genes. Each color represents the percentage of each part of the total (target genes number/total genes number) in the donut map. *Inhba*, inhibin beta-A. *Hsd17b7*, hydroxysteroid (17-beta) dehydrogenase 7. *Cyp17a1*, cytochrome P450, family 17, subfamily a, polypeptide 1. *Lif*, leukemia inhibitory factor. *Prap1*, proline-rich acidic protein 1. *H2-Ea*, histocompatibility 2, class II antigen E alpha. (n = 3).

implantation stage of both the ovary and uterus. Our findings revealed that superovulation treatment led to gene expression alterations in the ovary each time. Specifically, we observed that only 65 genes were altered between 2 dpc and 4 in normal pregnant mice, whereas superovulation resulted in a more substantial variance with 199 DEGs. Conversely, the normal uterus exhibited significant gene expression changes in pregnant mice during pre-implantation. However, only 319 differentially expressed genes were shown after superovulation, indicating that the uterus on 2 dpc was more similar to that on 4 dpc and was not yet prepared for implantation. While the ovary showed minimal gene expression changes, the uterus exhibited substantial alterations in natural pregnancy mice. Nevertheless, superovulation interferes with these changes and has the potential to lead to embryo implantation failure. These results underscore the

intricate impact of superovulation on the molecular processes involved in embryo implantation.

The gonadotropin-releasing hormone (GnRH) targets the pituitary as the primary organ for follicle-stimulating hormone (FSH) and luteinizing hormone (LH) synthesis and secretion. These hormones regulate estrus, ovulation, and other biological processes (32, 33). FSH could be regulated by inhibin in the ovary (34). GO analysis indicated that DEGs were enriched in the process of FSH secretion about inhibin, with a significant decrease in the expression of inhibin-related genes on 2 dpc, suggesting that it may increase FSH secretion. Typically, FSH induces the formation of LH receptors in granulosa cells, with LH surges triggering ovulation.

Additionally, examination of the FSH and LH receptor expression in the ovary revealed reduced *Fshr* abundance and a significant increase in the *Lhcgr* abundance. In previous superovulation protocols, PMSG induced the development of many follicles to the preovulatory stage, and hCG was directly bound to LHCGR to drive ovulation (35). A Study has demonstrated that superovulation protocols triggered by GnRH and hCG can elevate *Lhcgr* expression before embryo implantation, potentially impairing ovarian function (17). Unlike hCG directly binding to LHCGR, GnRHa stimulates gonadotropin secretion, promoting LHCGR expression more akin to the natural physiological cycle. It is well known that a low pregnancy rate after superovulation is linked to abnormal expression of estrogen and progesterone (36). Our results showed that GnRHa-triggered superovulation protocols consistently showed high estrogen and low progesterone expression. Genes involved in ovarian steroid hormone synthesis exhibited an increased abundance of crucial enzyme genes for progesterone synthesis and a decreased abundance of genes involved in converting progesterone to estrogens. These changes may be attributed to negative feedback regulation from abnormal estrogen and progesterone levels. Interestingly, hCG-triggered ovulation led to the elevated expression of estrogen and progesterone levels (37). However, GnRHa-triggered ovulation resulted in decreased progesterone levels during pre-implantation.

The sustained action of LH is essential for maintaining luteal function (38). Prolonged stimulation with hCG, which directly binds to LHCGR, leads to elevated progesterone levels (39, 40). As a result, hCG is commonly used as a supplement to support corpus luteum during pregnancy (41, 42). Research has indicated that the duration of LH stimulation triggered by GnRHa is shorter than the physiological state, which may result in insufficient luteal function and even premature dissolution of the corpus luteum (43). In the human fresh embryo transfer cycle, ovulation triggered by GnRHa has demonstrated a higher rate of early miscarriage and a lower rate of live birth compared to hCG triggered (6). In a mouse model, GnRHa-triggered ovulation resulted in smaller embryos and placentas than naturally mated embryos, with significantly higher embryo resorption in GnRHa-triggered mice than in naturally pregnant mice (17). Furthermore, rabbits receiving the GnRHa trigger exhibited a high abortion rate and persistently low P_4 concentration (44). GnRHa administration on day 7 after breeding in llamas, but there was no difference in P_4 concentration compared to individuals with a single corpus luteum (45). These changes may be attributed to insufficient luteal function. In present studies, the results regarding progesterone levels after GnRHa-triggered ovulation varied (6, 17), but estrogen expression remained consistently high. This evidence suggests that superovulation triggered by GnRHa may lead to abnormal hormone synthesis and gonadotropin receptor expression in the ovary, potentially hindering embryo implantation.

During embryo implantation, the uterus undergoes molecular and histological changes, including inhibiting proliferation, remodeling endometrial epithelial cells, and transforming stromal cells into decidual cells. These coordinated changes in estrogen, progesterone, and certain cytokines are primarily responsible for these alterations (3, 46, 47). In ovariectomized mice, estrogen and progesterone have been shown to induce decidualization, indicating the critical role of these hormones in establishing uterine receptivity (48). Our findings revealed abnormal expression of estrogen and progesterone following superovulation. It was further confirmed that *Esr1* abundance in the uterus of the PG group was up-regulated on 2 dpc and showed no significant difference on 4 dpc compared to the CTR group. *Pgr* abundance showed no significant difference between the two groups in either 2 dpc or 4 dpc. Our results suggest that the uterus responded to high estrogen signals, as evidenced by abnormally elevated levels of genes activated by estrogen in the uterus after superovulation, even in the absence of protein level validation. Intriguingly, a high estrogen level typically leads to increased *Lif* expression (49); however, our results demonstrated that the *Lif* expression was down-regulated in the superovulation group. Those results suggest that estrogen may have a complex regulatory network for *Lif*, although the specific mechanisms require further exploration.

Endometrial remodeling is a crucial process for preparing the uterus to receive embryo implantation, involving coordinated changes in intercellular junctions, extracellular matrix remodeling, and the loss of epithelial apical-base polarity (50, 51). In this study, GO and KEGG analyses revealed that terms related to extracellular matrix remodeling were mainly enriched on 2 dpc. Further analysis indicated the up-regulation of multiple mucin family members and the down-regulation of genes associated with gap junction and tight junction. However, extracellular matrix components were up-regulated, while multiple matrix metalloproteinase family members showed down-regulation. Mucin 1 (MUC1), a member of the mucin family, plays a critical role in embryo implantation (52). MUC1 expression in epithelial cells in mice begins to down-regulate on 3.5-4 dpc, with minimal expression at the time of embryo implantation (53, 54). Our results demonstrated the down-regulation of Muc1 on 2 dpc, and a similar expression trend was observed for three other mucin family members. The result indicates that intercellular junctions and extracellular matrix remodeling are not synchronized and fail to support endometrial remodeling. E-cadherin (CDH1) is expressed at the apex of epithelial cells, and its loss during implantation indicates the loss of the epithelial cell polarity required for embryo implantation (2). Our results indicated that *Cdh1* was down-regulated on 2 dpc after superovulation, although there was no difference on 4 dpc,

suggesting that premature CDH1 loss could also contribute to abnormal endometrial remodeling.

The maximum receptive period of the endometrium for the embryo is known as the implantation window (1). In mice, the implantation window typically opens on 4.5-5 dpc and lasts approximately 24 h. During this period, the embryo undergoes positioning, adhesion, and invasion, ultimately completing the implantation process (1, 55, 56). Even minor alterations in the uterine microenvironment during the implantation window can disrupt the process of embryo implantation. At the time of embryo implantation, there is a reduction in uterine cavity fluid, closure of the uterine cavity, and other changes that promote embryo attachment to the endometrium (57). Studies have shown that after superovulation, mice experience abnormal uterine fluid secretion and absorption during the implantation stage (23). GO analysis revealed significant enrichment of molecular functions related to ion transport, with many ion transport-related genes being up-regulated in the uterus on 4 dpc, including the cystic fibrosis transmembrane regulator (*Cftr*). CFTR is a chloride channel associated with uterine receptivity (58). Elevated CFTR expression during embryo implantation can lead to fluid accumulation in the uterine cavity, potentially resulting in implantation failure (57). In addition, research has indicated that increased estrogen levels induced by superovulation can lead to elevated CFTR expression and increased endometrial apoptosis.

The findings of this study indicate that superovulation can lead to changes in ion transport-related genes, possibly resulting in alterations in the uterine fluid environment that can impair embryo implantation. The immune environment of the uterus is also critical for successful implantation, as the embryo is initially perceived as a foreign body, triggering an immune response (59). However, this excessive immune response can lead to implantation failure (60–62). Our results revealed that DEGs were primarily enriched in immune-related pathways in the 4 dpc uterus. Immune-related genes were observed in up to 40% of the top 20 differential genes, with high expression levels in the superovulation group. These findings suggest that superovulation may result in excessive uterine immune defense, ultimately leading to embryo implantation failure.

5 Conclusions

In summary, our study has confirmed the detrimental impact of superovulation on embryo implantation through embryo transfer. Superovulation induces excessive ovarian production and prolonged presence of supraphysiological levels of estrogen. Through comprehensive transcriptome sequencing analysis, we have identified several factors contributing to implantation failure in mice following superovulation. These include abnormal gene expression related to endometrial remodeling, disrupted uterine cavity closure due to intrauterine fluid transport-related gene changes, and an intensified immune-related gene, all linked to elevated estrogen levels. As a result, future efforts aimed at mitigating the adverse effects of superovulation on pregnancy establishment should prioritize strategies to improve ovarian function and reduce the duration of elevated estrogen levels.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1064015.

Ethics statement

The animal study was approved by The Ethical Committee of Hebei Agricultural University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

ML: Conceptualization, Data curation, Methodology, Writing – original draft. JH: Conceptualization, Writing – original draft, Writing – review & editing. NY: Methodology, Writing – review & editing. XL: Writing – review & editing. XW: Conceptualization, Data curation, Project administration, 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.

Publisher's note

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EDITED BY
Jan Tesarik,
MARGen Clinic, Spain

REVIEWED BY
Lang Qin,
Sichuan University, China
Gedis Grudzinskas,
Independent Researcher, London, United Kingdom
Abdelhamid Benmachiche,
Université Frères Mentouri Constantine 1, Algeria

*CORRESPONDENCE
Haiying Liu
liuhaiying0606@163.com

†These authors have contributed equally to this work

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Effectiveness and safety of GnRH antagonist originator and generic in real-world clinical practice: a retrospective cohort study

Mingzhu Cao[1†], Yuqi Hu[2,3†], Jiaqi Xiao[4†], Sichen Li[1], Yanshan Lin[1], Jianqiao Liu[1] and Haiying Liu[1*]

[1]Department of Obstetrics and Gynecology, Center for Reproductive Medicine, Guangdong Provincial Key Laboratory of Major Obstetric Diseases, Guangdong Provincial Clinical Research Center for Obstetrics and Gynecology, Guangdong-Hong Kong-Macao Greater Bay Area Higher Education Joint Laboratory of Maternal-Fetal Medicine, The Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, China, [2]Department of Clinical Laboratory Medicine, The Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, China, [3]Department of Clinical Medicine, The Third Clinical School of Guangzhou Medical University, Guangzhou, China, [4]Department of Clinical Medicine, The Nanshan College of Guangzhou Medical University, Guangzhou, China

Objective: This study aims to determine whether the live birth rates were similar between GnRH antagonist original reference product Cetrotide® and generic Ferpront®, in gonadotropin-releasing hormone (GnRH) antagonist protocol for controlled ovarian stimulation (COS).

Methods: This retrospective cohort study investigates COS cycles utilizing GnRH antagonist protocols. The research was conducted at a specialized reproductive medicine center within a tertiary care hospital, spanning the period from October 2019 to October 2021. Within this timeframe, a total of 924 cycles were administered utilizing the GnRH antagonist originator, Cetrotide® (Group A), whereas 1984 cycles were undertaken using the generic, Ferpront® (Group B).

Results: Ovarian reserve markers, including anti-Mullerian hormone, antral follicle number, and basal follicular stimulating hormone, were lower in Group A compared to Group B. Propensity score matching (PSM) was performed to balance these markers between the groups. After PSM, baseline clinical features were similar, except for a slightly longer infertile duration in Group A versus Group B (4.43 ± 2.92 years vs. 4.14 ± 2.84 years, P = 0.029). The duration of GnRH antagonist usage was slightly longer in Group B than in Group A (6.02 ± 1.41 vs. 5.71 ± 1.48 days, P < 0.001). Group B had a slightly lower number of retrieved oocytes compared to Group A (14.17 ± 7.30 vs. 14.96 ± 7.75, P = 0.024). However, comparable numbers of usable embryos on day 3 and good-quality embryos were found between the groups. Reproductive outcomes, including biochemical pregnancy loss, clinical pregnancy, miscarriage, and live birth rate, did not differ significantly between the groups. Multivariate logistic regression analyses suggested that the type of GnRH antagonist did not independently

impact the number of oocytes retrieved, usable embryos, good-quality embryos, moderate to severe OHSS rate, clinical pregnancy, miscarriage, or live birth rate.

Conclusion: The retrospective analysis revealed no clinically significant differences in reproductive outcomes between Cetrotide® and Ferpront® when used in women undergoing their first and second COS cycles utilizing the GnRH antagonist protocol.

KEYWORDS

GnRH antagonist, generic, live birth rate, Cetrotide®, Ferpront®

1 Introduction

Infertility, defined as the failure to conceive within a year of unprotected sexual activity, remains a persistent global challenge (1). Assisted reproductive technologies (ART), such as *in vitro* fertilization (IVF) and intra-cytoplasmic sperm injection (ICSI), offer effective solutions for infertility (1). Controlled ovarian stimulation (COS) using exogenous gonadotropins stands as a critical step in ART, enabling the recruitment of a sufficient number of fertilizable oocytes and subsequent embryo formation. The two most widely used protocols in COS are the Gonadotropin Releasing Hormone (GnRH) antagonist and GnRH agonist protocols.

The GnRH antagonist protocol presents several advantages over the GnRH agonist protocol, including a shorter duration of antagonist treatment, reduced gonadotropin (Gn) stimulation, lowered risk of ovarian hyperstimulation syndrome (OHSS), and absence of flare-up effects and low estrogen impact (2, 3). GnRH antagonists inhibit luteinizing hormone (LH) release directly and swiftly by competitively binding to GnRH receptors in the pituitary (3). Notable antagonists used in clinics include cetrorelix and ganirelix.

Cetrotide®, also known as Cetrorelix acetate injection (patent expired in April 2019), is a synthetic decapeptide recognized for its stability, minimal variability, high bioavailability, and efficacy in preventing premature LH surges during COS in females (4, 5). It was the first GnRH antagonist introduced in clinical settings (6). Ferpront® (Ferring Pharmaceuticals, China) is a cetrorelix generic developed to emulate Cetrotide®'s physicochemical properties (7). In December 2018, Ferpront® received authorization from the Chinese Center for Drug Evaluation (8). Pre-clinical studies have demonstrated the safety and pharmacokinetics of Ferpront® compared to Cetrotide®7.

Despite their shared similar active pharmaceutical ingredients, there remains a lack of comparative studies evaluating the efficacy and safety between Ferpront® and Cetrotide®. To address this gap, the current retrospective cohort study aims to investigate the clinical efficacy and safety of Ferpront® as a generic of Cetrotide® in infertile women undergoing COS with the GnRH antagonist protocol.

2 Materials and methods

2.1 Study population

The study was a retrospective, single-center investigation conducted at the Guangzhou Medical University Third Affiliated Hospital. Notably, this hospital stands as one of the largest reproductive medicine centers in Southern China, performing nearly 10,000 ART cycles annually. The study protocol received approval from the ethical committee (approval number: 2023–121). Comprehensive clinical records, encompassing detailed demographic and treatment-related data, were extracted from the hospital's database for analysis. Clinical records from infertile couples undergoing IVF or ICSI between October 2019 and October 2021 were screened for eligibility criteria. Included participants met specific criteria: utilization of the GnRH antagonist protocol for COS, involvement in either the first or second COS cycle, females aged 20–40, and use of either Cetrotide® (Group A) or Ferpront® (Group B) as the GnRH antagonist. Exclusion criteria comprised compromised endometrial conditions, severe endometriosis, repeated miscarriages or implantation failures, pre-implantation genetic testing, fertility preservation, Micro-TESE sperm retrieval, oocyte or embryo banking cycles, and severe systemic diseases potentially impacting reproductive outcomes. Consecutive participants fulfilled the inclusion and exclusion criteria were included for further analysis.

2.2 Ovarian stimulation protocols and embryo transfer

Cycles with GnRH antagonist protocols were included, incorporating several types of gonadotropins (Gn), including recombinant follicular stimulating hormone (Gonal-F®, Merck & Co., Germany; Puregon®, Organon & Co., USA), urine FSH (LiShengBao®, Livzon Pharm, China), and human menopausal gonadotropin (LeBaoDe®, Livzon Pharm, China) for COS. In the fixed protocol, the GnRH antagonist—either 0.25 mg of Cetrotide® or Ferpront®—was administered daily on day 5 of Gn

administration. In the flexible protocol, the initiation of 0.25 mg GnRH antagonist occurred upon meeting at least one of the following criteria: 1) the dominant follicle reached an average diameter of 12 mm, 2) serum E2 levels were > 550–1400 pmol/L (150–400 pg/ml), 3) serum LH was elevated more than 2 times the baseline level or LH ≥ 10 IU/L.

GnRH antagonist administration continued until the day of ovulation trigger. Regular monitoring of follicle development through transvaginal ultrasound and serum FSH, LH, estradiol (E2), and progesterone (P) levels was performed. The ovulation trigger was administered if there were at least 2 leading follicles with a mean diameter of 18 mm or at least 3 leading follicles ≥ 17 mm, using of recombinant human chorion gonadotropin (Ovidrel®, Merck & Co., Germany), 2000 to 10000 IU of urine HCG (Livzon Pharm, China), or 0.2 mg of GnRHa (Decapeptyl®, Ferring Pharmaceuticals, Switzerland). Transvaginal oocytes recollection was arranged approximately 34 to 36 hours after the trigger, and fertilization with IVF or ICSI was performed based on semen quality.

The freeze-all policy was applied under several conditions: 1) if more than 20 oocytes were retrieved, 2) serum E2 levels were ≥ 18350 pmol/L on the trigger day, 3) other medical conditions deemed unsuitable for fresh embryo transfer as determined by physicians, 4) personal reasons prohibiting fresh embryo transfer. One or two cleavage stage embryos or blastocyst embryos were transferred either 3 or 5 days following oocytes pick-up (OPU) day, and the remaining usable embryos were vitrified. Embryo grading was conducted based on fragmentation levels (9) (Grade I: < 5%, Grade II: 5–20%, Grade III: 20–50%, Grade IV: < 50%). An embryo with good quality on day 3 was defined as 7–9 cells with < 20% cellular debris and uniformity in cell size. Blastocyst quality was evaluated based on the Gardner scoring system for trophectoderm and inner cell mass scores. Routine luteal phase support with dydrogesterone 20 mg/day (Duphaston®, Abbott Laboratories, USA), 90 mg/day vaginal progesterone gel (Crinone®, Merck, Germany), or 0.2 g/day of vaginal progesterone capsule (Utrogestan®, Besins Healthcare, Monaco) was administered post-oocyte retrieval and continued after fresh embryo transfer. Pregnancy was evaluated through serum HCG testing 14 days following embryo transfer and transvaginal ultrasound examination approximately 4 weeks after embryo transfer.

2.3 Outcomes measured

The study's primary endpoint was the live birth rate per embryo transfer cycle, defined as the delivery of live newborns after 28 weeks of gestation. The birth of twins or triplets was considered as one live birth. Secondary endpoints included: 1) the number of retrieved oocytes, 2) the number of usable embryos on day 3, 3) the number of good quality embryos, 4) clinical pregnancy rate, and 5) spontaneous miscarriage rate. Biochemical pregnancy was identified by detecting serum HCG > 10 mIU/ml two weeks post-embryo transfer, while clinical pregnancy was confirmed by observing an intrauterine gestational sac via ultrasonography around 6 weeks of gestation. Spontaneous miscarriages were characterized by pregnancy losses with detectable intrauterine gestational sacs before 28 weeks of gestation. Biochemical pregnancy rate, clinical pregnancy rate, and live birth rate was calculated as the percentage of cycles meeting these criteria out of cycles with fresh embryo transfer. The spontaneous miscarriage rate was determined as the proportion of cycles experiencing spontaneous miscarriage among those resulting in clinical pregnancy.

Safety outcomes were measured by evaluating the incidence of moderate/severe OHSS. The diagnosis criteria followed recommendations from a consensus of Chinese experts (10). Moderate OHSS was identified by the presence of abdominal discomfort, nausea, vomiting, diarrhea; ovarian enlargement (8–12 cm) and ascites detected through ultrasound; and specific laboratory findings including a hematocrit < 0.45 and elevated leukocyte count (10–15 × 10^9/L). Severe OHSS presented symptoms such as severe nausea, vomiting, dyspnea, significant abdominal pain, oliguria or anuria (< 300 ml/d or < 30 ml/h), rapid weight gain (> 1 kg/24 h), enlarged ovaries (> 12 cm) with sonographic evidence of tension ascites, pleural effusion, vascular embolism, low blood pressure, or low central venous pressure. Additionally, it included elevated hematocrit (> 0.45), increased leukocyte levels (> 15 × 10^9/L), hyperkalemia (potassium >5 mmol/L), hyponatremia (sodium < 135 mmol/L), impaired renal function (creatinine > 1.0 g/L), and altered liver function (increased levels of glutamic oxaloacetic transaminase and glutamic pyruvic transaminase).

2.4 Statistical analysis

In the current study, Cetrotide® was used as the reference medication. All statistical analyses were carried out with SPSS (version 22.0, IBM Inc., US). Quantitative variables with a normal distribution were described as mean ± standard deviation (SD) and compared using Student's t-test, while those with a skewed distribution were depicted as median (25th and 75th quartiles) and compared with the Mann-Whitney U test. Comparisons of frequencies and proportions were performed using the Chi-squared test.

Several baseline clinical parameters, such as baseline follicle-stimulating hormone (FSH), anti-müllerian hormone (AMH), and antral follicle count (AFC) differed significantly between Group A and Group B. To minimize the influence of these confounders, propensity score matching (PSM) was conducted to align these parameters. The two groups were matched 1:1 using nearest neighbor matching. The standardized mean difference (SMD) before and after PSM was calculated and presented in Supplementary Table 1, showing a reduced SMD after matching to less than 0.1, considered balanced (11).

To determine if the type of GnRH antagonist independently impacted various reproductive outcomes, multivariate logistic regression analyses were conducted with these outcomes as dependent factors before and after PSM. Possible confounders, including female age, duration of infertility, infertility factors, AMH, AFC, baseline FSH, BMI, duration and dosage of Gn and GnRH antagonist, number of oocytes collected, and trigger type, were included in the multivariable logistic regression before PSM.

After PSM, additional potential confounders included in the analyses were female age, duration of infertility, serum AMH, AFC, BMI, duration and total dosage of Gn and GnRH antagonist, and the number of oocytes retrieved. The likelihoods of reproductive outcomes were displayed as adjusted odds ratios (OR) with their 95% confidence intervals (95% CI). Multiple linear regressions using a stepwise selection approach were utilized in a multivariate statistical model to assess the impact of GnRH antagonist type on the number of oocytes retrieved, usable embryos, and good-quality embryos. A significance level of less than 0.05 was considered statistically significant for all analyses.

3 Results

3.1 Baseline clinical characteristics

Overall, 2908 cycles with the first or second cycles of GnRH antagonist protocol for COS were included and further divided into two groups based on the type of GnRH antagonist used (n=924 for Group A with Cetrotide® and n=1984 for Group B with Ferpront®). The flow chart depicting data collection was presented in Figure 1.

As presented in Table 1, detailed participants' baseline characteristics, infertile factors in Group B slightly differed from those in Group A. Notably, several ovarian reserve tests, including serum AMH levels, AFC, and baseline FSH levels, were significantly lower in Group B than in Group A. Considering the substantial clinical value of ovarian reserve markers, balancing these markers using PSM was performed. As revealed in Supplementary Table 1, the SMD in the ovarian reserve markers after PSM was noticeably less than before PSM, thus achieving a well-balanced status for the ovarian reserve markers post-PSM. After PSM, all other parameters were comparable between the two groups, except for the infertile duration, which was slightly higher in Group A than in Group B, but with minimal clinically substantive significance.

3.2 Ovarian stimulation outcomes and embryological results

Ovarian stimulation outcomes and embryological results were presented in Table 2. The total dose and duration of Gn, the proportion of urinary Gn, and the duration of GnRH antagonist were significantly higher in Group B compared to those in Group A. There were fewer cycles with E2 > 18350 pmol/L on trigger day in Group B (13.05% vs. 21.65%, $P < 0.001$), and significantly more cycles with HCG for trigger (71.77% vs. 64.39%, $P < 0.001$), and more fresh embryo transfers (54.00% vs. 61.44%, $P < 0.001$) observed in Group B, possibly due to differences in ovarian reserve tests. After PSM for balancing ovarian reserve tests, the previously mentioned differences were minimal, although the duration of GnRH antagonist in Group B was significantly longer than that in Group A, and more urinary Gn was administered in Group B compared to Group A ($P < 0.001$). Before PSM, serum levels of LH and P on trigger day were slightly lower in Group B compared to those in Group A, which remained comparable after PSM. In Group A, more cycles had higher E2 levels (E2 > 18350 pmol/L) on trigger day than those in Group B (21.65% vs. 13.05%), and less cycles had low levels of E2 (E2 < 3670 pmol/L) than those in Group B (1.95% vs. 4.74%), and this trend remined even after PSM ($P < 0.001$). Premature LH surge poses a recognized risk in GnRH antagonist protocols and is a crucial parameter under assessment. While various studies present differing specifics, most commonly cite the LH threshold at LH ≥ 10 IU/L (12). Remarkably, in this study, instances where LH ≥ 10 IU/L were so rare that they were negligible and did not require attention or intervention. However, we did notice a reduced number of oocytes retrieved in Group B than in Group A (13.96 ± 7.19 vs. 14.97 ± 7.76 oocytes, $P = 0.001$), and this difference persisted even after PSM (14.17 ± 7.30 vs. 14.96 ± 7.75 oocytes, $P = 0.024$). The number of usable embryos and good-quality embryos was comparable between the two groups before and after PSM.

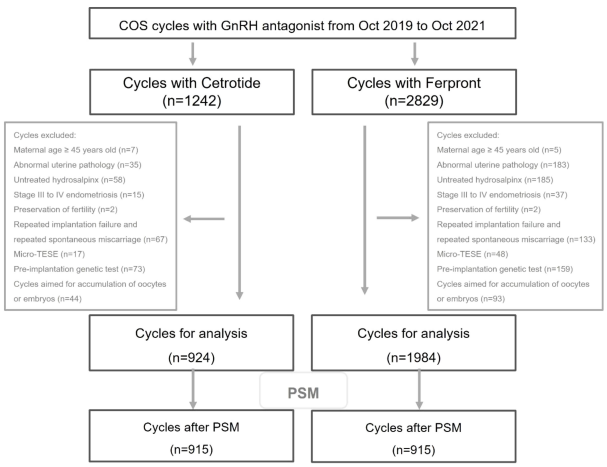

FIGURE 1
Flow chart of cycles inclusion and exclusion.

TABLE 1 Comparison of basic characteristics.

	Before PSM				After PSM			
	Group A	Group B	t/χ^2	*P* value	Group A	Group B	t/χ^2	*P* value
n	924	1984			915	915		
Female age	31.45 ± 4.37	31.51 ± 4.43	-0.302	0.762	31.47 ± 4.36	31.25 ± 4.46	1.066	0.287
Male age	33.49 ± 5.26	33.72 ± 5.30	-1.088	0.277	33.49 ± 5.23	33.43 ± 5.34	0.234	0.815
Infertile duration (years)	4.44 ± 2.93	4.24 ± 2.89	1.784	0.075	4.43 ± 2.92	4.14 ± 2.84	2.19	0.029
Infertile type			0.831	0.362			0.179	0.673
Primary infertility	509/55.09%	1057/53.28%			504/55.08%	496/54.10%		
Secondary infertility	415/44.91%	927/46.72%			411/44.92%	420/45.90%		
Infertile factors			21.568	0.001			10.97	0.052
Male factor	190/20.56%	404/20.36%			188/20.55%	171/18.69%		
Tubal/pelvic factor	301/32.58%	735/37.05%			298/32.57%	332/36.28%		
Endometriosis	14/1.52%	5/0.25%			14/1.53%	3/0.33%		
Ovulation disorder	93/10.06%	184/9.27%			92/10.05%	91/9.95%		
Mixed factors	236/25.54%	452/22.78%			234/25.57%	218/23.83%		
Unexplained	90/9.74%	204/10.28%			89/9.73%	100/10.93%		
BMI (kg/m^2)	22.37 ± 3.42	22.28 ± 3.26	0.68	0.496	22.36 ± 3.40	22.20 ± 3.22	1.08	0.28
AMH (ng/ml)	5.59 ± 4.13	5.22 ± 3.72	2.459	0.014	5.59 ± 4.13	5.48 ± 3.89	0.559	0.576
Basal FSH level (IU/L)	6.11 ± 2.39	5.91 ± 2.11	2.247	0.025	6.07 ± 2.29	6.03 ± 2.28	0.423	0.672
AFC (n)	22.97 ± 10.17	22.20 ± 9.48	1.944	0.047	22.97 ± 10.17	22.99 ± 10.28	-0.053	0.958
Source of sperm			0.624	0.43			1.028	0.311
Husband's	895/96.86%	1932/97.38%			887/96.94%	894/97.70%		
Sperm bank	29/3.14%	52/2.62%			28/3.06%	21/2.30%		

PSM, propensity score matching; BMI, body mass index; AMH, anti-Mullerian hormone; FSH, follicular stimulating hormone; AFC, antral follicle counting.

TABLE 2 Comparison of cycle characteristics.

	Before PSM				After PSM			
	Group A	Group B	t/Z	*P* value	Group A	Group B	t/Z	*P* value
Cycle number	924	1984			915	915		
COS cycle number			0.096	0.757			0.179	0.672
First cycle	751/81.28%	1622/81.75%			744/81.31%	751/82.08%		
Second cycle	173/18.72%	362/18.25%			171/18.69%	164/17.92%		
Gn type			48.317	< 0.001			41.322	< 0.001
Recombinant	781/84.52%	1637/82.51%			775/84.70%	756/82.62%		
Urinary	44/4.76%	227/11.44%			42/4.59%	106/11.58%		
Combined	99/10.71%	120/6.05%			98/10.71%	53/5.79%		
Gn starting dose	169.16 ± 56.53	169.34 ± 57.53	-0.079	0.937	168.99 ± 56.35	168.70 ± 56.88	0.108	0.914
Gn total dose	1500 (1200, 2025)	1500 (1200, 2025)	-2.775	0.006	1500 (1200, 2100)	1500 (1200, 2025)		0.111
Gn duration	9 (8,10)	9 (8, 10)	-2.724	0.006	9 (9, 10)	9 (8, 10)		0.107
GnRHant duration	5.72 ± 1.47	6.01 ± 1.42	-5.162	< 0.001	5.71 ± 1.48	6.02 ± 1.41	-4.521	< 0.001
GnRHant dose	1.52 ± 0.47	1.54 ± 0.43	-1.137	0.256	1.52 ± 0.47	1.55 ± 0.43	-1.164	0.245

(Continued)

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TABLE 2 Continued

	Before PSM				After PSM			
	Group A	Group B	t/Z	*P* value	Group A	Group B	t/Z	*P* value
LH level on trigger day (IU/L)	1.54 (0.96, 2.66)	1.32 (0.83, 2.21)	-4.585	< 0.001	2.10 ± 1.85	1.93 ± 3.39	1.258	0.208
P level on trigger day (nmol/L)	2.40 (1.75, 3.50)	2.30 (1.60, 2.10)	-4.115	< 0.001	2.78 ± 1.50	2.63 ± 1.69	1.841	0.066
E2 level on trigger day (pmol/L)			45.287	< 0.001			24.688	< 0.001
< 3670	18/1.95%	94/4.74%			18/1.97%	46/5.03%		
3670–18350	581/62.88%	1361/68.60%			577/63.06%	615/67.21%		
> 18350	200/21.65%	259/13.05%			196/21.42%	135/14.75%		
Type of trigger			18.67	< 0.001			0.33	0.848
HCG	595/64.39%	1424/71.77%			591/64.59%	586/64.04%		
GnRHa	219/23.70%	400/20.16%			217/23.72%	214/23.39%		
Dual trigger	110/11.90%	160/8.06%			107/11.69%	115/12.57%		
Freeze-all cycles			0.161	0.689			0.639	0.424
Reasons for freeze-all								
OHSS risk	349/82.12%	621/81.18%			344/81.90%	310/79.69%		
Others	76/17.88%	144/18.82%			76/18.10%	79/20.31%		
OPU number in COS cycle	14.97 ± 7.76	13.96 ± 7.19	4.456	0.001	14.96 ± 7.75	14.17 ± 7.30	2.255	0.024
Fertilization type			2.024	0.364			0.53	0.767
IVF	724/78.35%	1514/76.31%			717/78.36%	705/77.05%		
ICSI	154/16.67%	374/18/85%			162/17.70%	174/19.01%		
IVF+ICSI	46/4.87%	96/4.84%			36/3.93%	36/3.93%		
Fertilization rate (%)	76.21 ± 20.95	77.68 ± 19.95	-1.812	0.07	76.31 ± 20.92%	77.51 ± 20.17	-1.251	0.211
Cleavage rate (%)	74.96 ± 21.02	76.41 ± 20.13	-1.796	0.073	75.04 ± 21.00%	76.26 ± 20.31	-1.258	0.209
Number of 2PN embryo(s)	7.93 ± 5.04	7.66 ± 4.66	1.409	0.159	7.92 ± 5.03	7.75 ± 4.72	0.767	0.443
Number of usable embryos (D3)	6.22 ± 4.55	5.98 ± 4.09	1.422	0.155	6.23 ± 4.54	6.18 ± 4.15	0.226	0.821
Number of good quality embryo	2.20 ± 2.28	2.18 ± 2.16	0.206	0.836	1.34 ± 0.48	1.38 ± 0.49	-0.483	0.629
Cycles with fresh embryo transfer	499/54.00%	1219/61.44%	14.423	< 0.001	495/54.10%	526/57.49%	2.129	0.145
Endometrial thickness	10.39 ± 1.98	10.46 ± 1.96	-0.786	0.432	10.40 ± 1.98	10.58 ± 1.99	-1.744	0.081
Number of embryos for ET			1.963	0.161			1.815	0.178
N = 1	327/65.53%	755/61.94%			325/65.66%	324/61.60%		
N = 2	172/34.47%	464/38.06%			180/34.34%	202/38.40%		
Embryo stage			0.003	0.956			0.025	0.874
Cleavage	329/65.93%	802/65.79%			327/66.06%	345/65.59%		
Blastocyst	170/34.07%	417/34.21%			168/33.94%	181/34.41%		

PSM, propensity score matching; COS, controlled ovarian stimulation; Gn, gonadotropin; LH, luteinizing hormone; P, progesterone; E2, estrogen; HCG, human chorionic gonadotropin; GnRHa, gonadotropin releasing hormone agonist; OHSS, ovarian hyper-stimulation syndrome; OPU, oocytes pick up; IVF, in vitro fertilization; ICSI, intra-cytoplasmic sperm injection; 2PN, 2 pronucleus; D3, day 3; ET, embryo transfer.

3.3 Reproductive and safety outcomes

Reproductive outcomes, such as implantation rate, biochemical pregnancy loss, clinical pregnancy, spontaneous miscarriage, multiple pregnancy, and live birth rate, along with the safety outcome, moderate to severe OHSS rate, were presented in Table 3. Notably, no adverse events were reported. The table revealed similar reproductive and safety outcomes between the two groups before and after PSM.

3.4 Multivariate regression analyses

After adjusting for several confounders, the multivariate regression analyses in Table 4 found that the types of GnRH antagonists were not independent factors influencing the number of oocytes retrieved, usable embryos, and good-quality embryos on day 3, as well as multiple reproductive and safety outcomes before and after PSM.

3.5 Subgroup analysis of fixed and flexible protocol of GnRH antagonist

The subgroup analysis of both fixed and flexible protocol of GnRH antagonist as demonstrated in Table 5 found no obvious differences of reproductive outcomes between the two groups. Multivariate regression analysis of fixed and flexible protocol as shown in Table 6 further confirmed that the type of GnRH antagonist had no independent impact on the number of oocytes retrieved, usable embryos, and good-quality embryos, as well as reproductive and safety outcomes regardless of before or after PSM.

4 Discussion

ART services have shown a continuous growth trend in recent years. According to statistics released by the International Committee for Monitoring Assisted Reproductive Technologies (ICMART) in 2022, a total of 3.19 million ART cycles were reported globally, with 1.07 million occurring in China (13). Considering the increasing demand for ART services, effective, safe, and financially viable treatment options are highly needed. Particularly, high treatment burden stands as a critical factor leading to ART treatment discontinuation and poor treatment experiences (14).

The GnRH antagonist protocol stands as the predominant protocol for Controlled Ovarian Stimulation (COS) worldwide. According to the Deutsches IVF Register Annual Report, more than 77.5% of patients underwent COS using the GnRH antagonist protocol, in contrast to only 14.5% who received the GnRH agonist protocol (15). In China, the use of GnRH antagonist regimens for COS has increased substantially, rising from 6% in 2014 to 37% in 2021 (16). The GnRH antagonist is a crucial component of this protocol. Preclinical studies of GnRH antagonists have shown no detrimental effects on the fetus, no mutagenic or teratogenic impacts on the human body. GnRH antagonists exhibit comparable implantation rates, clinical pregnancy rates, and live

TABLE 3 Comparison of reproductive outcomes.

	Before PSM				After PSM			
	Group A	Group B	χ^2/t	P	Group A	Group B	χ^2/t	P
Implantation	42.47% (285/671)	41.53% (699/1683)	0.175	0.676	42.56% (283/665)	40.38% (294/728)	0.676	0.411
Biochemical pregnancy	4.01% (20/499)	4.76% (58/1219)	0.460	0.498	4.04% (20/495)	5.13% (27/526)	0.693	0.405
Clinical pregnancy	51.70% (258/499)	50.29% (613/1219)	0.284	0.594	51.72% (256/495)	49.43% (260/526)	0.534	0.465
Miscarriage	13.19% (34/258)	12.89% (79/613)	0.014	0.907	12.89% (33/256)	11.54 (30/260)	0.220	0.639
Live birth	43.09% (215/499)	42.33% (516/1219)	0.083	0.773	43.23% (214/495)	41.63% (219/526)	0.266	0.903
Multiple pregnancy	12.02% (31/258)	15.01% (92/613)	1.341	0.247	12.11% (31/256)	13.85% (36/260)	0.344	0.557
Birth weight of newborns (kg)	3.00 ± 0.53	3.00 ± 0.53	0.078	0.938	3.00 ± 0.53	3.00 ± 0.52	-0.546	0.585
Birth height of newborns (cm)	49.07 ± 2.66	48.95 ± 2.63	0.529	0.597	49.09 ± 2.65	49.15 ± 2.74	-0.258	0.797
Malformation of newborns	0.93% (2/215)	0.78% (4/516)	/	1.000	0.93% (2/214)	0.91% (2/219)	/	1.000
Moderate/severe OHSS rate	3.57% (33/924)	2.87% (57/1984)	1.025	0.311	3.61% (33/915)	2.51% (23/915)	1.622	0.203

PSM, propensity score matching; OHSS, ovarian hyper-stimulation syndrome.

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TABLE 4 Multivariate regression analysis of the impact of GnRH antagonist type.

	Before PSM			After PSM		
	Coefficient	t	*P*	Coefficient	t	P
OPU number	-0.002	-0.126	0.900	-0.002	-0.105	0.917
Number of usable embryos	0.008	0.429	0.668	0.033	1.412	0.158
Number of good quality embryos	0.018	0.942	0.346	0.038	1.566	0.117
	Wald value	95% CI	*P*	Wald value	95% CI	*P*
OHSS	1.181	0.211, 1.561	0.277	3.309	0.958, 3.174	0.069
Clinical pregnancy	0.363	0.729, 1.182	0.547	1.252	0.647, 1.126	0.263
Live birth	0.001	0.787, 1.279	0.980	0.321	0.697, 1.220	0.571
Multiple pregnancy	3.642	0.987, 2.732	0.056	2.134	0.853, 2.967	0.144
Miscarriage	0.182	0.542, 1.481	0.669	0.402	0.443, 1.515	0.526

PSM, propensity score matching; OPU, oocytes pick up; OHSS, ovarian hyper-stimulation syndrome; 95% CI, 95% confidential interval.

TABLE 5 Subgroup analysis of reproductive outcomes from fixed and flexible protocol of GnRH antagonist.

	Before PSM				Before PSM			
Fixed protocol	Group A	Group B	χ^2	P	Group A	Group B	χ^2	P
N	342	706			341	328		
Implantation	40.96% (111/271)	39.18% (250/638)	0.250	0.617	40.96% (111/271)	40.81% (111/282)	0.147	0.702
Biochemical pregnancy	2.99% (6/201)	4.54% (21/463)	0.864	0.353	2.99% (6/201)	4.46% (9/202)	0.608	0.436
Clinical pregnancy	50.25% (101/201)	47.95% (222/463)	0.297	0.586	50.25% (101/201)	47.52% (96/202)	0.299	0.584
Miscarriage	13.86% (14/101)	9.91% (22/222)	1.094	0.295	13.86% (14/101)	6.25% (6/96)	3.126	0.077
Live birth	40.30% (81/201)	39.96% (185/463)	0.007	0.934	40.30% (81/201)	42.57% (86/202)	0.215	0.643
Moderate/severe OHSS rate	4.09% (14/342)	2.55% (18/706)	1.855	0.173	3.81% (13/341)	1.83% (6/328)	2.383	0.123
multiple pregnancy	11.88% (12/101)	13.06% (29/222)	0.087	0.767	11.88% (12/101)	16.67% (16/96)	0.924	0.336
Flexible protocol								
N	582	1278			574	587		
Implantation	43.50% (174/400)	42.97% (449/1045)	0.924	0.336	43.65% (172/394)	41.26% (184/446)	0.493	0.483
Biochemical pregnancy	4.70% (14/298)	4.89% (37/756)	0.018	0.894	4.76% (14/294)	5.56% (18/324)	0.198	0.657
Clinical pregnancy	52.68% (157/298)	51.72% (391/756)	0.080	0.778	52.72% (155/294)	50.62% (164/324)	0.273	0.601
Miscarriage	12.74% (20/157)	14.58% (57/391)	0.314	0.575	12.26% (19/155)	14.63% (24/164)	0.386	0.535
Live birth	42.62% (127/298)	41.93% (317/756)	0.924	0.336	44.22% (130/294)	41.05% (133/324)	0.633	0.426
Moderate/severe OHSS rate	3.26% (19/582)	3.05% (39/2178)	0.06	0.806	3.31% (19/574)	2.90% (17/587)	0.166	0.684

PSM, propensity score matching; N, number; OHSS, ovarian hyper-stimulation syndrome.

TABLE 6 Multivariate regression analysis of fixed and flexible protocol of GnRH antagonist.

	Before PSM			After PSM		
Fixed protocol	Coefficient	t	*P*	Coefficient	t	P
OPU number	0.003	0.126	0.900	-0.002	-0.061	0.951
Number of usable embryos	-0.020	-0.780	0.436	0.008	0.237	0.813
Number of good quality embryos	0.029	0.968	0.333	0.039	1.037	0.300
	Wald value	95% CI	*P*	Wald value	95% CI	*P*
OHSS	0.759	0.097, 2.451	0.384	2.633	0.837, 6.637	0.105
Clinical pregnancy	0.486	0.626, 1.250	0.486	0.388	0.581, 1.325	0.877
Live birth	0.001	0.700, 1.417	0.982	3.531	0.958, 7.604	0.060
Multiple pregnancy	0.189	0.560, 2.485	0.664	2.402	0.798, 6.881	0.121
Miscarriage	2.006	0.266, 1.238	0.157	3.757	0.128, 1.012	0.053
Flexible protocol	Coefficient	t	*P*	Coefficient	t	P
OPU number	0.000	-0.023	0.982	-0.034	-1.444	0.149
Number of usable embryos	0.031	1.576	0.115	0.041	1.826	0.068
Number of good quality embryos	0.023	0.979	0.328	0.044	1.538	0.124
	Wald value	95% CI	*P*	Wald value	95% CI	*P*
OHSS	0.076	0.516, 1.648	0.783	0.163	0.555, 2.444	0.686
Clinical pregnancy	0.521	0.679, 1.196	0.470	0.820	0.610, 1.199	0.365
Live birth	0.296	0.695, 1.228	0.587	0.247	0.420, 1.677	0.619
Multiple pregnancy	1.273	0.783, 2.485	0.259	0.027	0.457, 1.940	0.942
Miscarriage	0.260	0.656, 2.048	0.610	0.307	0.630, 2.286	0.580

PSM, propensity score matching; OPU, oocytes pick up; OHSS, ovarian hyper-stimulation syndrome; 95% CI, 95% confidential interval.

birth rates, with a lower risk of OHSS compared to GnRH agonists (17).

Cetrotide® stands as one of the initial GnRH antagonist preparations approved by the European Medicines Agency (EMA) in Europe. It is used to prevent premature ovulation as part of COS treatment by inhibiting LH secretion (18). The generic product is nearly identical to an existing EMA-approved reference product, showing no meaningful differences in terms of clinical efficacy, side effects, and immunogenicity (19). Fewer clinical trials are required compared to the reference biologics, significantly reducing the cost of generics (19, 20). Despite of the near interchangeability of generics and reference biologic products, the benefit-risk profiles of generics remain unclear due to limited pre-marketing trials on efficacy and safety information (20). Ferpront® is the first generic of Cetrotide® in China and has been utilized in numerous major reproductive centers across the country since its market introduction. However, there is a lack of clinical data regarding the efficacy and safety of Ferpront®. To address clinicians' needs for evidence-based information, we introduce one of the first piece of real-world evidence to compare the clinical efficacy and safety between generic Ferpront® and its original product Cetrotide®.

This comparability study involving the two types of GnRH antagonist extends the understanding of the therapeutic efficacy and safety of GnRH antagonists. Here, we have demonstrated the therapeutic equivalence of Ferpront® and Cetrotide® in controlled ovarian stimulation and reproductive outcomes in infertile women undergoing IVF/ICSI using the GnRH antagonist protocol. Patients receiving these two types of GnRH antagonists showed comparable numbers of 2PN embryos, usable embryos, and good-quality embryos on day 3, as well as similar incidences of moderate/severe OHSS, clinical pregnancy rates, miscarriage rates, and live birth rates.

The present study is powered by the primary outcome, live birth rate, one of the most critical objectives of ART therapy. Other reproductive outcomes, including clinical pregnancy rate and miscarriage rate, were also similar between the two types of GnRH antagonists. The study findings revealed that women in group B yielded similar reproductive outcomes compared to those in group A concerning embryo implantation, clinical pregnancy, miscarriage, and live birth rates. The duration of GnRH antagonist in group B is a little longer than that of group A (5.72 ± 1.47 vs. 6.01 ± 1.42 days before matching, and 5.71 ± 1.48 vs. 6.02 ± 1.41 days after matching), but the differences showed minimal clinical values. However, we observed approximately 0.8 fewer oocytes retrieved in cycles with group B than in group A (14.17 ± 7.30 vs. 14.96 ± 7.75 oocytes). Notably, women in group A showed higher levels of serum E2 on trigger day than in group B, which could probably lead to

more yield of oocytes (21). Although the precise reasons remain unknown, this phenomenon likely holds little clinical significance, given that the fertilization and cleavage rates, as well as the amounts of usable embryos and good-quality embryos on day 3, remained equivalent between the two groups. Multivariate linear regression analysis showed no obvious impact of the type of GnRH antagonist on the number of oocytes retrieved. The duration of GnRH antagonist usage in group B was 0.3 days longer than in group A. Although statistically significant, this difference revealed minimal clinically substantive value, especially when considering the nearly equivalent duration and dose of Gn and the dose of GnRH antagonist. These results were further supported by data from propensity score matching, demonstrating similar effectiveness and safety of the two types of GnRH antagonist.

Likewise, other studies have also demonstrated that most generics/biosimilars do not significantly differ from their originators. For instance, follitropin alfa original (Gonal-f®) and generic (Ovaleap®) showed similar safety and efficacy in infertile ovulatory women undergoing ART (22). Hu et al (23) reported an equivalent effect of Gonal-f® and its generic QL1012. Although there are few reports of GnRH antagonist generics available at present, we believe that there is no significant difference between the generics and the original product of GnRH antagonist.

This study represents one of the initial comparative examinations between Ferpront® and Cetrotide®. It encompassed a substantial number of participants across a wide spectrum of infertile couples. The primary focus on live birth rate as the key endpoint aligns with one of the pivotal goals in assisted reproduction. The inclusion of a relatively large sample from one of the most voluminous reproductive centers, characterized by a standardized treatment regimen, bolsters the study's credibility. Moreover, to mitigate potential selection biases and confounding factors, PSM and multivariate regression analyses were conducted to assess the independent impact of the GnRH antagonist type, further enhancing the solidity of the results. A limitation lies in the retrospective design, and several parameters including the number of mature oocytes retrieved was not analyzed, and possible selection bias cannot be avoided completely. However, despite of this aspect, this real-world study provides valuable insights into the effectiveness and safety of these treatments in routine ART practice.

5 Conclusion

This study supports the conclusion that there are no clinically significant differences between Ferpront® and Cetrotide® concerning clinical efficacy and safety when used in GnRH antagonist protocols for COS. The study's results indicate the therapeutical equivalence and safety alignment of Ferpront® and Cetrotide®.

Data availability statement

The raw data underlying this article will be shared on reasonable request to the corresponding author.

Ethics statement

The studies involving humans were approved by the local hospital ethics committee (Approval number is 2023-121). 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

MC: Data curation, Formal analysis, Funding acquisition, Writing – original draft, Writing – review & editing. YH: Formal analysis, Writing – review & editing. JX: Formal analysis, Writing – review & editing. SL: Data curation, Funding acquisition, Writing – review & editing. YL: Data curation, Writing – review & editing. JL: Data curation, Funding acquisition, Investigation, Writing – review & editing. HL: Conceptualization, 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1358278/full#supplementary-material

SUPPLEMENTARY TABLE 1
SMD of ovarian reserve markers before and after PSM. Denotes; PSM, propensity score matching; SMD, standardized mean difference; n, number; AMH, anti-Mullerian hormone; FSH, follicular stimulating hormone; AFC, antral follicle counting.

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EDITED BY
Emanuele Garzia,
Santi Paolo e Carlo Hospital, Italy

REVIEWED BY
Huihang Wang,
First Affiliated Hospital of Fujian Medical University, China
Da Li,
China Medical University, China

*CORRESPONDENCE
Shaodi Zhang
✉ zhangshd@126.com
Cuilian Zhang
✉ luckyzcl@qq.com

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The effect of gonadotropin-releasing hormone agonist downregulation in conjunction with hormone replacement therapy on endometrial preparation in patients for frozen–thawed embryo transfer

Haoying Hao, Meng Li, Cuilian Zhang* and Shaodi Zhang*

Reproductive Medicine Center, Henan Provincial People's Hospital, People's Hospital of Zhengzhou University, Zhengzhou, China

Objective: To investigate the effects of combining gonadotropin-releasing hormone agonist (GnRHa) downregulation with hormone replacement therapy (HRT, GnRHa-HRT) on the clinical outcomes of patients undergoing frozen–thawed embryo transfer (FET).

Methods: In this retrospective study, we included patients who had FET between January 2018 and December 2022. They were categorized into HRT and GnRHa-HRT groups based on the endometrial preparation protocol. The study compared the clinical outcomes of patients in two groups. Possible factors affecting clinical outcomes were analyzed using univariate analysis. To analyze the impact of two endometrial preparation methods on clinical outcomes, multifactorial logistic regression was performed.

Results: The rates of clinical pregnancy (47.31% vs. 59.60%), embryo implantation (37.58% vs. 49.65%), biochemical pregnancy (52.36% vs. 64.31%), and early abortion (7.07% vs. 10.77%) were statistically different between the two groups ($p < 0.05$). Analysis using multifactorial logistic regression showed that there was a 1.65-fold increase in clinical pregnancy rates (OR = 1.65, 95% CI: 1.29–2.12, $p < 0.001$) and a 1.55-fold increase in embryo implantation rates (OR = 1.55, 95% CI: 1.27–1.90, $p < 0.001$) in the GnRHa-HRT group when compared to the HRT group. For blastocyst transfer, the clinical pregnancy and implantation rates of the GnRHa-HRT group were significantly higher than those of the HRT group (OR = 1.75, 95% CI: 1.30–2.37, $p < 0.001$; OR = 1.73, 95% CI: 1.35–2.21, $p < 0.001$).

Conclusion: In FET cycles, leuprorelin (as a GnRHa) downregulation combined with HRT may improve the clinical outcome of patients compared to the HRT cycle, especially for the clinical pregnancy and embryo implantation rates of patients with blastocyst transfer.

KEYWORDS

frozen–thawed embryo transfer, GnRHa, HRT, leuprorelin, clinical outcomes

1 Introduction

Frozen–thawed embryo transfer (FET) has steadily grown in importance as a supplementary technique in the advancement of human-assisted reproductive technology due to its simplicity of operation and high safety features (1). Endometrial preparation is a critical stage of the FET cycle, which is crucial to the success of embryo implantation and influences the pregnancy outcomes following the transfer. It is available in several protocols, including natural, ovulation promotion, hormone replacement therapy (HRT), and downregulation of the HRT cycles (2).

For different endometrial preparation protocols, the natural cycle is simple, economical, and suitable for patients with normal ovulation. Patients with irregular menstruation and ovulation disorders are suitable for the ovulation promotion cycle. The HRT cycle is suitable for patients who need to cancel the natural cycle or the ovulation induction cycle for various reasons. Studies have shown that the HRT cycle increases the possibility of pregnancy compared to patients with natural cycles and the same results have been found in patients with a thin endometrium (3).

The combination of gonadotropin-releasing hormone agonist downregulation and HRT (GnRHa-HRT) prevents unexpected ovulation during the HRT cycle (4). In addition, GnRHa has been used for long-term pituitary suppression in FET cycles (5). The affinity of GnRHa to the receptor is much higher than gonadotropin-releasing hormone (GnRH) secreted by the hypothalamic, which can lower pituitary sensitivity and reduce or inhibit the occurrence of spontaneous luteinizing hormone (LH) surge. It not only synchronizes follicular development but also improves the receptivity of the endometrium (6). Leuprorelin, also called leuprolide, is a GnRHa that has been studied for the treatment of endometriosis, adenomyosis, and uterine fibroids (7–9). For patients with FET, uterine conditions are closely related to pregnancy, and leuprorelin is commonly used for uterine disorders or in combination with other medications (10, 11).

For the different endometrial preparation protocols commonly used, studies have shown that there is no significant difference in the HRT cycle or GnRHa-HRT cycle and the latter protocol increases the cost of treatment (12, 13). On the contrary, studies have also shown that GnRHa-HRT effectively improved clinical pregnancy (14) and live birth rates (15). Based on the results of the above studies, some controversies about the advantages and disadvantages of the HRT cycle or GnRHa-HRT cycle in endometrial preparation protocols still exist. Therefore, we retrospectively gathered clinical data from patients who underwent FET after the cancelation of fresh cycle transfer or non-pregnancy after the first embryo transfer due to variable factors such as abnormal endometrium or hormone levels. This research aimed to evaluate the effects of HRT vs. GnRHa-HRT on the clinical outcomes in patients who underwent FET.

2 Materials and methods

2.1 Study design and population

This study was a retrospective cohort analysis conducted on patients who had FET with HRT cycles between January 2018 and December 2022 at the Reproductive Medicine Center of Henan Provincial People's Hospital. The inclusion criteria for patients were as follows: (1) patients who underwent FET after the cancelation of the fresh cycle due to their endometrium or hormone levels, or non-pregnancy after the first embryo transfer; (2) patients who received HRT or GnRHa plus HRT (downregulation + HRT, with leuprorelin). The exclusion criteria were as follows: (1) recurrent miscarriages and repeated implantation failures; (2) frozen eggs and egg recipients; (3) intrauterine adhesions, adenomyosis, uterine fibroids, endometrial polyps, and congenital uterine malformation; (4) either spouse with abnormal chromosomes; (5) presence of internal medical diseases such as diabetes and hypertension. This research was granted ethical approval by the Ethics Committee of Henan Provincial People's Hospital (Approval No. SYSZ-LL-2019110401). Prior to treatment, all patients provided informed consent. Patients were categorized into the HRT group and the GnRHa-HRT group.

2.2 Embryo cryopreservation and thawing

Blastocyst embryos were graded based on the Gardner and Schoolcraft criteria (16), and cleavage-stage embryos were evaluated according to the scoring system as described by Dale et al. (17). All embryos were preserved by vitrification (Vitrification Kit, Kitazato, Japan) and thawed when transferred.

2.3 Endometrial preparation protocol

HRT cycle: Patients orally took estradiol valerate (1 mg/tablet, 4–6 mg/d × 7 days) from the 2nd to 4th day of their menstrual cycle or withdrawal bleeding. After 7 days, ultrasound was used to monitor the endometrial thickness (EMT), and the dose of estradiol valerate was modified accordingly. The maximum dose was 8 mg/day, and the total time of use was 11 to 20 days. When the EMT was measured at least 8 mm or human chorionic gonadotropin (hCG) was administered in the ovulation cycle, the endometrium was transformed with progestogen. The dose of estradiol valerate was kept unchanged, and progesterone was given in the form of a vaginal slow-release gel (90 mg/capsule, 1 capsule/d) in conjunction with oral dydrogesterone tablets (10 mg/tablet, 20 mg/d). Cleavage embryos were transferred 4 days post-transformation, and blastocysts were transferred on the 6th day.

GnRHa-HRT cycle: Between the 2nd and 3rd days of the menstrual cycle, the long-acting GnRHa (leuprorelin, Beijing Biote Pharmaceutical Co., Ltd., 3.75 mg) was subcutaneously injected. When the endothelium was <5 mm and progesterone <1.0 ng/mL after 14–21 days, the estradiol valerate (8 mg/day) was given and was administered for ≥9 days. Endometrium was transformed when EMT was ≥8 mm, cleavage embryos were transferred 4 days post-transformation, and blastocysts were transferred on the 6th day. The luteal support of GnRHa-HRT was the same as HRT.

The serum β-human chorionic gonadotropin (β-hCG) levels were checked 2 weeks following the transfer of the embryo. If the result was positive, the patient would continue taking estradiol valerate and progesterone daily until a fetal heartbeat was seen on ultrasound, and the dosage would be reduced gradually and discontinued by the 10th week of pregnancy.

2.4 Outcome indicators

The primary outcomes included clinical pregnancy and live birth rates. Clinical pregnancy was determined using ultrasound to confirm the presence of a gestational sac 4–6 weeks after the embryo transfer. A live birth refers to the successful delivery of a living baby after 28 weeks of pregnancy. The secondary outcomes were embryo implantation, multiple pregnancy, biochemical pregnancy, and early abortion rates. Successful embryo implantation refers to the interaction between the embryo and the endometrium, and implanting into the endometrium. Multiple pregnancy refers to more than one fetus in a pregnancy. Biochemical pregnancy was characterized as β-hCG >25 IU/L in serum at 12–14 days of transplantation. Early abortion was defined as miscarriage or the cessation of embryonic growth before 12 weeks of gestation.

2.5 Statistical analysis

Statistical analysis was conducted using SPSS 27.0. The Shapiro–Wilk test was utilized to assess normal distribution, and non-normally distributed continuous variables were presented as medians (IQR). Group comparisons were conducted using the Wilcoxon test. Categorical variables were evaluated utilizing either the chi-square test or Fisher's exact test. Possible factors affecting clinical outcomes were analyzed using univariate analysis. The effects of the two endometrial preparation protocols on clinical outcomes were analyzed using multifactorial logistic regression after adjusting for confounding factors. A statistical significance was determined if the $p < 0.05$.

Propensity score matching was used to match the study objects according to HRT and GnRHa-HRT protocols using 1:1 nearest neighbor matching method, and the matching tolerance was set at 0.0005. Female age, male age, endometriosis, anti-Müllerian hormone (AMH), developmental stages of transferred embryos, embryo quality, the number of embryos transferred, and EMT on the day of transfer were used as matching variables. According to the data from our center, it is assumed that the clinical pregnancy rate in the GnRHa-HRT group is 66% (p_1), while in the HRT group it is 56% (p_2). The hypothesis testing is conducted with a type I error (α) set at 0.05 and a type II error (β) at 0.1. The sample size ratio (k) of the two groups is 1:1. Utilizing the sample size formula: $n_2 = (z_{1-\alpha/2} + z_{1-\beta})^2 \times [p_1 (1-p_1)/k + p_2 (1-p_2)]/(p_1-p_2)^2$, $n_1 = k \times n_2$, where $z_{1-\alpha/2} = 1.96$, $z_{1-\beta} = 1.28$, yielding a sample size of 495 for the GnRHa-HRT group and 495 for the HRT group.

3 Results

3.1 Baseline characteristics

There were 9,200 patients in the conventional HRT group who met the criteria and 637 patients in the GnRHa-HRT group. After PSM, there were 594 patients in each of the two groups (Figure 1). The baseline characteristics of the matched patients are shown in Table 1. Statistical differences were observed in the EMT, developmental stages of transferred embryos, and the number of embryos transferred among the two groups ($p < 0.05$). However, there were no significant differences in male factors, female age, female BMI, duration of infertility, presence of endometriosis, infertility types, AMH, fertilization mode, and quality of transferred embryos.

3.2 Clinical outcomes

There were significant differences in the rates of clinical pregnancy (47.31% vs. 59.60%), embryo implantation (37.58% vs. 49.65%), biochemical pregnancy (52.36% vs. 64.31%), and early abortion rates (7.07% vs. 10.77%) between HRT and GnRHa-HRT groups ($p < 0.05$). The differences in the rates of live birth (38.55% vs. 43.10%) and multiple pregnancies (10.94% vs. 12.29%) did not show statistical significance ($p > 0.05$). Furthermore, there were no ectopic pregnancies in the HRT group and two in the GnRHa-HRT group (Table 2).

3.3 Univariate analysis

According to the univariate analysis, male age, female age, duration of infertility, AMH, endometrial preparation protocols, embryo development stage, embryo quality, EMT on the transplantation date, and the presence of endometriosis may influence the clinical pregnancy ($p < 0.05$). Male age, female age, AMH, embryo development stage, embryo quality, and EMT may influence the live birth rate ($p < 0.05$). In addition, male age, female BMI, female age, duration of infertility, AMH, endometrial preparation protocols, embryonic development stage, number of transplanted embryos, EMT on the transplantation date, and the presence of endometriosis may influence embryo implantation ($p < 0.05$) (Table 3).

3.4 Multifactorial logistic regression analysis

In the adjusted model, the results revealed that in the GnRHa-HRT group, the clinical pregnancy and embryo implantation rates were 0.55 times higher (OR = 1.55, 95% CI: 1.27–1.90, $p < 0.001$) and 0.65 times higher (OR = 1.65, 95% CI: 1.29–2.12, $p < 0.001$) than those in the HRT group, respectively. However, there was no significant difference in the live birth rate (OR = 1.21, 95%CI:0.93–1.52, $p = 0.175$) (Table 4).

Furthermore, the embryo implantation and clinical pregnancy rates were also significantly higher with GnRHa-HRT in the group of patients who were transferred blastocyst-stage embryos (OR = 1.73, 95% CI: 1.35–2.21, $p < 0.001$; OR = 1.75, 95% CI: 1.30–2.37, $p < 0.001$). However, in the group of patients transferred with cleavage-stage embryos, there was no significant difference (OR = 1.18, 95% CI: 0.72–1.91, $p = 0.513$; OR = 1.07, 95% CI: 0.73–1.56, $p = 0.728$). Meanwhile, in the groups of blastocyst-stage embryos transferred and cleavage-stage embryos transferred, there were no significant differences in the live birth rate between HRT and GnRHa-HRT (Table 5).

4 Discussion

The patients involved in this research were those who underwent FET following the cancelation of fresh cycle transfer or non-pregnancy after the initial embryo transfer, attributed to factors such as their endometrium or hormone levels, which is different

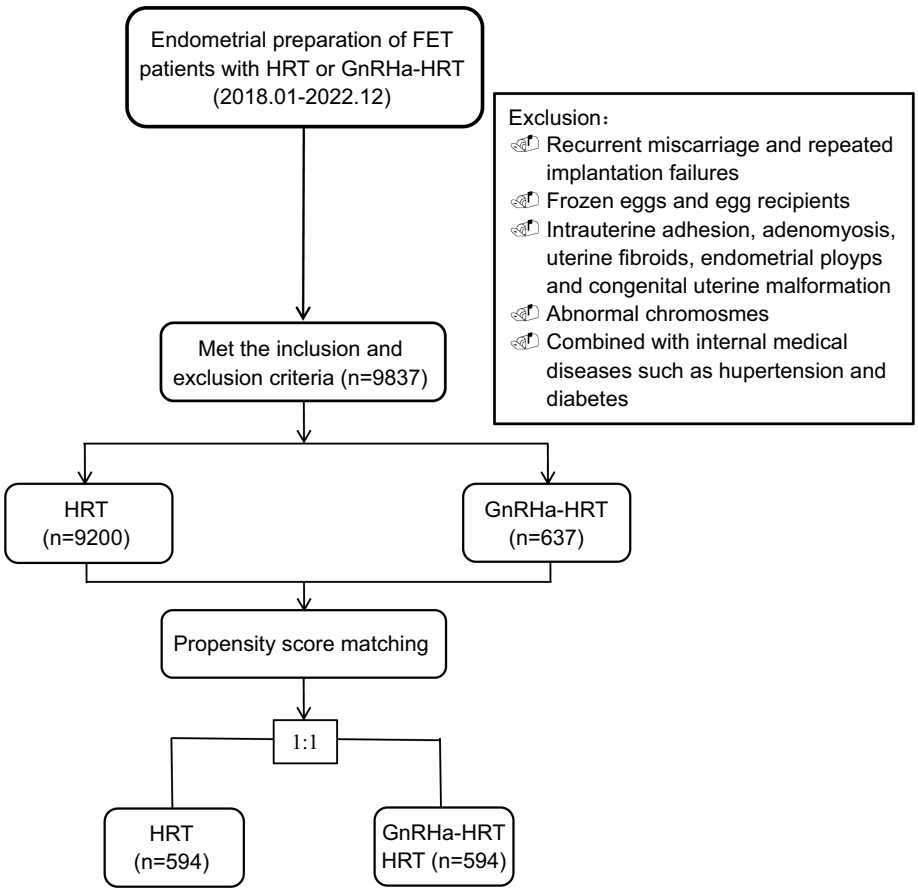

FIGURE 1
Patient inclusion flowchart. FET, frozen–thawed embryo transfer; HRT, hormone replacement therapy; GnRHa-HRT, gonadotropin-releasing hormone agonist downregulation and HRT.

from the inclusion scope of other studies (11, 18, 19). Endometrium factors may include thin endometrium and endometriosis. Hormone levels involve estrogen, progestin, follicle-stimulating hormone, luteinizing hormone, and androgen, and any abnormality of these factors can lead to infertility. Leuprorelin may improve the clinical symptoms of infertility patients by affecting the above hormones and is commonly used in treating endometriosis (20). Previous studies have not examined the clinical outcomes of downregulation with leuprorelin alone in the same range as included in this study.

This research included patients over a period of nearly 5 years and had relatively complete baseline data. The PSM was performed on the study population according to the HRT and GnRHa-HRT protocols using the 1:1 nearest neighbor matching method to avoid potential confounders and selection bias. The reliability of the results was therefore enhanced. EMT affects endometrial receptivity, some studies have shown better FET outcomes with the EMT > 7 mm. The rate of clinical pregnancy for an EMT of 7 mm or less is 23.3%, which is significantly lower than the 48.1% rate observed in cases where the EMT exceeds 7 mm (21). Liu et al. also found that patients with an EMT < 8 mm exhibited a heightened likelihood of giving birth to infants classified as small for gestational age (22). Therefore, the threshold for EMT on the day of transformation was set at 8 mm in this study.

Currently, studies have shown that the GnRHa-HRT cycle has a positive therapeutic effect on FET patients with thin endometrium or adenomyosis (23, 24). It can also improve the reproductive outcomes for older patients (aged 36–43 years) who in experience recurrent implantation failure in FET cycles (25). Prior treatment with GnRHa in FET can enhance the chances of successful clinical pregnancy, live birth, and implantation, particularly in individuals who have experienced multiple failed implantation attempts (26). A study also showed that there were no statistically significant differences in clinical pregnancy, live birth, abortion, multiple pregnancy, and biochemical pregnancy rates in patients with endometriosis between the two protocols (12). Our results showed that the GnRHa-HRT cycle could improve clinical pregnancy, live birth rate, and embryo implantations, although there were no statistically significant differences in the rate of live birth. In addition, the biochemical pregnancy and early abortion rates of GnRHa-HRT are significantly higher than HRT, but the sample size of miscarriages was small. Overall, GnRHa-HRT showed better clinical outcomes.

Multifactorial logistic regression analysis showed that the clinical pregnancy rate was significantly higher in the GnRHa-HRT group than in the HRT group although there was no significant difference in the live birth rate. As successful embryo implantation is also important for clinical outcomes, we also performed further analysis of embryo implantation, and the results showed that the implantation rate was higher in the GnRHa-HRT group. Although there have been previous studies comparing GnRHa-HRT and HRT, there is a lack of stratified

TABLE 1 Baseline characteristics of patients with HRT cycle and GnRHa-HRT cycle [Mean (SD) Median (Q1-Q3) / *N* (%)].

Variable	HRT (*n* = 594)	GnRHa-HRT (*n* = 594)	*p*-value
Age, years	31 (28, 36)	31 (29, 35)	0.547
BMI, kg/m^2	23 (20.8, 25.4)	22.58 (20.69, 25.28)	0.136
Male factors			
Age, year	32 (28, 37)	32 (29, 35)	0.853
BMI, kg/m^2	25.08 (22.86, 27.51)	25.25 (22.86, 27.68)	0.737
Endometriosis, *n* (%)			0.560
Yes	37 (6.23%)	42 (7.07%)	
No	557 (93.77%)	552 (92.93%)	
Duration of infertility, years	3 (2, 5)	3 (1.5, 5)	0.123
Infertile type, *n* (%)			0.680
Secondary infertility	343 (57.74%)	350 (58.92%)	
Primary infertility	251 (42.26%)	244 (41.08%)	
AMH, ng/ml	3.36 (1.64, 5.54)	3.5 (1.85, 5.7)	0.244
EMT, cm	9.6 (8.8, 10.78)	10 (9, 11)	0.009
Mode of fertilization, *n* (%)			0.787
IVF	448 (75.42%)	452 (76.09%)	
ICSI	146 (24.58%)	142 (23.91%)	
Type of embryo transferred, *n* (%)			0.004
Cleavage-stage embryo	207 (34.85%)	161 (27.1%)	
Blastocyst-stage embryo	387 (65.15%)	433 (72.9%)	
No. of embryos transferred			<0.001
1	270 (45.45%)	334 (56.23%)	
2	324 (54.55%)	260 (43.77%)	
Embryo quality, *n* (%)			0.448
High	334 (56.23%)	321 (54.04%)	
Not high	260 (43.77%)	273 (45.96%)	

HRT, hormone replacement therapy; GnRHa-HRT, gonadotropin-releasing hormone agonist downregulation and HRT; BMI, body mass index; AMH, anti-Müllerian hormone; EMT, endometrial thickness; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection.

TABLE 2 Clinical outcomes of patients with HRT cycle and GnRHa-HRT cycle.

Variable	HRT (*n* = 594)	GnRHa-HRT (*n* = 594)	*P*-value
Primary outcome indicators			
Clinical pregnancy, *n* (%)			<0.001
Yes	281 (47.31%)	354 (59.60%)	
No	313 (52.69%)	240 (40.40%)	
Live birth rate			0.111
Yes	229 (38.55%)	256 (43.10%)	
No	365 (61.45%)	338 (56.90%)	
Secondary outcome indicators			
Embryo implantation, *n* (%)			<0.001
Yes	345 (37.58%)	424 (49.65%)	
No	573 (62.42%)	430 (50.35%)	
Biochemical pregnancy, *n* (%)			<0.001
Yes	311 (52.36%)	382 (64.31%)	
No	283 (47.64%)	212 (35.69%)	
Multiple pregnancy, *n* (%)			0.469
Yes	65 (10.94%)	73 (12.29%)	
No	529 (89.06%)	521 (87.71%)	
Early abortion, *n* (%)			0.025
Yes	42 (7.07%)	64 (10.77%)	
No	552 (92.93%)	530 (89.23%)	

analysis of embryo transfer types for the two protocols. Some studies have shown that transferring embryos at the blastocyst stage leads to a higher pregnancy success rate compared to transferring embryos at the cleavage stage (27, 28). To avoid the impact of differences in embryo type transferred on clinical outcomes, this study compared the outcomes of transferring blastocyst-stage and cleavage-stage embryos. We found that patients who received blastocyst-stage embryos, not cleavage-stage embryos, had significantly higher rates of clinical pregnancy and embryo implantation in the GnRHa-HRT cycle group compared to the HRT group, although there was no significant difference in the live birth rate.

Previous studies had inconsistent conclusions about the two protocols, which may stem from differences in study populations and methodology between different research teams, and the limitation of the sample size is also an influencing factor. Our study had a broader inclusion, was not limited to a single cause of disease, and had a relatively large sample size, further providing confirmation and supplement for favorable clinical outcomes in the GnRHa-HRT cycle. In previous studies, GnRHa demonstrated a notable ability to decrease the inflammatory response and formation of new blood vessels in women with endometriosis, uterine myoma, and adenomyosis (29). GnRHa may have a direct immunomodulatory effect by disrupting the imbalance between Th17 and Treg cells, thereby enhancing endometrial receptivity (30). In addition, it can enhance endometrial receptivity by enhancing the expression of αγβ3 integrin in the endometrium or through IL-6 and IL-11 expression levels of endometrial stromal cells regulated by the miR-124-3p, and increase the number of pinopodes to favor embryo implantation (31, 32). These may account for the favorable clinical outcomes of the GnRHa-HRT protocol.

Meanwhile, as a retrospective study, this study also has some limitations. Since the protocols are decided by physicians and based on patient characteristics, differences in initial parameters between these two groups are inevitable. A significant difference in the mean EMT between the two groups was observed (9.6 mm vs. 10 mm). However, this difference was considered clinically non-significant, as most previous studies have defined an EMT of more than 8 mm as ideal. Second, some confounding factors may inevitably affect the statistical analysis results, although the confounding factors were adjusted, and stratified analysis was performed based on the type of embryo transferred. Furthermore,

TABLE 3 Univariate analysis affecting embryo implantation rate, clinical pregnancy rate, and live birth rate in thawing cycles.

Variable	Clinical pregnancy rate		Live birth rate		Embryo implantation rate	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Age	0.91 (0.89, 0.93)	<0.001	0.90 (0.88, 0.92)	<0.001	0.91 (0.89, 0.93)	<0.001
BMI	0.97 (0.94, 1.01)	0.112	0.98 (0.95, 1.02)	0.32	0.97 (0.94, 1.00)	0.026
Male factors						
Male age	0.93 (0.91, 0.95)	<0.001	0.92 (0.90, 0.94)	<0.001	0.93 (0.91, 0.95)	<0.001
Male BMI	1 (0.97, 1.03)	0.993	1.01 (0.98, 1.04)	0.690	0.99 (0.96, 1.01)	0.290
Duration of infertility	0.95 (0.92, 0.99)	0.010	0.97 (0.93, 1.00)	0.084	0.94 (0.91, 0.97)	<0.001
Infertile type						
Secondary infertility	Ref		Ref		Ref	
Primary infertility	0.86 (0.68, 1.09)	0.212	0.88 (0.69, 1.11)	0.277	0.84 (0.7, 1.02)	0.083
AMH	1.09 (1.05, 1.12)	<0.001	1.08 (1.04, 1.11)	<0.001	1.08 (1.05, 1.12)	<0.001
Endometrial preparation protocols						
HRT	Ref		Ref		Ref	
GnRHa-HRT	1.64 (1.31, 2.07)	<0.001	1.21 (0.96, 1.52)	0.111	1.64 (1.36, 1.98)	<0.001
Developmental stages of transferred embryos						
Cleavage-stage embryo	Ref		Ref		Ref	
Blastocyst-stage embryo	1.55 (1.21, 1.98)	0.001	1.33 (1.03, 1.71)	0.028	1.9 (1.56, 2.32)	<0.001
Number of embryos transferred						
1	Ref		Ref		Ref	
2	1.19 (0.95, 1.5)	0.135	1.26 (1.00, 1.59)	0.050	0.61 (0.5, 0.75)	<0.001
Embryo quality						
High	Ref		Ref		Ref	
Not high	0.73 (0.58, 0.92)	0.007	0.73 (0.57, 0.92)	0.007	0.87 (0.72, 1.05)	0.157
EMT	1.2 (1.12, 1.29)	<0.001	1.18 (1.10, 1.26)	<0.001	1.18 (1.12, 1.25)	<0.001
Mode of fertilization						
IVF	Ref		Ref		Ref	
ICSI	1.14 (0.87, 1.49)	0.338	1.11 (0.85, 1.45)	0.455	1.14 (0.92, 1.42)	0.230
Endometriosis						
Yes	Ref		Ref		Ref	
No	0.61 (0.38, 0.98)	0.042	0.73 (0.46, 1.15)	0.175	0.69 (0.47, 1)	0.050

BMI, body mass index; AMH, anti-Müllerian hormone; HRT, hormone replacement therapy; GnRHa-HRT, gonadotropin-releasing hormone agonist downregulation and HRT; EMT, endometrial thickness; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection.

TABLE 4 Multifactorial logistic regression analysis of the effects of different endometrial preparation protocols on embryo implantation rate, clinical pregnancy rate, and live birth rate.

Variable	Clinical pregnancy rate				Live birth rate				Embryo implantation rate			
	Unadjusted		Adjusted		Unadjusted		Adjusted		Unadjusted		Adjusted	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
HRT	Ref		Ref		Ref		Ref		Ref		Ref	
GnRHa-HRT	1.64 (1.31, 2.07)	<0.001	1.65 (1.29, 2.12)	<0.001	1.21 (0.96, 1.52)	0.111	1.19 (0.93, 1.52)	0.175	1.64 (1.36, 1.98)	<0.001	1.55 (1.27, 1.90)	<0.001

[a]Adjustment: male/female age, male/female BMI, endometriosis, AMH, developmental stages of transferred, number of embryos transferred, EMT, duration of infertility, infertility type, and fertilization method.

TABLE 5 Multifactorial logistic regression analysis of the effects of endometrial preparation protocols on embryo implantation rate, clinical pregnancy rate, and live birth rate of different developmental stages of embryos transferred.

Variable	Clinical pregnancy rate				Live birth rate				Embryo implantation rate			
	Unadjusted		Adjusted		Unadjusted		Adjusted		Unadjusted		Adjusted	
	OR (95%CI)	*P*-value	OR (95%CI)	*p*-value	OR (95%CI)	*p*-value	OR (95%CI)	*p*-value	OR (95%CI)	*p*-value	OR (95%CI)	*p*-value
Cleavage-stage group												
HRT	Ref		Ref		Ref		Ref		Ref		Ref	
GnRHa-HRT	1.43 (0.95,2.17)	0.09	1.18 (0.72,1.91)	0.513	1.20 (0.78, 1.84)	0.404	1.14 (0.69, 1.90)	0.609	1.33 (0.96,1.85)	0.09	1.07 (0.73,1.56)	0.728
Blastocyst-stage group												
HRT	Ref		Ref		Ref		Ref		Ref		Ref	
GnRHa-HRT	1.68 (1.27, 2.22)	<0.001	1.75 (1.30, 2.37)	<0.001	1.18 (0.89, 1.55)	0.251	1.16 (0.87, 1.56)	0.315	1.67 (1.32, 2.11)	<0.001	1.73 (1.35, 2.21)	<0.001

[a]Adjustment: male/female age, male/female BMI, endometriosis, AMH, number of embryos transferred, EMT, duration of infertility, infertility type, and fertilization method.

additional future studies that are prospective and randomized are necessary to confirm the optimal protocol for FET cycles.

5 Conclusion

In FET cycles, leuprorelin (as a GnRHa) downregulation combined with HRT may be effective in improving the clinical outcome of patients compared to the HRT cycle. This combination may be more beneficial for patients undergoing blastocyst transfer as it can increase the chances of clinical pregnancy and successful embryo implantation.

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 the Ethics Committee of Henan Provincial People's 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

HH: Data curation, Formal analysis, Investigation, Software, Writing – original draft. ML: Data curation, Investigation, Writing – original draft. CZ: Data curation, Methodology, Supervision, Validation, Writing – original draft. SZ: Data curation, Methodology, Supervision, Validation, Writing – original draft, Formal analysis, Investigation, 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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EDITED BY
Jan Tesarik,
MARGen Clinic, Spain

REVIEWED BY
Qingxue Zhang,
Sun Yat-sen Memorial Hospital, China
Martin Stimpfel,
University Medical Centre Ljubljana, Slovenia

*CORRESPONDENCE
Guimin Hao
haoguiminhgm@163.com

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Risk factors, management, and future fertility of empty follicle syndrome: a retrospective study with real-world data

Zhuoye Luo[1,2,3,4], Suxin Xu[1,2,3,4] and Guimin Hao[1,2,3,4]*

[1]Department of Reproductive Medicine, The Second Hospital of Hebei Medical University, Shijiazhuang, China, [2]Hebei Center for Quality Control and Management of Human Assisted Reproductive Technology, Shijiazhuang, China, [3]Hebei Key Laboratory of Infertility and Heredity, Shijiazhuang, China, [4]Hebei Clinical Research Center for Birth Defects, Shijiazhuang, China

Background: Empty follicle syndrome (EFS) is a challenging clinical problem. This study aims to identify the risk factors for EFS, to present pregnancy outcomes in both EFS cycle as well as subsequent cycles, and to summarize an effective rescue protocol to improve outcomes.

Methods: A retrospective analysis between 2016 and 2020 was conducted at our center. Stricter criteria were applied to diagnose EFS. Logistic regression analysis was used to identify the risk factors for EFS. Further analyses were performed within the EFS cycle to present pregnancy outcomes and to find optimal rescue protocols. Long-term follow-up was conducted until live birth was achieved, covering at least two complete oocyte retrieval cycles.

Results: Among 14,066 patients, 54 (0.38%) were identified as EFS. Patients with polycystic ovary syndrome (PCOS) had a significantly higher risk of developing EFS than non-PCOS ones (aOR = 2.67; 95% CI, 1.47 to 4.83). Within EFS patients, delaying the second oocyte retrieval by 3–6 h significantly improved the rates of obtaining oocyte (97.4% *versus* 58.3%, $P = 0.002$), getting embryo available for transfer (92.3% *versus* 33.3%, $P < 0.001$), and pregnancy (48.7% *versus* 8.3%, $P = 0.017$) compared to other delayed retrieval times. Overall, 31.5% (17/54) and 46.7% (7/15) EFS patients achieved live birth in the EFS cycle and the future cycle, respectively.

Conclusions: PCOS is an independent risk factor for EFS, indicating that longer exposure time to human chorionic gonadotropin (hCG) may be necessary. Delaying the second oocyte retrieval by 3–6 h is an effective rescue protocol for EFS patients to achieve optimal outcomes. EFS in a single cycle does not necessarily indicate future fertility decline, but repeated EFS may result in poor outcomes.

KEYWORDS

empty follicle syndrome, oocyte trigger, hCG exposure time, polycystic ovary syndrome, cumulative live birth rate

1 Introduction

Empty follicle syndrome (EFS) was first described by Coulam et al. in 1986 (1). It is characterized by the failure to retrieve oocytes during repeated follicular aspiration and flushing despite appropriate follicular development and estradiol levels. The prevalence of EFS varies widely, as there is debate over whether low ovarian response patients should be included. Some studies define EFS as a no-oocyte obtained status that fits all patients (2, 3). However, other studies recommend excluding low responders from the definition of EFS (4, 5). They argue that low ovarian response is a sign of ovarian aging and a lower oocyte yield can be anticipated, thus justifying oocyte retrieval failure as a reasonable outcome rather than an indicator of EFS (6, 7). With stricter criteria, the incidence of EFS is estimated to be between 0.045% and 0.59% of cycles (4, 8, 9). Although rare, EFS can cause significant psychological distress for both healthcare providers and patients. Therefore, it is a challenging clinical problem that requires further investigation.

EFS is classified into genuine EFS (GEFS) and false EFS (FEFS) according to whether it has optimal beta human chorionic gonadotropin (β-hCG) levels after hCG injection. FEFS with negligible β-hCG levels indicates the possibility of injection mistake or pharmaceutical problem. However, no consensus has been reached on the etiologies of GEFS. The reasons are commonly speculated to be hCG factor (inadequate hCG exposure time, dosage, or activity) (10), inadequate ovarian response to hCG (11), and genetic or gene mutation factors (12, 13).

Due to the extremely low incidence of EFS, most original studies were reported as case reports. While the meta-analysis or reviews compensated for the inadequate sample size of each study, the significant heterogeneity of each study made it challenging to draw consistent and convincing conclusions about the risk factors, preventive measures, rescue protocols, and prognosis of EFS.

In this study, we applied rigorous EFS diagnostic criteria to analyze a large-sample dataset, aiming at present the risk factors for EFS and pregnancy outcomes in both EFS cycle as well as subsequent cycles. We also aim to summarize an effective rescue protocol to improve oocyte obtainment and pregnancy outcome.

2 Materials and methods

2.1 Study design and patients

This study was approved by the ethics committee of the Second Hospital of Hebei Medical University (No. 2024-R106). There is no requirement for informed consent.

A retrospective analysis was conducted on 21,567 cycles of oocyte retrieval performed in our center between January 2016 and December 2020. The study included patients with adequate follicular development in their gonadotropin-releasing hormone (GnRH) antagonist or GnRH agonist downregulation protocols triggered using hCG. Adequate follicular development was regarded as the presence of at least four follicles with a diameter of ≥14 mm, including at least two follicles with a diameter of ≥18 mm on the trigger day (4). EFS was defined as no oocyte obtained after repeated aspiration and flushing despite adequate follicular development. Repeated cycles, cycles with female abnormal chromosomes, and those who underwent preimplantation genetic testing (PGT) were excluded. Repeated cycles refer to multiple cycles with oocyte retrieval for one patient during the analysis. Finally, 14,066 patients were eligible, and among them, 54 cases of EFS were identified.

2.2 Procedures

Ovarian stimulation was routinely performed as we have mentioned previously (14). Oocyte retrieval was initiated 36–38 h after administering 6,500–10,000 IU of hCG (which included urinary hCG (u-hCG) and/or recombinant hCG (r-hCG); 250 μg of r-hCG was equivalent to 6,500 IU of u-hCG) to induce ovulation when at least two follicles with a diameter of ≥18 mm were present. Insemination was determined based on the infertility reason. Embryos got transferred or vitrified cryopreservation 3–5 days following oocyte retrieval. Embryo culture and luteal phase support were routinely conducted (15).

2.3 Rescue protocol

Oocyte retrieval was interrupted immediately if no oocyte was obtained after thorough aspiration and flushing during unilateral or six to eight follicles ≥14 mm in mean diameter puncture. Urinary β-hCG was then tested (8). If the urinary β-hCG was positive, and the average E_2 levels per follicle ≥14 mm was less than 200 pg/mL as well as the number of such follicles was less than 15 on the trigger day, an additional 2,000–4,000 IU of hCG was administered, or there was no additional hCG injection otherwise. The second oocyte retrieval was delayed, varying from 1.6 to 7.1 h depending on the actual situation. If the urinary β-hCG was negative, blood β-hCG was tested to determine rescue hCG injection dosage and the second oocyte retrieval time. When no oocytes were retrieved during the EFS cycle, change in stimulation protocol, higher hCG dosage, and longer hCG exposure time were considered in the next cycle.

2.4 Pregnancy outcomes

Clinical pregnancy was confirmed by visualization of an intrauterine gestational sac with transvaginal ultrasound 4 to 5 weeks after embryo transfer. Miscarriage was defined as suffering pregnancy loss before 28 weeks of gestation after achieving clinical pregnancy. Live birth was defined as delivering at least one living child.

2.5 Statistical analysis

SPSS version 26.0 software package (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Continuous variables were expressed as mean ± standard deviation (mean ± SD) or median ±

quartile range (median ± QR) according to distribution, with Student's *t*-test or paired *t*-test for comparison in normal distribution and Mann–Whitney *U*-test or Wilcoxon paired test for comparison in non-normal distribution. Pearson's chi-square analysis or Fisher's exact test was applied in categorical variables. Logistic regression analysis was conducted to manifest the risk factors for EFS. Univariate logistic regression was conducted, and five variables with *P*-value less than 0.1 were included in the multivariate analysis. The sample size of EFS was 51, which met the minimum standard of 10 events per variable (EPV) to fit a model (16). When performing self-controlled comparison between EFS cycles and their normal cycles, paired *t*-test or Wilcoxon paired test was used to compare continuous variables and McNemar's test was used to compare categorical variables. A two-sided *P*-value <0.05 means statistically significant.

3 Results

A total of 14,066 patients were eligible, and 54 (0.38%) of them were identified as EFS (Figure 1).

3.1 Etiology of EFS

The causes of EFS were analyzed according to clinical manifestations and medical history. Specifically, one case was attributed to an hCG injection mistake, two cases were due to oocyte developmental disorder, and inadequate ovarian response to hCG was suspected in 51 other cases (Figure 1) (Supplementary Table S1).

Urinary β-hCG was found to be negative in only one patient. On the day of oocyte retrieval, her blood levels of β-hCG, luteinizing hormone (LH), and progesterone (P) were 1.4 IU/L, 4.58 IU/L, and 1.38 ng/mL, respectively. The patient self-reported a shallow hCG injection with the skin surface moist. We categorized this as an hCG injection error, and a second oocyte retrieval was performed 36 h later following the administration of 10,000 IU of hCG for rescue. The patient successfully obtained oocytes and achieved a live birth.

Two patients had suffered EFS during a previous cycle. All oocytes obtained were in metaphase I (MI) stage with abnormal zona pellucida. They were both primary infertility cases and denied a family history of infertility. We classified the condition as oocyte

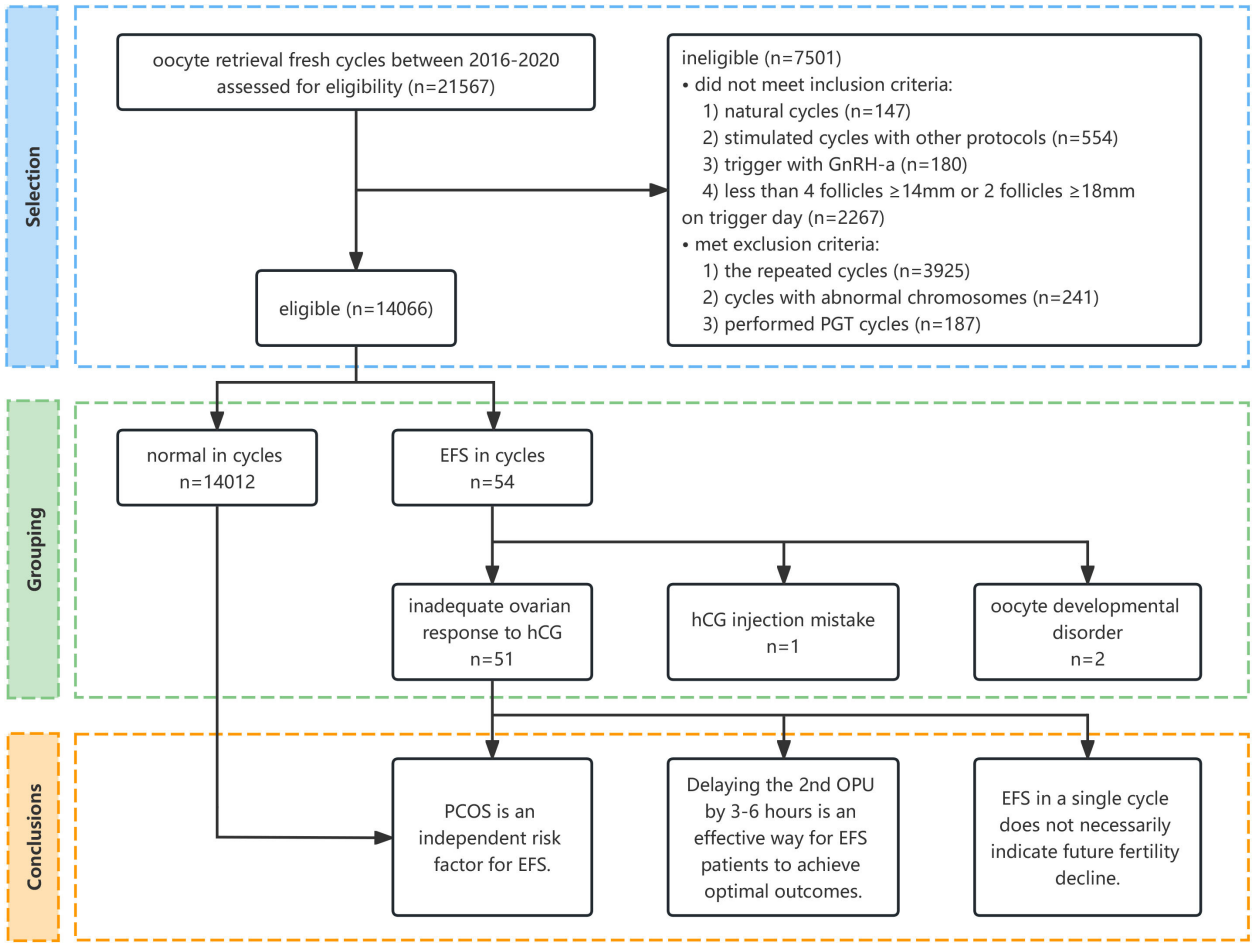

FIGURE 1
Flow diagram of the selection process and conclusion. GnRH-a, gonadotropin-releasing hormone agonist; PGT, preimplantation genetic testing; EFS, empty follicle syndrome; hCG, human chorionic gonadotropin; PCOS, polycystic ovary syndrome; OPU, oocyte pick-up.

developmental disorder and suspected genetic abnormalities, but the patients declined further genetic testing.

We could not find an obvious reason for EFS in the other 51 cases, so they were classified into inadequate ovarian response to hCG tentatively.

3.2 Risk factors for EFS

The EFS group had a higher body mass index (BMI) (25.3 ± 4.4 *versus* 23.6 ± 3.6, $P = 0.001$), a higher proportion of PCOS (45.1% *versus* 18.6%, $P < 0.001$), a higher proportion of downregulated protocols (86.3% *versus* 77.7%, $P = 0.004$), and a lower E_2 per ≥14-mm follicle (265.5 ± 99.3 *versus* 333.4 ± 118.9, $P < 0.001$) than the group with normal situation for oocyte retrieval (non-EFS group). However, there were no significant differences in other factors between the two groups. After adjusting for confounding factors, PCOS was found to be a significant risk factor for EFS (aOR = 2.67; 95% CI, 1.47 to 4.83) (Table 1).

3.3 Optimal delayed time for second oocyte retrieval

We classified the delayed time by each hour and analyzed the clinical outcomes of each group. Subsequently, we merged the groups into two categories, namely, the 3–6h group and the non-3–6h group. Although the demographic data and the number of ≥14 mm follicles on the trigger day were similar in both groups, patients in the 3–6h group showed a significantly higher rate of obtaining oocyte (97.4% *versus* 58.3%, $P = 0.002$), obtaining embryo available for transfer (92.3% *versus* 33.3%, $P < 0.001$), and achieving pregnancy (48.7% *versus* 8.3%, $P = 0.017$) compared with those in the non-3–6h group (Table 2).

TABLE 1 Risk factors for EFS.

	Non-EFS (n = 14,012)	EFS (n = 51)[a]	Crude OR (95% CI)	P	Adjusted OR (95% CI)	P
Age (years)	30.5 ± 4.2	29.9 ± 3.1	0.97 (0.90, 1.00)	0.315	–	–
BMI (kg/m^2)	23.6 ± 3.6	25.3 ± 4.4	1.13 (1.05, 1.21)	0.001	1.07 (0.99, 1.16)	0.064
Basal FSH (IU/L)	7.1 ± 2.2	6.7 ± 1.4	0.91 (0.79, 1.05)	0.192	–	–
Basal LH (IU/L)	6.9 ± 3.3	7.4 ± 4.1	1.04 (0.96, 1.12)	0.315	–	–
Infertility types				0.790	–	–
Primary infertility	7,707 (55.0%)	29 (56.9%)	Reference			
Secondary infertility	6,305 (45.0%)	22 (43.1%)	0.93 (0.53, 1.62)			
Infertility factors				<0.001		0.001
Non-PCOS	11,400 (81.4%)	28 (54.9%)	reference		reference	
PCOS	2,612 (18.6%)	23 (45.1%)	3.59 (2.06, 6.23)		2.67 (1.47, 4.83)	
Protocols				0.004		0.164
GnRH-ant	3,122 (22.3%)	7 (13.7%)	reference		reference	
GnRH-a long	5,023 (35.8%)	30 (58.8%)	2.66 (1.17, 6.07)		2.07 (0.89, 4.79)	
GnRH-a short	5,867 (41.9%)	14 (27.5%)	1.06 (0.43, 2.64)		1.31 (0.52, 3.26)	
Gn dosage per day (IU)	232.4 ± 74.9	230.1 ± 60.7	1.00 (0.99, 1.01)	0.784	–	–
E_2 per ≥14-mm follicle (pg/mL)	333.4 ± 118.9	265.5 ± 99.3		<0.001		0.065
<200	1,789 (13.3%)	14 (27.5%)	2.47 (1.34, 4.59)		1.84 (0.96, 3.50)	
≥200	11,697 (86.7%)	37 (72.5%)	Reference		Reference	
hCG types				0.376	–	–
Urinary hCG	4,411 (31.5%)	19 (37.3%)	Reference			
Recombined hCG	9,601 (68.5%)	32 (62.7%)	0.77 (0.44, 1.37)			
hCG dosage (IU)/1,000	7.7 ± 1.1	8.0 ± 1.2	1.26 (0.99, 1.61)	0.063	1.26 (0.98, 1.60)	0.067
hCG exposure time (h)	36.6 ± 0.7	36.5 ± 0.5	0.76 (0.52, 1.11)	0.152	–	–

Data are presented as either means ± SD or number (%).
EFS, empty follicle syndrome; BMI, body mass index; FSH, follicle-stimulating hormone; PCOS, polycystic ovary syndrome; GnRH-a, gonadotropin-releasing hormone agonist; Gn, gonadotropin; hCG, human chorionic gonadotropin.
[a]Only EFS cases due to inadequate ovarian response to hCG were included.

TABLE 2 Clinical outcomes during the empty follicle syndrome cycle of patients with different delayed time for the second oocyte retrieval.

Variables	Delayed time (h)					Group of delayed time (h)		
	<3	[3, 4]	(4, 5]	(5, 6]	>6	<3 or >6	[3, 6]	*P*
*N**	9	11	19	9	3	12	39	–
Age (years)	30.6 ± 2.1	30.3 ± 3.7	30.0 ± 3.2	29.6 ± 2.6	27.0 ± 4.0	29.7 ± 3.0	30.0 ± 3.2	0.747
BMI (kg/m^2)	25.3 ± 4.3	24.4 ± 4.0	26 ± 4.8	24.9 ± 4.3	24.7 ± 5.9	25.2 ± 4.4	25.3 ± 4.4	0.924
Basal FSH (IU/L)	6.9 ± 1.5	6.4 ± 1.2	6.6 ± 1.5	7.2 ± 1.4	7.1 ± 1.3	7.0 ± 1.4	6.7 ± 1.4	0.532
No. of ≥14-mm follicles on trigger day	10.9 ± 2.2	14.7 ± 4.4	12.8 ± 5.7	12.4 ± 4.4	14.3 ± 4.9	11.8 ± 3.2	13.3 ± 5.0	0.334
Patients with oocyte obtained (%)	5 (55.6)	11 (100.0)	18 (94.7)	9 (100.0)	2 (66.7)	7 (58.3)	38 (97.4)	0.002
Patients with embryo for transfer (%)	3 (33.3)	11 (100.0)	18 (94.7)	7 (77.8)	1 (33.3)	4 (33.3)	36 (92.3)	<0.001
No. of pregnancies (%)	0 (0.0)	5 (45.5)	9 (47.4)	5 (55.6)	1 (33.3)	1 (8.3)	19 (48.7)	0.017

Data are presented as either means ± SD or number (%). Continuous variables were compared using Student's t test. Categorical variables were compared using Exact Fisher test.
*Only EFS cases due to inadequate ovarian response to hCG were included.

3.4 Self-controlled comparison between EFS cycles and their normal cycles

A total of 16 EFS patients who had previously normal cycles were analyzed. The protocols, E_2 per ≥14 mm follicle, hCG dosage, and exposure were all similar between the previous normal cycle and EFS cycle, but the EFS cycle showed significantly lower numbers of oocyte obtained (4.3 ± 3.8 *versus* 9.4 ± 5.3, P = 0.003), two polar nucleus (PN) embryos (3.2 ± 3.0 *versus* 5.5 ± 3.2, P = 0.021), and embryos available for transfer (1.3 ± 1.2 *versus* 1.9 ± 1.1, P = 0.023) (Table 3).

Moreover, 15 EFS patients who failed to achieve live birth in their EFS cycle underwent a subsequent normal cycle, which showed significantly higher numbers of oocytes obtained (10.5 ± 4.6 *versus* 3.7 ± 2.7, P = 0.001), two PN embryos (6.0 ± 3.7 *versus* 2.5 ± 2.2, P = 0.006), and embryos available for transfer (2.9 ± 2.5 *versus* 1.1 ± 0.9, P = 0.006). No patient repeated EFS after performing less downregulated protocol and longer hCG exposure time. The live birth rate in the subsequent normal cycle was 46.7% (7/15) (Table 3) (Figure 2).

4 Discussion

Our study indicated that PCOS patients are more prone to EFS and may require a longer hCG exposure for ovulation. Delaying the second oocyte retrieval by 3–6 h may be an effective way to achieve optimal pregnancy outcomes in EFS cases. EFS that occurred once did not suggest a fertility decline in future cycles.

4.1 Etiology of EFS

Studies have shown that mature metaphase II (MII) oocytes can be obtained 28–38 h after the onset of LH peak, and hCG exposure time less than 36 h significantly decreases the oocyte number and

TABLE 3 Self-controlled comparison between EFS cycles and their future normal cycles.

Variables	Previous normal cycle (n = 16)	EFS cycle (n = 16)	*P*	EFS cycle (n = 15)	Subsequent normal cycle (n = 15)	*P*
Age (years)	29.5 ± 3.1	30.4 ± 2.4	0.184	30.8 ± 3.0	31.5 ± 3.2	0.012
Downregulated protocol	13 (81.3%)	11 (68.8%)	0.727	13 (86.7%)	2 (13.3%)	0.001
hCG dosage (IU)	8,156 ± 1,044	8,438 ± 1,276	0.402	8,167 ± 1,249	8,800 ± 1,811	0.270
hCG exposure time (IU)	36.4 ± 0.3	36.3 ± 0.4	0.253	36.5 ± 0.5	38.0 ± 1.2	<0.001
E_2 per ≥14-mm follicle (pg/mL)	328.1 ± 94.4	339.6 ± 101.6	0.778	264.1 ± 101.7	336.1 ± 106.0	0.062
No. of oocytes	9.4 ± 5.3	4.3 ± 3.8	0.003	3.7 ± 2.7	10.5 ± 4.6	0.001
No. of 2PN embryos	5.5 ± 3.2	3.2 ± 3.0	0.021	2.5 ± 2.2	6.0 ± 3.7	0.006
No. of embryos for transfer	1.9 ± 1.1	1.3 ± 1.2	0.023	1.1 ± 0.9	2.9 ± 2.5	0.006
Clinical pregnancy rate	3 (18.8%)	3 (18.8%)	1.000	1 (6.7%)	8 (53.3%)	0.039
Live birth rate	3 (18.8%)	2 (12.5%)	1.000	0 (0%)	7 (46.7%)	0.016

Data are presented as either means ± SD or number (%). Continuous variables were compared using paired t-test or Wilcoxon paired test. Categorical variables were compared using McNemar's test.
EFS, empty follicle syndrome; hCG, human chorionic gonadotropin; PN, polar nucleus.

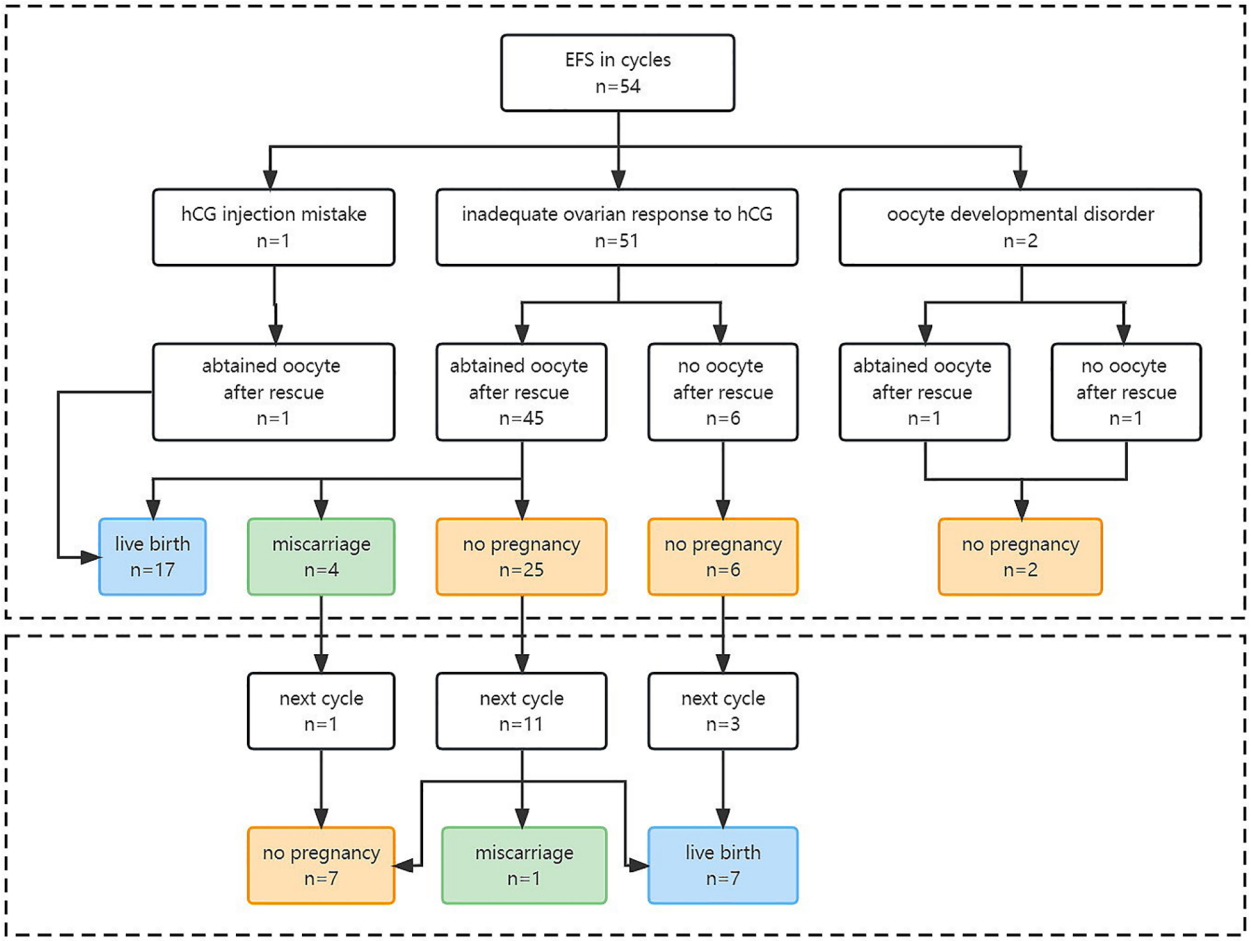

FIGURE 2
Pregnancy outcomes of EFS patients in EFS cycle and the future cycle. EFS, empty follicle syndrome; hCG, human chorionic gonadotropin.

maturity (17, 18). The optimal interval time remains unclear, but 36–38 h is widely accepted (19, 20). Our center administered a minimum hCG dosage of 6,500 IU to induce ovulation, surpassing the recommended minimum dosage of 5,000 IU in previous studies (21). Thus, the hCG exposure time and dosage both met the routine clinical criteria for all patients.

An accidental hCG injection mistake, as seen in our study, can halt ovarian stimulation without triggering ovulation, which was similar to "coasting". Previous studies showed that 1 to 2 days of "coasting" is harmless (22, 23). Stevenson reported live births in six out of 14 similar cases after rescue hCG injection, suggesting that oocyte and embryo quality may not be compromised (24). However, another study presented an increased rate of embryonic triploidy and compromised outcomes after rescue (4). Additionally, we should pay attention to spontaneous LH surge in GnRH-antagonist protocol. Blood LH and P levels should be tested after EFS to determine the appropriate time for the second oocyte retrieval.

Repeated EFS may be associated with premature oocyte atresia or oocyte maturation disorders, with the oocytes obtained presented as germinal vesicle (GV) or MI stage (25), immature oocytes without zona pellucida (26), or with an identifiable zona but devoid of oocytes (27). Our study identified two patients with repeated EFS, whose oocytes were all in the MI stage with abnormal zona pellucida, consistent with previous findings. GnRH-a triggering can stimulate FSH surge simultaneously, and dual trigger combined with hCG may be an alternative to improve oocyte maturation (28, 29). However, these two patients failed to obtain mature oocyte by dual triggering in previous EFS cycles. These suggested that they possibly had oocyte developmental disorders related to genetic factors, such as luteinizing hormone/choriogonadotropin receptor (LHCGR) (12) or zona pellucida (ZP) subtype (13).

However, most EFS cases in our study lacked an obvious cause. We speculated that individual hCG thresholds vary, and routine hCG dosage/exposure time may be insufficient for patients with higher thresholds, resulting in EFS. Additional HCG injections or extended exposure to HCG may improve oocyte retrieval, indicating inadequate or delayed response to HCG in these patients. Blazquez et al. also mentioned similar cases and

hypothesized that EFS in these patients might be attributed to temporary signal conduction delay rather than an ovarian pathological problem (8). Experimental evidence is needed to prove this speculation further.

4.2 Risk factors for EFS

PCOS patients were found to be more susceptible to EFS, possibly due to a persistently higher LH level and the inadequate or delayed expression of LH receptors. Thus, they may require more dosage of or exposure time to hCG to be triggered. Previous studies have supported this conclusion (11, 30). Daichi et al. speculated that the significantly fewer oocytes collected from the group of patients with higher LH were due to their insufficient FSH receptor (31).

Gambini et al. found that a higher BMI was associated with an increasing risk of oocyte immaturity after GnRH-a triggering (32). Pharmacokinetics changes associated with high BMI may partly explain the difference (33). In our study, BMI was significantly higher in the EFS group; however, the difference failed to reach statistical significance after adjustment. Singh et al. suggested a higher EFS occurrence in GnRH-antagonist protocols (34), but other studies have shown no impact of stimulation protocol on EFS prevalence (5, 35). Our findings align with the latter despite a higher proportion of EFS cases in the downregulated protocol. Differences in race and EFS criteria may account for this discrepancy, necessitating further investigation.

4.3 Rescue protocol

E_2 per mature follicle typically ranges from 200 to 300 pg/mL before ovulation (36), and lower levels indicate oocyte immaturity and poor prognosis. Thus, in EFS cases with E_2 less than 200 pg/mL per follicle, an additional 2,000–4,000 IU of hCG was administered. To reduce the risk of ovarian hyperstimulation, patients with over 15 follicles ≥14 mm were not given additional hCG (37).

It was reported that delaying the second retrieval by over 6 h can rescue 70% of EFS cases (38), but we found that delaying by 3–6 h help in achieving optimal outcomes. Too short or long of a delay may result in retrieval failure. Discrepancies in results may be due to differences in trigger standards and race.

4.4 Pregnancy outcomes in EFS cycles and the future cycles

Our study found that 31.5% (17/54) of EFS patients achieved live birth in the same cycle. For those who had a failed pregnancy in the EFS cycle, altering stimulation protocols, increasing hCG dosage or exposure time, or using a dual trigger of hCG combined with GnRH-a (39, 40) in the next cycle resulted in a live birth rate of 46.7% (7/15). This rate was comparable to the overall cumulative live birth rate of one retrieval cycle for all patients (50%) in our center. Thus, we speculated that the compromised outcome in the EFS cycle maybe due to the insufficient oocyte number obtained rather than fertility decline. Our findings are consistent with Revelli's opinion (35) but in conflict with Lorusso's (41), which found in three patients that EFS could predict less optimistic outcomes of the subsequent cycle. Larger studies are needed to resolve this discrepancy.

Obtaining a favorable pregnancy outcome with repeated EFS is challenging (42). *In vitro* maturation offers promise for patients with oocyte maturation problems (43, 44), while oocyte donation is a last-resort option.

4.5 Strength and limitations

This single-center study had a large sample size and low heterogeneity. It is the first study to analyze the risk factors for EFS with hCG trigger using multivariate logistic regression, and patients with PCOS were found to be more susceptible to EFS. We just found that delaying the second retrieval for 3–6 h may be an effective way for EFS to achieve optimal outcomes. Additionally, our long-term follow-up, including at least two complete oocyte retrieval cycles until live birth, is a novel contribution not mentioned in previous studies. However, the retrospective nature of our study may limit its statistical power due to potential biases and incomplete data. Further studies are required to confirm our findings and provide stronger evidence.

5 Conclusion

PCOS is an independent risk factor for EFS, possibly requiring a longer hCG exposure time. Delayed second oocyte retrieval by 3–6 h is an effective way for EFS to achieve optimal outcomes. The occurrence of EFS in a single cycle does not necessarily indicate future fertility decline, but repeated instances of EFS are associated with poor outcomes.

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 the ethics committee of the Second Hospital of Hebei Medical University (No. 2024-R106). 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 because the study was retrospective and patients' information was anonymous, which is in accordance with the national legislation and the institutional requirements.

Author contributions

ZL: Funding acquisition, Software, Writing – original draft. SX: Conceptualization, Data curation, Methodology, Supervision, Writing – review & editing. GH: Conceptualization, Funding acquisition, Investigation, Methodology, 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1424837/full#supplementary-material

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EDITED BY
Jan Tesarik,
MARGen Clinic, Spain

REVIEWED BY
Yujia Zhang,
Centers for Disease Control and Prevention (CDC), United States
Ravikrishna Cheemakurthi,
Krishna IVF Clinic, India

*CORRESPONDENCE
Chao Chin Hsu
tube2363808@gmail.com

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

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Ovarian sensitivity index affects clinical pregnancy and live birth rates in gonadotropin-releasing hormone agonist and antagonist *in vitro* fertilization cycles

Chao Chin Hsu[1,2*†], Isabel Hsu[2†], Sonam Dorjee[1], Yi Chun Chen[1], Tzu Ning Chen[1] and Yu Lin Chuang[1]

[1]Taiwan United Birth-Promoting Experts Fertility Clinic, Tainan, Taiwan, [2]Department of Obstetrics and Gynecology, National Taiwan University Hospital, Taipei, Taiwan

Objectives: This study aimed to investigate the correlation of ovarian sensitivity index (OSI) and clinical parameters in IVF treatments.

Methods: IVF data files between January 2011 and December 2020 in a single unit were included. The primary outcome measure was the correlation between the OSI and clinical pregnancy and live birth rates. A generalized linear model was employed to assess group differences while controlling for age. Correlations between the OSI and clinical parameters were analyzed using Pearson's correlation test.

Results: In total, 1,627 patient data were reviewed, comprising 1,160 patients who received GnRH antagonists and 467 who received GnRH agonists. There was no difference in the incidence of premature ovulation and LH surge in women receiving either GnRH antagonists or agonists. A higher number of mature oocytes and good embryos were obtained in the GnRH agonist cycles. No differences were observed in pregnancy and live birth rates between both groups. Regarding the correlation of the OSI with clinical parameters, serum anti-Müllerian hormone, cycle day 2 follicle-stimulating hormone, LH, and estradiol concentrations, numbers of larger follicles, fertilization rate, and the incidence of premature LH surge were positively correlated with the OSI. Whereas the body mass index, mature oocytes obtained, embryo transfer number, and dose of GnRH antagonists were negatively correlated with the OSI. In the GnRH antagonists group, an OSI of 225.75 significantly distinguished pregnancy from non-pregnancy ($p < 0.001$), with an AUC of 0.615, and an OSI of 208.62 significantly distinguished live births from non-live births ($p < 0.001$), with an AUC of 0.637. As for the GnRH agonist group, an OSI of 228 significantly distinguished live births from non-live births, ($p = 0.020$) with an AUC of 0.569.

Conclusion: We demonstrated the capability of employing OSI to distinguish the clinical pregnancy and live birth outcomes in IVF cycles.

KEYWORDS

GnRH-agonist, GnRH-antagonist, ovarian sensitivity index, controlled ovarian hyperstimulation, *in vitro* fertilization, clinical pregnancy rate, live birth rate

1 Introduction

The efficiency of controlled ovarian hyperstimulation (COH), which directly affects the outcomes of treatments, including clinical pregnancy and live birth rates, is a major objective for assisted reproduction. Currently, personalized treatment based on an individual's response to exogenous gonadotropin (Gn) is the main focus in clinical practice. The goal of COH is not only to obtain enough oocytes to achieve better clinical outcomes but also to prevent the incidence of ovarian hyperstimulation syndrome (OHSS) and manage poor ovarian response, particularly in many older women (1–3). Traditionally, women's age, serum follicle-stimulating hormone (FSH) and anti-müllerian hormone (AMH) levels, and antral follicle count (AFC) have been used for this purpose, and the starting dose of Gn in the COH cycle has been estimated (4, 5). Employing different doses of Gns for each patient is the most important clinical practice in individualized therapy (6).

The dynamic ovarian response to COH has attracted considerable attention in recent years. Different dynamic aspects of the ovarian response that correlate follicular growth to Gn have been studied, for example, ovarian sensitivity index (OSI, the dose of Gn used divided by the number of mature oocytes obtained) (7) and follicular output rate (FORT, the ratio of pre-ovulatory follicle count (14–22 mm in diameter) on human chorionic gonadotropin (hCG) day × 100/small antral follicle count (3–8 mm in diameter) at baseline (8), and the follicle-to-oocyte index (FOI, the ratio between the number of oocytes obtained and number of antral follicles at the beginning of COS) (9). The study and application of dynamic ovarian responses to COH are based on the premise that ovarian responses rely on multiple parameters. Few studies have attempted to integrate the dynamic ovarian response to different clinical parameters with the advantages of employing Gn-releasing hormone-agonist (GnRH-a) and/or GnRH-antagonists (GnRH-antag) in COH (10, 11). In this study, we investigated the correlation between OSI and multiple clinical parameters in GnRH-a and GnRH-antag cycles. The relationship between OSI and clinical outcomes of *in vitro* fertilization (IVF), especially clinical pregnancy and live birth rates, was determined.

2 Methods

2.1 Study population and design

This retrospective study analyzed the assisted reproduction files of all women in our IVF unit between January 2011 and December 2020. Data from those using the natural cycle or GnRH agonist ultra-long protocol, frozen embryo replacements, preimplantation genetic screening, or preimplantation genetic diagnosis were excluded. Only data from the first IVF treatment were included if the patients consecutively received several cycles of IVF in our unit.

2.2 Ethics approval

This study was approved by the Institutional Review Board (TSMH IRB/Protocol No: 18-115-B). All assisted reproductive processes were performed in accordance with the Declaration of Helsinki, Good Clinical Practice guidelines, and local regulatory requirements. All patients included in the study were treated at the IVF unit at the TUBE Fertility Clinic, Tainan, Taiwan, under a license from the Taiwan Department of Health Authority. Written consent was obtained from each patient to receive different administration modes of COH. All women receiving IVF treatments were informed about the benefits, risks, and potential adverse reactions of the entire procedure, including the different administration modes of COH (Gn, GnRH-a, and GnRH-antag). Possible risks of OHSS, allergic reactions, and local transitory effects, such as ecchymosis, itching, discomfort, and irritation were explained.

2.3 Controlled ovarian stimulation

The administration of GnRH-a, GnRH-antag, and Gn followed established protocols (12, 13). For the GnRH-a protocol: starting from day 3 of the preceding menstrual cycle, oral contraceptive pills (Marvelon, containing 0.03 mg ethinyl estradiol and 0.15 mg desogestrel, NV Organon, Oss, The Netherlands) were used. From day 18, a GnRH-a nasal spray (200 mg buserelin acetate, Aventis Pharma Deutschland GMBH, Frankfurt, Germany) was administered three times daily to achieve pituitary suppression. The GnRH agonist was maintained throughout the COH until the onset of hCG triggering. Gonadotropin (Gonal-f Prefilled Pen 300 IU rhFSH in 0.5 mL, Merck Serono S.p.A., Modugno, Italy) in combination with menopur (300 IU Menopur 75 IU, corresponding to 75 IU of FSH and luteinizing hormone (LH) 75 IU; Ferring GmBH, Kiel, Germany) was initiated on day 2 of the IVF cycle once pituitary suppression was achieved as manifested by serum estradiol (E2) <50 pg/mL, LH <2.5 mIU/mL, and FSH <10 mIU/mL. Intermittent injections of Gn on cycle days 2, 5, and 8 were performed in accordance with our previously established method (13). In brief, Gonal-f 300 IU in combination with 300 IU menopur was initiated on day 2 of the IVF cycle. Follow-up of ovarian follicular growth by ultrasound scanning was mostly performed on days 5 and 8–11. On days 5 and 8, if follicular growth did not meet the criteria (≥2 follicles ≥17 mm) for egg retrieval, a second and third dose of Gn injection were administered. The dosage for the second and third dose of Gn injection was based on the number and size of the follicles detected: 450 IU Gn if ≥2 follicles were >12 mm, and 600 IU Gn if most follicles were ≤12 mm.

For the GnRH-antag protocol, the third-generation GnRH antag ganirelix (orgalutron 0.25 mg, NV Organon, Oss, The Netherlands) was initiated once the ovarian follicle reached 12 mm in size on day 5 or 8 of the COH cycle. The GnRH-a was maintained throughout the COS until the day of hCG triggering. The mode of Gn administration in the GnRH-antag cycle was similar to that described for the GnRH-a cycle protocol.

Thus, two groups of patients were identified: group A received GnRH-a, and group B received GnRH-antag injections to suppress the premature LH surge. Follicular growth was detected using two-dimensional ultrasound scanning (Aloka 900, Tokyo, Japan) and performed by the same observer (C.C. Hsu) using a 5.0-MHz transvaginal transducer. The follicle diameter was calculated as the mean diameter measured in two dimensions. Serum levels of

FSH, LH, progesterone (P4), and E2 on day 2 of the menstrual cycle and the day of hCG were assessed.

2.4 Oocyte retrieval and clinical outcomes

Oocytes were retrieved in accordance with our previously established method (13, 14). In brief, oocyte retrieval took place 36 h after triggering the final follicular maturation using 2 mg GnRH-a (Leuprolide acetate, FAMAR L'AIGLE, Saint Remy Sur Avre, France) in combination with 6,000 IU hCG (Ovidriel, Merck Serono) when two or more follicles reached ≥17 mm in diameter. Mature oocytes were fertilized *in vitro* or by intracytoplasmic sperm injection (ICSI). Fertilized pre-embryos were cultured to day 3 cleavage-stage embryos or day 5–6 blastocyst stage for embryo transfer. The number of embryos transferred was based on the age of the women: one embryo for ≤35 years old, two embryos for 35–40 years old, or three embryos for ≥40 years old. Additional embryos were cryopreserved at day 3 of cleavage stage or at the blastocyst stage. Micronized P4 (utrogesterone; Besins Healthcare, Ayutthaya, Thailand) 100 mg three times daily was used for luteal support from the day after oocyte retrieval for 15 days until pregnancy was confirmed by serum hCG determination. Clinical pregnancy was confirmed using ultrasound at 4 weeks after embryo transfer. The safety endpoints included the proportion of women with moderate/severe-grade OHSS and preventive interventions for early OHSS (i.e., cycle cancellation due to excessive ovarian response). Adverse events, such as pain or skin reactions, were also recorded during Gn and GnRH-a/GnRH-antag injections.

2.5 Study outcome measures

The primary outcome was the correlation between the OSI and clinical parameters, including clinical pregnancy and live birth rates during fresh embryo transfer cycles. The secondary outcomes included mature oocytes retrieved and the incidence of premature ovulation.

2.6 Measurement of serum hormone levels

The Beckman Coulter ACCESS immunoassay system was used in the hormone assay (UniCelDxl 800, Beckman Coulter, Brea, CA, RRID: FSH: AB_2750983, LH: AB_2750984, AMH: AB_2892998, estradiol:AB_2892997, progesterone:AB_2756883). However, FSH and LH were measured using a sequential two-step immunoenzymatic "sandwich" assay. The lowest detectable level was 0.2 IU/L, and the assay exhibited a total imprecision of ≤10% for both FSH and LH. AMH levels were measured in serum samples using a simultaneous 1-step immunoenzymatic "sandwich" assay. The assay has a limit of detection at ≤0.02 ng/mL, with total imprecision ≤10.0% at concentrations of ≥0.16 ng/mL. A competitive binding immunoenzymatic assay was used to measure serum E2 and P4 levels. The lowest detectable level of E2 was 20 pg/mL, and that of P4 was 0.10 ng/mL.

2.7 Statistical analysis

Continuous variables were described as mean ± standard deviation (SD), and comparisons between groups of women were conducted using the Student's t-test. Categorical variables were expressed as frequencies and percentages, with the chi-square test applied to analyze their distributions. A generalized linear model (GLM) was employed to assess group differences while controlling for age and to evaluate the relationships between categorical and continuous variables. Correlations between the OSI and clinical parameters were analyzed using Pearson's correlation test. Partial correlation analysis, adjusted for age, was also performed. Additionally, receiver operation characteristic (ROC) curve analysis was conducted to distinguish clinical pregnancy and live birth outcomes between the GnRH-a and GnRH-antag groups, utilizing the pROC package in R. Statistical analyses were performed using JMP Statistics version 22.0 and various R packages in R Studio.

3 Results

3.1 Participant demographics

From the data of 3,012 cases, 1,385 were excluded: 764 due to frozen embryo replacement cycles; 534, repeated treatment cycles; and 87, other exclusion factors. In total, 1627 patient data files were analyzed, of which 1,160 patients received GnRH-antag and 467 received GnRH-a IVF cycles. The demographic patterns of the infertile women are presented in Table 1. The average age of the study population was 36.68 ± 4.60 years, with a body mass index (BMI) of 22.32 ± 3.46 kg/m^2. The average serum AMH was 2.67 ± 2.88 ng/mL, with AFC of 9.01 ± 6.89. Younger age and better AMH and AFC parameters were noted in GnRH-a group (Table 1). The cycle day 2 serum hormones FSH, E2, and LH were higher in those received GnRH-antag (Table 2).

3.2 Clinical response after COH using GnRH-antag or GnRH-a cycles

Elevated serum concentrations of LH, >2.5 times the baseline level and surpassing 17 IU/L, were not different between the two groups of women. Serum E2 levels on the day of hCG triggering were 2043.32 ± 2815.32 and 2052.65 ± 1914.49 pg/mL in women who received GnRH-antag and GnRH-a, respectively, with a significant difference ($p = 0.036$). Premature luteinization (P4 >2 ng/mL) was noted in 13.19% (153/1160) and 3.86% (18/466) of women who received GnRH-antag and GnRH-a, respectively ($p < 0.001$). However, the incidence of premature ovulation, indicated by the disappearance of growing follicles before oocyte retrieval, did not differ between the two groups. The number of medium-to-large-sized follicles (12–14 mm and >15 mm) and the incidence of OHSS were higher in women who received GnRH-a (Table 2).

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TABLE 1 Demographic characteristics of the participants.

	GnRH-antagonist N = 1160	GnRH-agonist N = 467	p value	p value[1]
age	37.33 ± 4.71	35.05 ± 3.87	<.0001	
Years infertile	4.51 ± 3.3	4.69 ± 3.31	0.441	0.001
previous IVF	1.15 ± 1.94	0.64 ± 1.02	<0.001	0.008
Primary infertility	53.79% (624/1160)	57.60% (269/467)	0.141	0.891
BMI	22.44 ± 3.47	22.03 ± 3.41	0.032	0.199
AMH	2.55 ± 2.96	2.99 ± 2.64	<0.001	0.001
AFC	8.13 ± 6.43	11.28 ± 7.46	<0.001	<0.001

Data are expressed as Mean ± Standard Deviation. The statistical significance shows the results of Student's t-test and Chi-squared test. The p value[1] is obtained by generalized linear model (GLM) after adjustment for age.
BMI, body mass index (kg/m^2); AMH, anti-mullerian hormone (ng/mL); AFC, antral follicle count.

3.3 Embryology and clinical outcomes in GnRH-antag or GnRH-a cycles

In the embryo laboratory, higher total oocyte numbers, mature oocytes, two pronuclear pre-embryos, and good embryo numbers were obtained following GnRH-a treatment cycles. Linear regression analysis of receiver operating characteristics indicated that higher numbers of oocytes were obtained from younger women, especially in the GnRH-a group (area under curve = 0.63), in comparison to area under curve = 0.52 in the GnRH-antag group. However, in both GnRH-a and GnRH-antag cycles, age was significantly correlated with total oocytes and mature oocytes (p <0.0001) (Figure 1).

Higher OSI of 282.67 ± 277.42 was noted in GnRH-antag treatment cycles in comparison to 201.74 ± 176.65 in GnRH-a treatment cycles (p <0.0001), indicating that higher Gns is required

TABLE 2 Endocrinology parameters in ovarian hormones and follicle growth were expressed.

	GnRH-antagonist N = 1160	GnRH-agonist N = 467	p value	p value[1]
Total dose Gn	1962.24 ± 778.87	2054.39 ± 795.30	0.031	0.009
D2 FSH	7.70 ± 3.80	5.67 ± 3.32	<0.001	<0.001
D2 E2	31.20 ± 18.71	18.07 ± 13.54	<0.001	<0.001
hCGd E2	2043.32 ± 2815.32	2052.65 ± 1914.49	0.997	0.036
Drop E2	3.71% (43/1160)	2.78% (13/467)	0.344	0.237
D2 LH	3.16 ± 2.26	1.57 ± 1.45	<0.001	<0.001
hCGd LH	3.85 ± 5.29	1.75 ± 2.16	<0.001	<0.001
Premature LH surge	3.71% (43/1160)	2.2% (10/454)	0.002	0.981
D2 P4	0.58 ± 0.44	0.56 ± 0.44	0.399	0.173
hCGd P4	1.41 ± 2.73	1.52 ± 3.41	0.689	0.855
hCGd P4 > 2	13.19% (153/1160)	3.86% (18/466)	<0.001	<0.001
Premature ovulation	0.34% (4/1160)	0.0% (0/467)	0.204	0.994
hCGd f < 11 mm	1.80 ± 2.07	1.58 ± 2.07	0.049	<0.001
hCGd f 12-14 mm	3.09 ± 3.28	4.18 ± 3.77	<0.001	0.005
hCGd f > 15 mm	5.78 ± 5.13	8.23 ± 6.18	<0.001	<0.001
hCGd Em (mm)	9.59 ± 2.72	9.83 ± 2.05	0.084	0.632
OHSS	7.84% (91/1160)	15.42% (72/467)	<0.001	0.003
Moderate to Seveve OHSS	2.76% (32/1160)	7.92% (37/467)	<0.001	0.021

Data are expressed as Mean ± Standard Deviation. The statistical significance shows the results of Student's t-test and Chi-squared test. The p value[1] is obtained by generalized linear model (GLM) after adjustment for age.
Gn, gonadotropin; GnRH, gonadotropin releasing hormone; D2, cycle day 2; hCGd, day of hCG injection; FSH, follicle stimulating hormone (IU/L); E2, estradiol (pg/mL); LH, luteinizing hormone (IU/L); P4, progesterone (ng/mL); f, follicle; Em, endometrium thickness; OHSS, ovarian hyperstimation syndrome.

to stimulate one mature oocyte in GnRH-antag cycles. No difference was noted based on the FORT and FOI indices between the two groups (Table 3). Thus, OSI is very useful as a predictive value than FORT and FOI and was used as the ovarian response factor for further analysis with other clinical parameters.

A total of 680 women conceived following fresh embryo transfer, with a clinical pregnancy rate of 40.85% and 40.38%, and a live birth rate of 32.53% and 28.69% in the GnRH-antag and GnRH-a cycles, respectively (Table 3). No differences were noted in clinical pregnancy and live birth rates between the two groups.

3.4 The correlation between OSI and clinical parameters

In all the participants, serum AMH, FSH, LH, and E2 at cycle day 2, E2, LH at hCG day, and patients' age, fertilization rate, and signs of uncontrolled COH (including drop of E2, premature LH surge), and numbers of larger follicles were positively correlated with the OSI. Whereas BMI, serum P4 at day 2, endometrium thickness at hCG day and numbers of mature oocytes, fresh cycle embryo transfer number and dose of GnRH-antag were negatively correlated with the OSI (Tables 4, 5). Among those received GnRH-antag, which represent 71.3% of our participants, the correlation between OSI and clinical parameters studied were similar to the total population. Compared with GnRH-antag cycles, higher negative correlation between numbers of mature oocytes and OSI were noted in women received GnRH-a (Tables 4, 5; Figure 2). In the GnRH-antag group (Figures 3A, C), an OSI of 225.75 significantly distinguished pregnancy from non-pregnancy ($p < 0.001$), with an AUC of 0.615. It also revealed that an OSI of 208.62 significantly distinguished live births from non-live births, ($p < 0.001$), with an AUC of 0.637. As for the GnRH-a group (Figures 3B, D), an OSI couldn't differentiate pregnant from non-pregnant individuals (p=0.320), while an OSI of 228 significantly distinguished live births from non-live births, (p =0.020) with an AUC of 0.569.

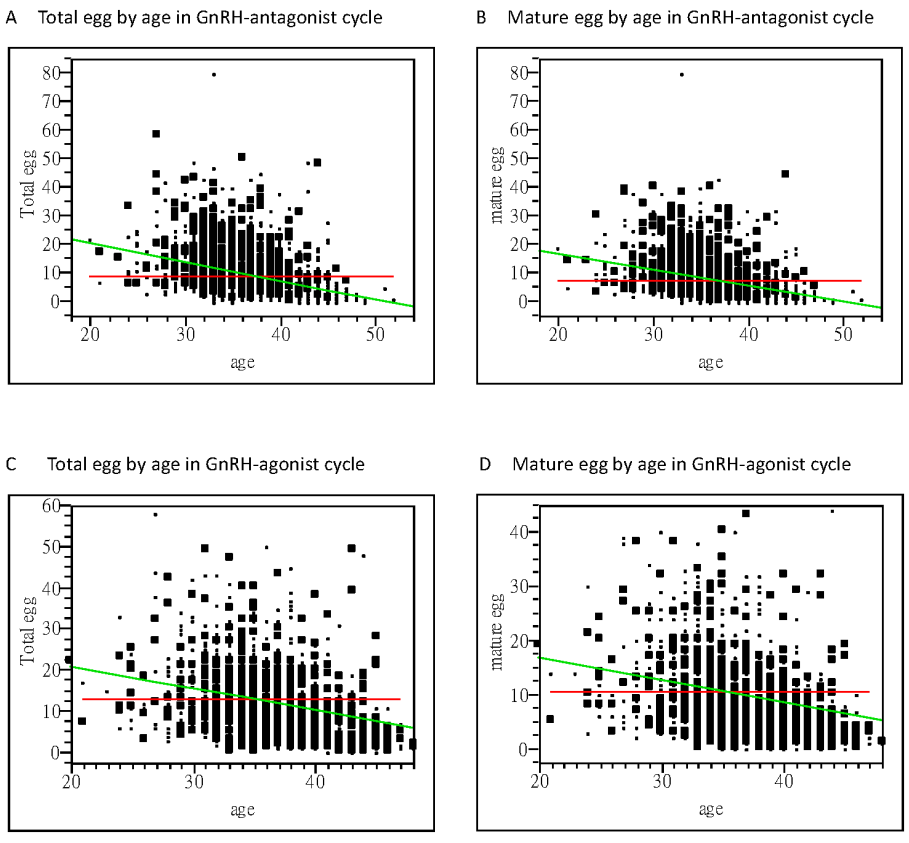

FIGURE 1
Bivariate fit of oocytes retrieved and women's age. **(A)** A significant correlation between the total oocytes obtained and age is noted with a correlation coefficient of −0.65 using linear regression analysis ($p < 0.0001$) in the GnRH antagonist cycle. **(B)** A significant correlation between the mature oocytes obtained and age is noted, with a correlation coefficient of −0.56 using linear regression analysis ($p < 0.0001$) in the GnRH antagonist cycle. **(C)** A significant correlation between the total oocytes obtained and age is noted, with a correlation coefficient of−0.54 using linear regression analysis ($p < 0.0001$) in the GnRH-agonist cycle. **(D)** A significant correlation between the mature oocytes obtained and their age was noted, with a correlation coefficient of −0.42 using linear regression analysis ($p < 0.0001$) in the GnRH-agonist cycle. GnRH, gonadotropin-releasing hormone.

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TABLE 3 Oocytes retrieved, embryo and clinical outcomes in this study.

	GnRH-antagonist N = 1160	GnRH-agonist N = 467	p value	p value[1]
Total eggs	9.51 ± 8.04	13.32 ± 8.92	<0.001	<0.001
mature eggs	7.59 ± 6.82	10.90 ± 7.64	<0.001	<0.001
2 PN number	6.46 ± 5.52	7.91 ± 6.18	<0.001	0.016
Fertilization rate %	73.2 ± 42.5	74.7 ± 50.0	0.562	0.719
Good embryo number	4.10 ± 3.94	5.32 ± 4.61	<0.001	0.001
freeze embryo number	2.22 ± 4.20	2.48 ± 3.49	0.231	0.607
OSI	282.67 ± 277.42	201.74 ± 176.65	<0.001	<0.001
FORT	80.03 ± 89.91	79.41 ± 48.95	0.861	0.857
FOI	123.48 ± 75.20	125.73 ± 65.66	0.593	0.983
ET no.	1.84 ± 0.52	1.89 ± 0.52	0.166	0.544
Biochemical pregnancy	45.28% (470/1038)	45.45% (210/462)	0.651	0.585
Clinical pregnancy	40.85% (424/1038)	40.38% (187/462)	0.614	0.707
Live birth	32.53% (338/1038)	28.69% (136/462)	0.674	0.15

Data are expressed as Mean ± Standard Deviation. The statistical significance shows the results of Student's t-test and Chi-squared test. The p value[1] is obtained by generalized linear model (GLM) after adjustment for age.
PN, pronuclear; hCGd, day of hCG injection; OSI, ovarian sensitivity index, the dose of Gn used divided by number of mature oocytes obtained; FORT, follicular output rate, the ratio of pre-ovulatory follicle count (14–22 mm in diameter) on hCG day ×100/small antral follicle count (3–8 mm in diameter) at baseline.; FOI, follicle to oocyte index, the ratio between the number of oocytes obtained and the number of antral follicles at the beginning of stimulation. ET, embryo transfer.

TABLE 4 Correlation of OSI and clinical parameters.

	Overall				GnRH-antagonist				GnRH-agonist			
	N=1627				N=1160				N=467			
	R^2	p	R^2*	p*	R^2	p	R^2*	p*	R^2	p	R^2*	p*
Age	0.060	0.022			0.037	0.219			-0.012	0.792		
BMI	-0.060	0.023	-0.063	0.012	-0.061	0.043	-0.065	0.031	-0.081	0.083	-0.080	0.085
AMH	0.060	0.027	0.087	0.002	0.103	0.001	0.125	<0.001	0.002	0.964	0.000	0.995
AFC	-0.020	0.49	0.004	0.866	0.014	0.630	0.031	0.295	0.002	0.958	0.000	0.996
Infertile years	0.010	0.64	-0.003	0.919	0.008	0.791	-0.002	0.935	0.047	0.312	0.051	0.278
Previous IVF	0.010	0.66	-0.007	0.770	-0.017	0.572	-0.031	0.311	0.059	0.208	0.062	0.186
D2 FSH	0.080	0.001	0.071	0.007	0.046	0.142	0.038	0.229	0.062	0.185	0.064	0.171
D2 E2	0.280	<0.001	0.271	<0.001	0.266	<0.001	0.265	<0.001	0.118	0.011	0.118	0.011
D2 LH	0.270	<0.001	0.269	<0.001	0.244	<0.001	0.246	<0.001	0.218	<0.001	0.218	<0.001
D2 P4	-0.090	0.001	-0.091	0.001	-0.089	0.008	-0.086	0.011	-0.127	0.007	-0.127	0.007
hCGd f <11	-0.010	0.718	-0.001	0.975	-0.025	0.411	-0.018	0.539	0.008	0.867	0.007	0.888
hCGd f 12–14	-0.030	0.311	-0.006	0.814	-0.010	0.740	0.004	0.899	0.004	0.927	0.001	0.975
hCGd f >15	0.100	<0.001	0.129	<0.001	0.156	<0.001	0.182	<0.001	0.085	0.068	0.084	0.071
hCGd E2	0.450	<0.001	0.474	<0.001	0.449	<0.001	0.471	<0.001	0.463	<0.001	0.474	<0.001
hCGd LH	0.220	<0.001	0.210	<0.001	0.219	<0.001	0.217	<0.001	0.096	0.332	0.097	0.332
hCGd P4	0.030	0.332	0.032	0.282	0.029	0.351	0.031	0.324	0.043	0.645	0.041	0.656
hCGd Em	-0.070	0.018	-0.058	0.038	-0.068	0.037	-0.062	0.054	-0.032	0.561	-0.032	0.551

(Continued)

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TABLE 4 Continued

	Overall				GnRH-antagonist				GnRH-agonist			
	N=1627				N=1160				N=467			
	R^2	p	R^{2*}	p*	R^2	p	R^{2*}	p*	R^2	p	R^{2*}	p*
Total egg	-0.020	0.343	-0.003	0.916	0.007	0.806	0.023	0.437	-0.008	0.863	-0.011	0.809
Mature egg	-0.090	0.001	-0.070	0.006	-0.040	0.183	-0.028	0.352	-0.128	0.006	-0.133	0.004
2 PN	-0.010	0.779	0.009	0.723	0.032	0.316	0.044	0.162	-0.059	0.207	-0.064	0.176
Good embryo	-0.040	0.146	-0.027	0.344	0.007	0.847	0.018	0.603	-0.082	0.090	-0.085	0.077
Total Gn dose	-0.010	0.584	-0.016	0.516	-0.027	0.373	-0.028	0.343	0.064	0.170	0.065	0.162
FORT	0.160	<0.001	0.164	<0.001	0.170	<0.001	0.170	<0.001	0.135	0.004	0.135	0.004
FOI	-0.010	0.818	-0.002	0.929	0.002	0.935	0.005	0.868	-0.038	0.409	-0.039	0.404
Freeze Embryo	0.040	0.138	0.049	0.052	0.058	0.052	0.067	0.024	-0.032	0.488	-0.033	0.474
Fresh ET No	-0.100	0.003	-0.089	0.006	-0.095	0.020	-0.092	0.024	-0.089	0.084	-0.093	0.071
Fertilization rate	0.050	0.032	0.056	0.026	0.075	0.012	0.076	0.011	0.008	0.860	0.008	0.859

The data presented consist of the coefficients and p-values for both Pearson's correlation (coefficients: R^2, p-values: p) and partial correlation (adjusted for age) (coefficients: R^{2*}, p-values: p*) in the analysis of the relationship between OSI and the indicated clinical parameters.
BMI body mass index (kg/m^2); AMH, anti-mullerian hormone (ng/mL); AFC, antral follicle count; Gn, gonadotropin; GnRH, gonadotropin releasing hormone; D2, cycle day 2; hCGd, day of hCG injection; FSH, follicle stimulating hormone (IU/L); E2, estradiol (pg/mL); LH, luteinizing hormone (IU/L); P4, progesterone (ng/mL); f, follicle; Em, endometrium thickness (mm); OHSS, ovarian hyperstimation syndrome; PN, pronuclear; hCGd, day of hCG injection; OSI, ovarian sensitivity index, the dose of Gn used divided by number of mature oocytes obtained; FORT, follicular output rate, the ratio of pre-ovulatory follicle count (14–22 mm in diameter) on hCG day ×100/small antral follicle count (3–8 mm in diameter) at baseline.; FOI, follicle to oocyte index, the ratio between the number of oocytes obtained and the number of antral follicles at the beginning of stimulation. ET, embryo transfer.

3.5 Adverse reactions

OHSS was noted in 7.84% (91/1160) and 15.42% (72/467) of patients in the GnRH-antag and GnRH-a treatment cycles, respectively. Among them, 4 and 8 patients experienced severe OHSS, 28 and 29 experienced moderate OHSS, and 59 and 35 experienced mild OHSS in those who received GnRH-antag and GnRH-a, respectively. Thus, moderate-to-severe OHSS was experienced by 2.76% (32/1160) and 7.92% (37/467) in the GnRH-antag and GnRH-a cycles, respectively. No cycle cancellation due to excessive ovarian response was noted in this study.

4 Discussion

In the present study, more mature oocytes and good embryos were obtained in the GnRH-a treatment cycles, which is similar to the results of most previous studies, including many systematic

TABLE 5 Relationship of OSI according to the clinical parameters.

	Overall				GnRH-antagonist				GnRH-agonist			
	(N=1627)				(N=1160)				(N=467)			
	R^2	p	R^{2*}	p*	R^2	p	R^{2*}	p*	R^2	p	R^{2*}	p*
Primary infertility	0.057	0.035	0.079	0.08	0.073	0.054	0.095	0.075	0.014	0.713	0.036	0.805
Drop of E2	0.101	<0.001	0.102	<0.001	0.119	0.001	0.123	0.001	0.065	0.035	0.066	0.034
Premature LH surge	0.229	0.001	0.266	0.002	0.228	0.002	0.259	0.003	0.008	1	0.008	1
Premature ovulation	0.722	0.133	0.808	0.093	0.719	0.172	0.801	0.104	0.009	1	0.009	1
Elevated P4	0.063	0.066	0.064	0.065	0.07	0.335	0.071	0.314	0.017	0.184	0.017	0.199
OHSS	0.015	0.165	0.098	0.313	0.014	0.987	0.112	0.726	0.017	0.111	0.054	0.102
Biochemical pregnancy	0.011	0.015	0.044	0.051	0.013	0.029	0.071	0.077	0.008	0.324	0.014	0.375
Clinical pregnancy	0.007	0.065	0.034	0.158	0.009	0.067	0.055	0.144	0.004	0.726	0.01	0.801
Live Birth	0.009	0.029	0.047	0.092	0.012	0.042	0.07	0.106	0.006	0.35	0.023	0.429

The data are presented as the coefficients and p-values from the generalized linear model (GLM) analysis, both before (coefficients: R^2, p-values: p) and after adjustment for age (coefficients: R^{2*}, p-values: p*), to examine the relationship between OSI and the specified clinical parameters.
GnRH, gonadotropin releasing hormone; E2, estradiol (pg/mL); LH, luteinizing hormone (IU/L); P4, progesterone (ng/mL); OHSS, ovarian hyperstimation syndrome.

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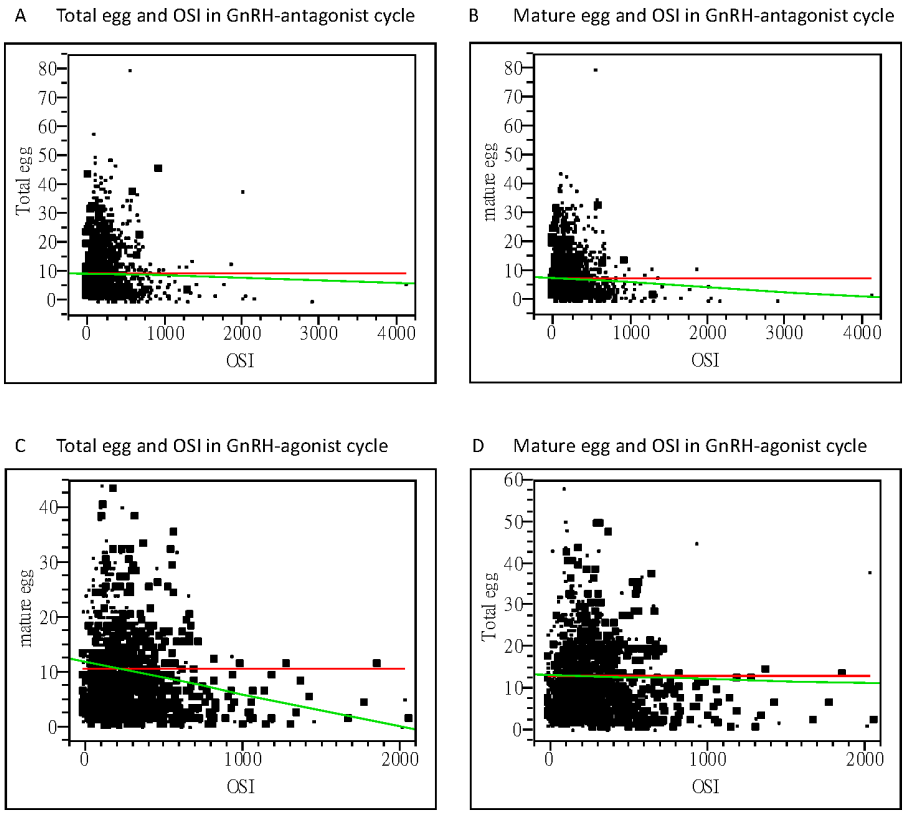

FIGURE 2
Bivariate fit of oocytes retrieved and OSI. **(A)** Correlation between the total oocytes obtained and OSI, with a correlation coefficient of −0.98 using linear regression analysis (p=0.3359) in the GnRH antagonist cycle. **(B)** Correlation between the mature oocytes obtained and OSI, with a correlation coefficient of −2.76 using linear regression analysis (p=0.0215) in the GnRH antagonist cycle. **(C)** Correlation between the total oocytes obtained and OSI, with a correlation coefficient of −0.39 using linear regression analysis (p=0.6715) in the GnRH-agonist cycle. **(D)** Correlation between the mature oocytes obtained and OSI, with a correlation coefficient of −3.13 using linear regression analysis (p=0.0034) in the GnRH-agonist cycle. OSI, ovarian sensitivity index; GnRH, gonadotropin-releasing hormone.

reviews and meta-analysis (15–19). A previous study indicated better synchronization of the follicular cohort with GnRH-a treatment and more natural recruitment of follicles in the follicular phase by employing the GnRH-antag cycle (20). They reported a strong correlation between patient age and the number of oocytes only in the GnRH-antag group (20). However, a strong correlation was noted between the woman's age and oocytes retrieved from our patients who received either GnRH-a or GnRH-antag COH in the present study. The GnRH-antag COH has been criticized for its relatively low pregnancy rate, and it may be used as a second-line treatment (15, 21). However, our data revealed similar clinical pregnancy and live birth rates when the GnRH-a and GnRH-antag protocols were used. Under equal demographic and clinical features, previous studies have shown similar pregnancy rates with either GnRH-a or GnRH-antag protocols (20, 22). Thus, the advantage of reducing the incidence of OHSS using GnRH-antag protocols without compromising clinical outcomes is encouraged based on our results.

Previous work showed the highest correlation between ovarian response (including OSI) and AFC, AMH, LH-to-FSH ratio, age, and FSH in GnRH-antag COH cycles (10). In the present study, including GnRH-a and GnRH-antag cycles, AMH, hormone status (FSH, E2, and LH levels) on cycle day 2 before COH and E2 and LH levels on the hCG day were positively correlated with OSI. However, BMI, and number of mature oocytes were negatively correlated with OSI in these women. Our results were also different from recent reports in which OSI was inversely related to age and BMI and directly related to AMH and AFC in their GnRH-a and GnRH-antag protocols (23), and another report indicated a negative correlation between OSI and age, FSH, basal FSH/LH, and Gn total dose, and a positive correlation between OSI and AMH, AFC, total oocytes, and mature oocytes (24). However, these studies did not compare the different clinical parameters relevant to OSI separately in either the GnRH-a or GnRH-antag protocols (23, 24). Among those received GnRH-antag in the present study, the correlation between OSI and clinical parameters studied were similar to the total population as described above. Compared with GnRH-antag cycles, negative correlation between numbers of mature oocytes and OSI were noted in women received GnRH-a in our study. For the parameters of AMH and AFC in the present study, only women who received GnRH-antag showed significant correlation with OSI in AMH (correlation coefficient of 0.125; $p < 0.001$), with no significant

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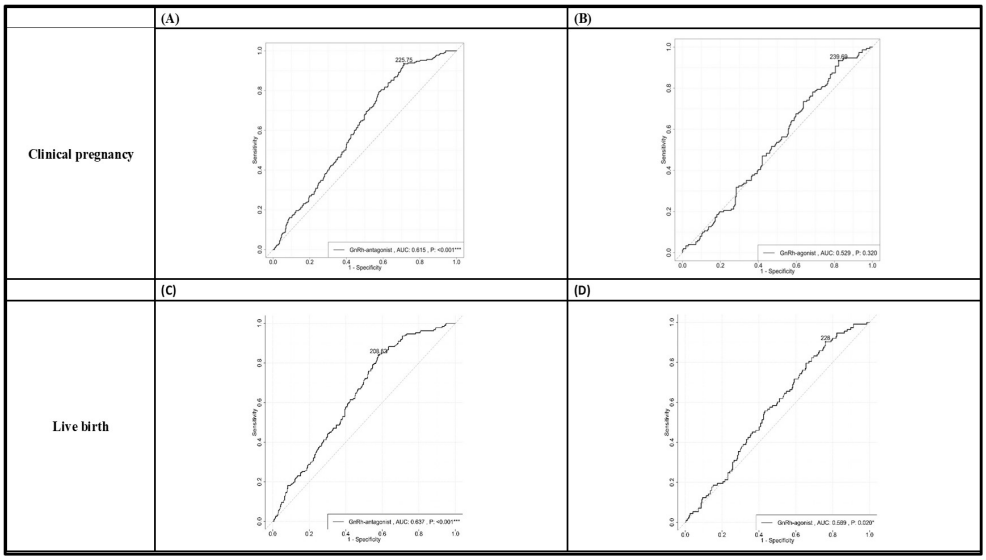

FIGURE 3
The receiver operating characteristic (ROC) curve plots demonstrated the ability to distinguish clinical pregnancy outcomes in both the GnRH antagonist group **(A)** and the GnRH agonist group **(B)**. Additionally, the plots also illustrated the ability to differentiate live birth outcomes in the GnRH antagonist group **(C)** and the GnRH agonist group **(D)**. We determined the optimal cutoff value for OSI through receiver operating characteristic (ROC) curve analysis. In the GnRH antagonist group **(A, C)**, an OSI of 225.75 significantly distinguished pregnancy from non-pregnancy ($p < 0.001$), with an AUC of 0.615. The sensitivity and specificity in this group were 0.935 and 0.286, respectively. It also revealed that an OSI of 208.62 significantly distinguished live births from non-live births, ($p < 0.001$), with an AUC of 0.637. The sensitivity and specificity in this case were 0.840 and 0.421, respectively. As for the GnRH agonist group **(B, D)**, an OSI couldn't differentiate pregnant from non-pregnant individuals ($p=0.320$), while an OSI of 228 significantly distinguished live births from non-live births, ($p =0.020$) with an AUC of 0.569. The sensitivity and specificity were 0.903 and 0.239, respectively. GnRH, gonadotropin-releasing hormone; OSI, ovarian sensitivity index.

correlation with OSI in AFC in either group of women. Thus, our results do not support the previous findings in which the OSI was strongly and significantly correlated with AMH and AFC (7, 25, 26).

A previous study suggested that instead of oocyte number, OSI is a better indicator of the ovarian response to Gn stimulation. For more personalized treatment, OSI has been suggested as an indicator of multiple confounding effects on oocyte number (26). The OSI has also been used as a tool to define poor, normal, and high response patterns in IVF cycles based on the long protocol GnRH-a COH (27). However, a recent study showed a marked intercycle variability of the OSI in 18% of women investigated, suggesting an intrinsic variability of ovarian sensitivity, both with the GnRH-a and GnRH-antag protocols (23). The most remarkable correlation between the OSI and clinical parameters in the present study was the demonstration of the ability to distinguish clinical pregnancy outcomes in both the GnRH-antag group and the GnRH-a group using the optimal cutoff value for OSI through receiver operating characteristic (ROC) curve analysis. Our results echo the recent study which showed a strong correlation between OSI values and the clinical pregnancy rate (23, 28). As the results were derived from data of our single institution, further investigations were warrented to confirm our finding using data from other sources. Further studies should be conducted to elucidate more consolidated clinical evidence of employing ovarian responses, including OSI, in IVF treatments, which might aid clinical decisions in the COH protocol.

There are limitations to this study, such as discrepancies in the baseline parameters of our participants; for example, the difference in the participants' age. One factor might be the accessibility of the medicine; for example, the GnRH-a (Supremone nasal spray; Buserelin acetate, Aventis Pharma Deutschland GMBH, Frankfurt, Germany) routinely used in the long protocol for our patients who underwent IVF was no longer available in Taiwan during the last 6 years. Additionally, the mean age of women receiving IVF treatment in Taiwan has increased from 32.7 to 37.8 years between 1998 and 2021 (29). These may be important factors causing the demographic patterns of the two groups of women to differ. Moreover, retrieving ovarian follicles through vaginal puncture, especially in those suffering marked pelvic and ovarian adhesion or distorted pelvic anatomy due to huge myoma/adenomyoma, and whether or not the operating clinician retrieves oocytes from small follicles may affect OSI accuracy (10). Furthermore, low correlations between patient parameters and OSI have been related to intercycle variations in ovarian responses using the same FSH doses in the same patients (30, 31). Thus, future larger randomized controlled studies should be carried out to achieve more accuracy in the determination of ovarian response to COH, such as OSI, and towards a better elucidation of the ovarian response relevant to clinical outcomes, including clinical pregnancy and live birth rates.

In conclusion, this study reconfirmed the efficiency of both GnRH-a and GnRH-antag in suppressing premature LH surges and premature ovulation in COH for IVF treatment. Similar clinical pregnancy and live birth rates were noted when using either the GnRH-a or GnRH-antag protocols. We further demonstrated the capability of employing OSI to distinguish the clinical pregnancy and live birth outcomes in both GnRH-a and GnRH-antag cycles.

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 Ethical Committe, Antai Tian-Sheng Memorial 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.

Author contributions

CH: Investigation, Conceptualization, Writing – original draft, Writing – review & editing. IH: Writing – original draft, Writing – review & editing, Conceptualization, Methodology. SD: Investigation, Writing – review & editing, Writing – original draft. YC: Writing – original draft, Data curation, Methodology. TC: Writing – original draft, Methodology, Data curation. YC: Methodology, Writing – review & editing, Data curation.

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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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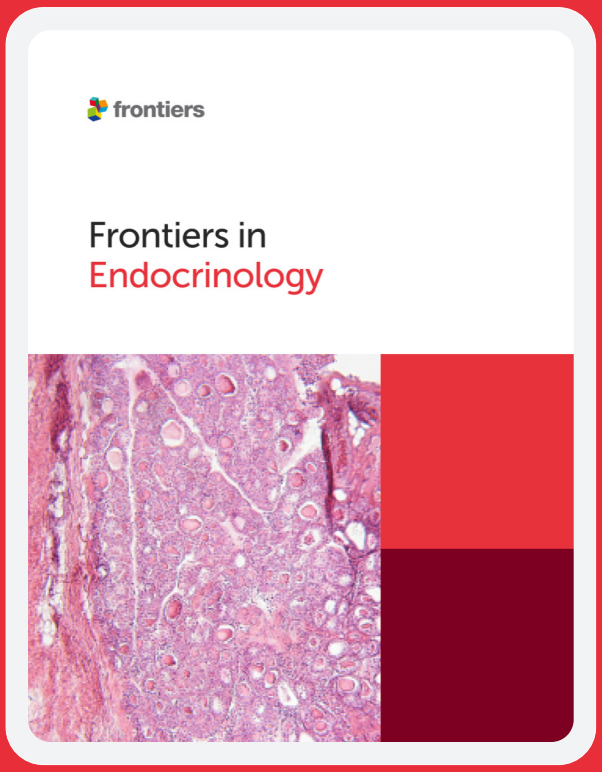

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