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Advances in the treatment of sexual precocity and infertility

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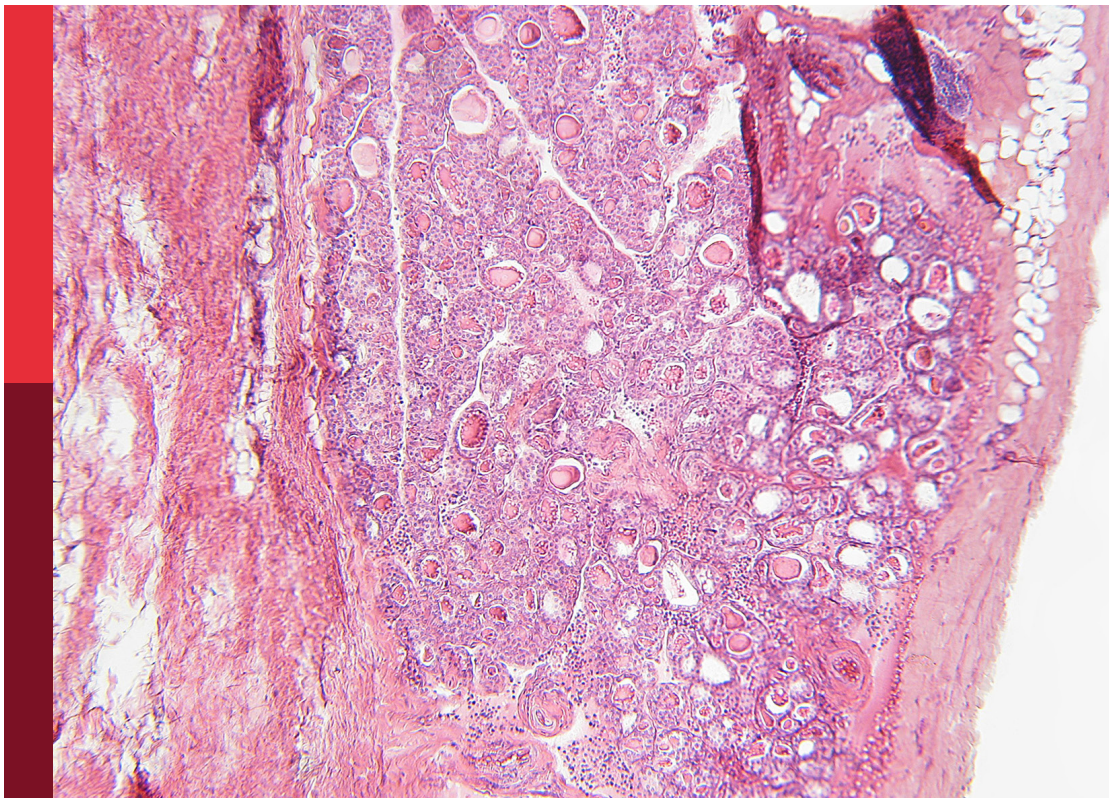

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Advances in the treatment of sexual precocity and infertility

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Editorial: Advances in the treatment of sexual precocity and infertility

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Editorial on the Research Topic
Advances in the treatment of sexual precocity and infertility

Introduction

Our topic "Advances in the Treatment of Sexual Precocity and Infertility" addresses critical advancements in pediatric-adolescent endocrinology, reproductive medicine, and gynecology. With genetic precision medicine becoming increasingly important (1), this collection of 27 articles explores the underlying mechanisms of sexual precocity, hypogonadism, infertility (2), and offers novel insights into therapeutic approaches, enhancing both clinical outcomes and quality of life for affected individuals. The research emphasizes innovations in reproductive treatments, personalized medicine, hormonal therapies involving aromatase inhibitors even in early maturing girls with a compromised growth potential (3), and management strategies for conditions like polycystic ovary syndrome (PCOS) (4), poor ovarian response, and hypogonadotropic hypogonadism.

Infertility treatments and protocol optimization

Several studies in this Research Topic focus on improving *in vitro* fertilization (IVF) protocols to optimize pregnancy outcomes while maintaining cost-effectiveness. A multicenter randomized controlled trial comparing a modified GnRH antagonist protocol based on luteinizing hormone (LH) levels with the conventional GnRH antagonist protocol found that both had similar clinical efficacy. However, the modified protocol was more cost-effective, reducing overall financial burden during assisted reproductive technology (ART) cycles (Liu et al.). The exploration of ovulation promotion protocols in young IVF patients with low anti-Müllerian hormone (AMH)

levels revealed that the GnRH antagonist protocol yielded better cumulative live birth rates (CLBR) than the progestin-primed ovarian stimulation (PPOS) regimen, particularly for patients with low but not very low AMH levels. For very low AMH levels, both protocols were comparable in CLBR outcomes, suggesting that age and AMH status should guide protocol selection (Li et al.). Further extending this focus on individualized protocols, a study evaluating different starting doses of recombinant human follicle-stimulating hormone (rhFSH) in patients with normal ovarian reserves found that a starting dose of 150 IU resulted in a similar pregnancy outcome compared to a 225 IU dose. The lower starting dose was also associated with shorter time to live birth and reduced cost, making it an attractive option for normal ovarian reserve patients (Jia et al.). Similarly, a real-world study on follitropin delta, an innovative rhFSH, confirmed its effectiveness in IVF protocols. The study highlighted its favorable pregnancy rates, minimized ovarian hyperstimulation syndrome (OHSS) risk, and reliable dosing patterns in routine clinical practice (Blockeel et al.). Letrozole cotreatment has also gained attention, especially in women with polycystic ovary syndrome (PCOS) and high body mass index (BMI), who often experience attenuated ovarian responses to IVF. This study found that letrozole combined with PPOS improved the follicular output rate (FORT), although the overall pregnancy outcomes did not differ significantly from those without letrozole (Liu et al.). The effects of glucocorticoids and androgens on ovarian follicle function were examined in a study exploring stress-induced diminished ovarian reserve. Findings indicated that excessive glucocorticoids impaired ovarian function, but androgens could improve early-stage follicle development through synergistic signaling with insulin-like growth factor 1 (IGF1) and follicle-stimulating hormone (FSH) (Gao et al.). Endometrial receptivity, a key factor in implantation success, was the subject of a study comparing outcomes of Day 7 blastocyst transfer to Day 5 and Day 6 transfers. The Day 7 group showed significantly lower live birth and pregnancy rates, while preimplantation genetic testing (PGT) was suggested as a strategy to enhance outcomes for Day 7 transfers (Liu et al.). A critical study on vanishing twin syndrome (VTS) identified intrauterine hematoma (IUH) as a risk factor for VTS in twin pregnancies following IVF. The presence of IUH was associated with increased risks of preterm birth, threatened abortion, and postpartum hemorrhage, emphasizing the need for close monitoring in twin pregnancies (Ge et al.).

Hormonal ratios and IVF outcomes

Several studies explored hormonal markers as predictors of IVF outcomes. A study examining the follicle-stimulating hormone-to-luteinizing hormone (FSH/LH) ratio in women undergoing GnRH antagonist protocols found that this ratio could predict poor ovarian response and reproductive potential. The basal FSH/LH ratio was the strongest predictor, particularly for identifying poor responders and women with limited embryo availability (Zhao et al.). The progesterone-to-retrieved oocyte (P/O) ratio during the late follicular phase also emerged as a significant predictor of pregnancy outcomes in fresh embryo transfer cycles. Higher P/O ratios were associated with reduced live birth rates, suggesting that this marker can guide decision-making on embryo transfer strategies (Zhang et al.).

Male infertility and advanced age

The role of the aging male in sperm quality and reproductive outcomes was addressed through a study assessing sperm chromatin integrity and hormonal markers in subfertile men. Older men (>40 years) exhibited increased sperm chromatin damage and chromatin immaturity compared to younger men, underscoring the impact of age on male fertility. These findings emphasize the importance of evaluating sperm integrity in older males undergoing ART (Bibi et al.).

Traditional and complementary approaches

The Research Topic also highlights alternative therapies. A prospective study on acupuncture examined its effect on endometrial receptivity and implantation success in rats undergoing controlled ovarian hyperstimulation (COH). Acupuncture improved pregnancy rates by restoring hormonal balance and extending the implantation window, indicating its potential as a complementary therapy in ART (Hu et al.). Traditional Chinese medicine (TCM), specifically the Dingkun pill, was evaluated for its role in improving fertility outcomes in women with thin endometrium. When combined with conventional hormonal treatments, TCM significantly improved endometrial thickness, luteal function, and cumulative pregnancy rates, demonstrating the utility of integrating TCM in fertility management (Jin et al.).

Sexual precocity and puberty suppression

In addressing sexual precocity, one notable study examined the combination of anastrozole and leuprorelin in treating early-maturing girls with compromised growth potential. The findings revealed that continuing anastrozole monotherapy after the combined treatment further improved near-adult height, offering a promising approach for managing growth outcomes in early puberty (Papadimitriou et al.). Puberty suppression (PS) in adolescents with gender dysphoria was another key focus. PS using GnRH analogs helps alleviate distress by halting the development of secondary sex characteristics, offering transgender adolescents more time to explore their gender identity. However, the long-term effects of PS, including bone health and reproductive potential, remain uncertain, necessitating further research (Betsi et al.).

Advances in reproductive surgery and machine learning

A novel web-based nomogram developed using machine learning models helps predict the likelihood of spontaneous pregnancy following reproductive surgery. This tool incorporates clinical indicators such as age, AMH levels, and infertility duration, providing an individualized prediction of natural conception and helping couples make informed decisions about their fertility options (Liu et al.).

Global trends and ethical considerations in ART

A bibliometric analysis of PCOS research over the past decade highlights trends in reproductive health, showing a growing focus on gut microbiota, microRNAs, and Vitamin D deficiency in PCOS management. This analysis provides insight into current research directions and identifies areas for future study, particularly in understanding the pathogenesis and treatment of PCOS (Shi et al.). In terms of ethical considerations, a survey of IVF add-ons in Japan revealed that many clinics offer treatments with limited supporting evidence. The study called for better regulatory oversight and patient counseling to ensure that ART patients receive evidence-based care, particularly when it comes to costly add-on treatments (Shionoya et al.). Another study addressing septate uterus correction through septum resection versus expectant management found no significant improvement in reproductive outcomes following septum resection. These results challenge the routine use of septum resection in patients with septate uterus, suggesting that a more cautious approach may be warranted (Liu et al.).

Conclusion

This extensive body of research on sexual precocity and infertility demonstrates significant progress in reproductive health. Innovations in ART protocols, personalized hormone therapies, and complementary treatments such as acupuncture and traditional medicine have shown promising results. However, challenges remain, particularly in optimizing treatments for specific subgroups such as those with poor ovarian response, high BMI, or advanced male age. Furthermore, ethical concerns regarding the use of unproven ART add-ons and the long-term safety of puberty suppression highlight the need for continued research and regulatory oversight. By embracing both modern and traditional approaches, as well as integrating technological advancements such as machine learning, the future of reproductive medicine holds great promise. Continued exploration into the genetic and hormonal mechanisms underlying reproductive disorders will pave the way for more effective, individualized therapies and improved clinical outcomes.

Author contributions

DP: Conceptualization, Writing – original draft. GM: Project administration, Writing – review & editing. CS: Supervision, Validation, Writing – review & editing.

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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Analysis of cumulative live birth rate and perinatal outcomes in young patients with low anti-müllerian hormone levels using two ovulation promotion protocols: A cohort study

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Objective: To compare cumulative live birth rates and perinatal outcomes of young IVF/ICSI patients with low anti-Mullerian hormone (AMH) levels on a gonadotropin-releasing hormone antagonist (GnRH-ant) regimen with those on a high progesterone state of ovulation (PPOS) regimen.

Methods: We retrospectively analyzed 798 patients who underwent *in vitro* fertilization (IVF) or intracytoplasmic sperm microinjection (ICSI) between January 2015 and December 2020 at the Third Affiliated Hospital of Zhengzhou University. A total of 798 cycles of complete clinical data from patients who underwent *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) at the Reproductive Medicine Center of Zhengzhou University Hospital between January 2015 and December 2020 and were eligible for AMH < 1.2 ng/ml at age < 35 years, Group A1: very low AMH levels (AMH < 0.5 ng/mL) and GnRH antagonist regimen; Group A2, very low AMH level (AMH < 0.5 ng/mL) and PPOS regimen; Group B1, low AMH level (0.5 ng/mL ≤ AMH < 1.2 ng/mL) and GnRH antagonist regimen; and Group B2, low AMH level (0.5 ng/mL ≤ AMH < 1.2 ng/mL), and the PPOS regimen.

Results: At very low levels of AMH (< 0.5 ng/mL), the CLBR of the GnRH antagonist regimen was not significantly different from that of the PPOS regimen (P > 0.05), at 0.5 ng/mL ≤ AMH < 1.2 ng/mL. Statistics showed that the CLBR of the GnRH antagonist regimen was significantly higher than that of the PPOS regimen (49.7% vs. 35.7%, P=0.002). Logistic regression analysis showed that in Group A: the younger the female partner, the higher the CLBR (OR = 0.972, 95% CI = 0.923–1.042, P = 0.022), and the more the AFC, the higher the CLBR (OR = 1.166, 95% CI = 1.091–1.336, P < 0.001). Group B: the higher the number of good-quality embryos, the higher the CLBR (OR = 2.227, 95% CI = 1.869–2.654, P < 0.001). Compared with PPOS regimens, the antagonist regimen was able to increase the CLBR. The analysis of Group A showed that the antagonist regimen had a shorter TTP than the PPOS regimen

(P < 0.001); however, the PPOS regimen had a lower cost of ovulation (4311.91 vs. 4903.81, P = 0.023). The antagonist regimen in Group B had a shorter TTP than the PPOS regimen, and there was no significant difference in the cost of ovulation. In the analysis of perinatal outcomes, there were no statistically significant differences in preterm birth, low birth weight, very low birth weight, and pregnancy complications among the four groups.

Conclusion: Young patients with very low AMH levels (< 0. 5 ng/mL), the GnRH antagonist regimen was comparable to the PPOS regimen in CLBR outcomes; the antagonist regimen shortens the time to clinical pregnancy, and the PPOS regimen is more cost-effective. In young patients with low AMH levels of 0.5 ng/mL and <1.2 ng/mL, the GnRH antagonist regimen can more appropriate to improve CLBR, and the perinatal outcomes were similar for both regimens.

KEYWORDS

anti-Mullerian hormone, *in vitro* fertilization/intracytoplasmic single sperm injection, GnRH antagonist protocol, progestin-primed ovarian stimulation protocol, cumulative live birth rate

1 Introduction

In assisted reproductive technology (ART)-assisted conception, the dose-response relationship between serum AMH and oocyte acquisition and its accuracy in predicting poor and excessive responses to ovarian stimulation has been well established (1). Poor ovarian response (POR) during controlled ovarian stimulation is one of the most challenging problems for reproductive clinicians (2). POR is often characterized by low AMH levels and low numbers of oocytes obtained using ovarian stimulation protocols. Owing to the low number of eggs obtained, patients with POR have higher cycle cancellation rates, lower pregnancy rates, and a lower cumulative live birth rate per starting cycle than normal responders (3). In many fertility centers, antagonist regimens and progestin-primed ovarian stimulation (PPOS) regimens are becoming ovulation regimens that are commonly used for patients with low ovarian response (4, 5). Research on POR has focused on the use of progesterone in the ovarian system. The literature on POR has focused on women aged > 35 years because of the age-related decline in ovarian reserve function. However, we have found that the incidence of POR in women aged under 35 years is similar to that in women older than 35 years (6). In this study, we performed a retrospective analysis of patients who underwent IVF/ICSI in the last 5 years at our center and were of age < 35 years with AMH < 1.2 ng/ml to explore the possible impact of different ovulation regimens on the final pregnancy outcome of IVF/ICSI in young patients with low AMH levels, to select the best ovulation regimen for young patients with different levels of low AMH in clinical practice. The results of this review are used to examine the possible impact of ovulation regimens on the final pregnancy outcome of young patients with different levels of low AMH and to improve the pregnancy outcome of patients with POR during ART.

2 Materials and methods

2.1 Study design and population

Patients who underwent *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) at the Department of Reproductive Medicine of the Third Affiliated Hospital of Zhengzhou University between January 2015 and December 2020 were selected for the retrospective analysis. The inclusion criteria were: 1) age < 35 years, 2) AMH < 1.2 ng/ml (= 8.568pmol/L), and 3) use of a gonadotropin-releasing hormone antagonist (GnRH-ant) regimen or a high progesterone state pro-ovulation (PPOS) regimen. The exclusion criteria were: 1) endometrial polyps, uterine adhesions, or abnormal uterine anatomy; 2) endocrine disorders namely, abnormal thyroid function, hyperprolactinemia; 3) systemic diseases such as reproductive tuberculosis; 4) women undergoing PGT and women with known chromosomal abnormalities; and 5) frozen oocytes or oocytes obtained through donation.

In total, 798 cycles of complete clinical data were available. Grouping was conducted according to the participants' AMH levels and different ovulation regimens: Group A1: very low

AMH levels (AMH < 0.5 ng/mL) and antagonist regimens for 54 cycles; Group A2, very low AMH levels (AMH < 0.5 ng/mL) and PPOS regimens, 214 cycles; Group B1: low AMH levels (0. 5 ng/mL ≤ AMH < 1.2 ng/mL) and antagonist regimens for 191 cycles; Group B2: low AMH levels (0. 5 ng/mL ≤ AMH < 1.2 ng/mL) and PPOS regimen groups,339 cycles.

2.2 Testing

(1) Blood AMH test: 4 mL of blood were drawn on empty stomach and sent to the test center for chemiluminescence testing.

(2) Sex hormones (follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2)): Venous blood (4 mL) was collected at 2–4 d of the menstrual cycle on empty stomach and assayed by electrochemiluminescence. The assay was performed by electrochemiluminescence using an E602 assay device, and the kit was purchased from the manufacturer.

(3) AFC test: The same vaginal ultrasound machine was used to measure sinus follicles—2–10 mm in diameter at 2–4 d of the menstrual cycle—and the total number of follicles in both ovaries was calculated.

2.3 Controlled ovarian hyperstimulation protocols

2.3.1 GnRH antagonist protocol

In the GnRH antagonist group, initiation was started on days 2 and 3 of the menstrual cycle, combining patient age, body mass index (BMI), AMH, basal FSH levels, and basal AFC, with a starting dose of Gn of 150–300 IU/d, added when the mean follicle diameter was 11–12 mm and the estradiol was > 500 ng/L (1835 pmol/L) GnRH-A (Stryker, Merck Serono, USA) or Ganirelix acetate injection, (N.V. Organon, The Netherlands), triggered when three follicles were > 17 mm in diameter or two follicles were >18 mm in diameter. Trigger drug selection: if women who were OHSS-prone, they can choose the plan A: GnRH-a [Dabigat, Pfizer (Switzerland) Pharma Ltd] 0.2 mg trigger + all embryos frozen, 33–36 h later puncture for egg retrieval under vaginal ultrasound guidance; for women who were not OHSS-prone, they can choose the plan B:Azer 250 μg trigger, and then 36 h later egg retrieval + fresh cycle transplantation. Conventional IVF/ICSI insemination was performed.

2.3.2 PPOS

For the PPOS regimen, a Gn initiation dose from 75–300 IU/day was administered on days 2 and 3 of the menstrual cycle, depending on the patient's age, body mass, BMI, AMH, and basal AFC, along with 6 mg medroxyprogesterone acetate (MPA) (Zhongxin Pharmaceuticals, Beijing, China). Follicle growth was monitored using vaginal ultrasound combined with serum hormone analysis after 4 d. If necessary, the dose of Gn was adjusted according to the follicle development. When three follicles were > 17 mm in diameter or two follicles were >18 mm in diameter, the final stage of ovulation triggering was performed with Daphne (Epsom, France) 0.1 mg and 2000 IU human chorionic gonadotropin (hCG) (Zhuhai Lizhu Group Lizhu Pharmaceuticals), followed by egg retrieval at 36 h.

2.4 Embryo transfer and endometrial preparation protocols

For patients on the antagonist regimen, fresh cycle transfer was possible if the endometrium was in good condition (thickness ≥ 7 mm; acceptable morphology) and there were no contraindications to transfer. Whole embryo freezing was performed in patients treated with PPOS.

Depending on the regularity of the menstrual cycle, a natural cycle or hormone replacement cycle was routinely selected to prepare the endometrium. Stimulation cycles were used if the menstrual cycle was regular, but follicular development was monitored using ovulation-promoting drugs.

2.5 Outcome measures and definition

The primary observation was the cumulative live birth rate (CLBR). The secondary observation was TTP: the time from the start of the prolotherapy program to clinical pregnancy. A clinical pregnancy rate [(number of clinical pregnancy cycles/number of transplant cycles) × 100%] determined by a positive blood test for β-hCG 14 d after transplantation was considered a pregnancy; a clinical pregnancy was diagnosed on an ultrasound 30 d after transplantation if a gestational sac was observed. Perinatal indicators: Gestational age (GA) is defined as the age from the first day of the last normal menstrual period to the time of delivery, usually expressed in weeks, for preterm babies (newborns with a GA < 37 weeks). The birth weight of newborns born alive was assessed as follows: low birth weight (LBW < 2500 g) and very low birth weight (VLBW < 1500 g) (7). Common pregnancy complications of IVF/ICSI comprised gestational hypertension, gestational diabetes, placenta previa, and premature membrane rupture (8, 9).

2.6 Assessment of CLBR

(1) CLBR: the number of patients who obtained a live birth in a fresh cycle after ovarian stimulation and the subsequent

thawing transfer cycle/number of patients who started an ovarian stimulation cycle; (2) Optimistic CLBR: assumes that women who do not return for a subsequent IVF cycle have the same chance of becoming pregnant as those who return for treatment; (3) Conservative CLBR: assumes that patients who do not return for subsequent IVF/ICSI treatment have no chance of a pregnancy resulting in a live birth (10, 11).

2.7 Statistical analysis

Statistical analysis was performed using SPSS 24.0, with normally distributed results expressed as mean ± standard deviation (mean ± SD); non-normally distributed measures were expressed as median (25th percentile, 75th percentile) [M (Q1, Q3)]; quantitative data between the two groups were tested using the rank sum test or independent samples *t* test; count data were expressed as frequency (%) using the $\chi 2$ test, and the factors influencing the CLBR were analyzed using logistic regression analysis. The test level was set at $P < 0.05$.

3 Results

A total of 798 egg retrieval cycles were included in this study, with 245 cycles in the antagonist group and 553 cycles in the PPOS group.

3.1 Baseline characteristics

In the antagonist regimen for young patients, the AMH level, AFC, Gn usage time, E2 level on HCG day, the total number of eggs obtained, 2PN number, number of available embryos, number of high-quality embryos and the cumulative live birth rate in group B1 were higher than those in group A1 (P<0.05). Details are provided in Table 1.

In the PPOS regimen for young patients, The AMH level, AFC, Gn initiation dose, E2 level on HCG day, the total number of eggs obtained, 2PN number, number of available embryos, number of high-quality embryos and the cumulative live birth rate in group B2 were higher than those in group A2 (P<0.05). Details are provided in Table 2.

Groups A1 and A2 showed no statistical difference in BMI, years of infertility, basal FSH, Gn initiation, total number of eggs gained, number of 2PN, number of available embryos, and number of good-quality embryos ($p > 0.05$). Patients on the PPOS regimen had a higher duration of Gn use than those on the antagonist regimen and a lower number of sinus follicles than those on the antagonist regimen. Details are provided in Table 3.

A comparison of the basic clinical data and laboratory results of patients in groups B1 and B2 showed no statistically significant differences in age, BMI, or years of infertility, and the PPOS regimen had a higher Gn initiation than the antagonist regimen. Details are provided in Table 4.

TABLE 1 Comparison of basic clinical information and laboratory results of patients with different AMH levels using antagonist regimens.

Projects	Antagonist regimen (A1)	Antagonist regimen (B1)	Z/t/x^2 Value	P-value
Number of cycles	54	191		
Female age (years)	30 (28, 32)	31.5 (29, 33)	0.374	0.709
Body mass index (kg/m2)	23.92 ± 0.27	23.68 ± 0.26	-0.732	0.465
Years of infertility (years)	3 (2, 5)	3(2,5)	0.007	0.995
Anti-Mullerian hormone (ng/ml)	0.32 (0.24, 0.42)	0.85(0.71,1.00)	-20.060	<0.001
Basal FSH (IU/L)	10(7,16)	8.32 (6.04, 9.58)	4.438	<0.001
Basal E2 (pmol/L)	160.2 (114.4, 193.8)	159.05 (119.96, 192.84)	-0.390	0.697
Sinus follicle count AFC	6(4,7)	8(6,11)	-5.756	<0.001
Gn initiation volume (U)	300.0(225.0, 300.0)	300 (225, 300)	-1.759	0.08
Gn usage time (d)	9 (7.25, 10)	10 (8, 11)	-2.208	0.028
hCG Day				
The endometrial thickness on hCG day (mm)	10.19 ± 0.33	10.36 ± 0.17	-0.770	0.442
hCG day LH (IU/L)	3.725 (2.03, 6.27)	1.8 (1.1125, 2.975)	5.071	<0.001
hCG day estradiol (pmol/L)	2171.5 (898.25, 3991.0)	3362 (1582, 6232.5)	-4.043	<0.001
Total number of eggs obtained (pieces)	3(2,4.75)	6 (4, 9)	-6.019	<0.001
2PN number (pieces)	2(1,4)	3 (2, 5)	-4.166	<0.001
Number of available embryos (pieces)	2(1,3)	3 (2, 4)	-4.231	<0.001
Number of high-quality embryos (pieces)	1 (0, 1)	1 (0, 2)	-3.140	<0.001
Cumulative live birth rate	22.2% (12/54)	49.7% (95/191)	12.957	<0.001
Optimistic cumulative live birth rate	22.0% (13/59)	49.3% (110/223)	14.133	<0.001
Conservative cumulative live birth rate	20.3% (12/59)	42.6% (95/223)	9.820	0.002

TABLE 2 Comparison of basic clinical information and laboratory results of patients with different AMH levels using PPOS protocol.

Projects	PPOS Program (A2)	PPOS Program (B2)	Z/t/x^2 Value	P-value
Number of cycles	214	339		
Female age (years)	31 (29, 33)	31 (29, 33)	-0.312	0.755
Body mass index (kg/m2)	23.00 ± 0.46	23.53 ± 0.19	1.706	0.089
Years of infertility (years)	3 (2, 6)	3(2,5)	0.255	0.799
Anti-Mullerian hormone (ng/ml)	0.32 (0.22, 0.43)	0.82(0.68,0.99)	-15.826	<0.001
Basal FSH (IU/L)	9(7,14)	8.37 (6.60, 10.71)	-0.178	0.859
Basal E2 (pmol/L)	138.8 (86.4, 200.4)	146.4 (104.35,196.4)	0.470	0.639
Sinus follicle count AFC	4(3,6)	7(5,10)	-9.137	<0.001
Gn initiation volume (U)	300.0(225.0, 300.0)	300 (300, 300)	-4.447	<0.001
Gn usage time (d)	9 (8.0, 12.0)	9 (8, 11)	1.623	0.105
hCG Day				
The endometrial thickness on hCG day (mm)	7.63 ± 0.15	7.93 ± 0.11	-0.631	0.528
hCG day LH (IU/L)	3.61 (2.01, 5.10)	3.035 (1.81, 4.608)	3.695	<0.001
hCG day estradiol (pmol/L)	2859.0 (1499.0, 4581.0)	5065(3319.5,7478.5)	-7.898	<0.001
Total number of eggs obtained (pieces)	3(1,5)	5 (3, 7)	-7.944	<0.001
2PN number (pieces)	2(1,3)	3 (2, 5)	-5.804	<0.001
Number of available embryos (pieces)	2(1,2)	2 (1, 4)	-4.807	<0.001
Number of high-quality embryos (pieces)	1 (0, 2)	1 (0, 3)	-4.507	<0.001
Cumulative live birth rate	26.6% (57/214)	35.7% (121/339)	4.931	0.026
Optimistic cumulative live birth rate	26.5% (69/260)	35.6% (154/432)	6.167	0.013
Conservative cumulative live birth rate	21.9% (57/260)	28.0% (121/432)	3.147	0.076

TABLE 3 Group A patients Comparison of basic clinical information and laboratory results [$\bar{x} \pm s$, M(Q1, Q3)].

Projects	Antagonist regimen (A1)	PPOS Program (A2)	*Z/t/* x^2 values	*P* value
Number of cycles	54	214		
Age of woman (years)	30 (28, 32)	31 (29, 33)	2.419	0.016
Body mass index (kg/m^2)	23.92 ± 0.27	23.00 ± 0.46	1.592	0.113
Years of infertility (years)	3 (2, 5)	3 (2, 6)	-0.219	0.827
Anti-Mullerian hormone (ng/ml)	0.32 (0.24, 0.42)	0.32 (0.22, 0.43)	-0.187	0.851
Basal FSH (IU/L)	10(7,16)	9(7,14)	-1.094	0.274
Basal E2 (pmol/L)	160.2 (114.4, 193.8)	138.8 (86.4, 200.4)	-1.397	0.163
Sinus follicle count AFC	6(4,7)	4(3,6)	-2.659	0.036
Gn initiation volume (U)	300.0 (225.0, 300.0)	300.0 (225.0, 300.0)	-1.388	0.165
Gn Duration of use (d)	9 (7.25, 10)	9 (8.0, 12.0)	-2.607	0.040
hCG Day				
hCG day endometrial thickness (mm)	10.19 ± 0.33	7.63 ± 0.15	-7.493	<0.001
hCG day LH (IU/L)	3.725 (2.03, 6.27)	3.61 (2.01, 5.10)	-0.468	0.64
hCG day estradiol (pmol/L)	2171.5 (898.25, 3991.0)	2859.0 (1499.0, 4581.0)	-2.012	0.047
Total number of eggs harvested (pieces)	3(2,4.75)	3(1,5)	-0.684	0.494
2PN number (pieces)	2(1,4)	2(1,3)	-0.350	0.726
Number of embryos available (pieces)	2(1,3)	2(1,2)	-0.253	0.800
Number of good-quality embryos (pieces)	1 (0, 1)	1 (0, 2)	-0.422	0.673
Cumulative live birth rate	22.2% (12/54)	26.6% (57/214)	0.439	0.507
Optimistic cumulative live birth rate	22.0% (13/59)	26.5% (69/260)	0.511	0.475
Conservative cumulative live birth rate	20.3% (12/59)	21.9% (57/260)	0.071	0.790

TABLE 4 Comparison of basic clinical information and laboratory results of patients in Group B [$\bar{x} \pm s$, M(Q1, Q3)].

Projects	Antagonist regimen (B1)	PPOS Program (B2)	$Z/t/x^2$ values	*P* value
Number of cycles	191	339		
Age of woman (years)	31.5 (29, 33)	31 (29, 33)	0.266	0.790
Body mass index (kg/m^2)	23.68 ± 0.26	23.53 ± 0.19	0.481	0.631
Years of infertility (years)	3(2,5)	3(2,5)	-0.491	0.623
Anti-Mullerian hormone (ng/ml)	0.85(0.71,1.00)	0.82(0.68,0.99)	-1.167	0.243
Basal FSH (IU/L)	8.32 (6.04, 9.58)	8.37 (6.60, 10.71)	-1.774	0.201
Basal E2 (pmol/L)	159.05 (119.96, 192.84)	146.4 (104.35,196.4)	-1.639	0.101
Sinus follicle count AFC	8(6,11)	7(5,10)	-4.119	<0.001
Gn initiation volume (U)	300 (225, 300)	300 (300, 300)	-6.558	<0.001
Gn Duration of use (d)	10 (8, 11)	9 (8, 11)	-0.914	0.361
hCG Day				
hCG day endometrial thickness (mm)	10.36 ± 0.17	7.93 ± 0.11	12.329	<0.001
hCG day LH (IU/L)	1.8 (1.1125, 2.975)	3.035 (1.81, 4.608)	-6.516	<0.001
hCG day estradiol (pmol/L)	3362 (1582, 6232.5)	5065 (3319.5,7478.5)	-5.373	<0.001
Total number of eggs harvested (pieces)	6 (4, 9)	5 (3, 7)	-3.284	0.001
2PN number (pieces)	3 (2, 5)	3 (2, 5)	-1.772	0.085
Number of embryos available (pieces)	3 (2, 4)	2 (1, 4)	-2.094	0.036
Number of good-quality embryos (pieces)	1 (0, 2)	1 (0, 3)	-0.413	0.680
Cumulative live birth rate	49.7% (95/191)	35.7% (121/339)	9.981	0.002
Optimistic cumulative live birth rate	49.3% (110/223)	35.6% (154/432)	11.439	<0.001
Conservative cumulative live birth rate	42.6% (95/223)	28.0% (121/432)	14.168	<0.001

3.2 Factors associated with CLBR

There was no significant difference in CLBR between the two regimens for patients with very low AMH levels, and the choice of antagonist regimen for patients with low AMH levels can improve the cumulative live birth rate (Tables 3, 4). To explore the possible factors affecting CLBR, we performed multiple regression analysis on clinical indicators such as female's partner's age, AFC, AMH, female BMI, number of good-quality embryos, and the two prolotherapy regimens. As shown in Table 5, Group A: the younger the female's partner, the higher the CLBR (OR = 0.972, 95% CI = 0.923–1.042, P = 0.022), and the more the AFC (OR = 1.166, 95% CI = 1.091–1.336, P < 0.001), while the higher the number of good-quality embryos, the higher the CLBR (OR = 2.654, 95% CI = 1.911–3.687, P < 0.001). Group B: the higher the number of good-quality embryos, the higher the CLBR (OR = 2.227, 95% CI = ~1.869–2.654, P < 0.001), the antagonist regimen was able to increase CLBR compared with PPOS regimens (OR = 0.499, 95% CI = 0.324–0.767, P = 0.002 for the PPOS regimen with the antagonist regimen as the control group).

3.3 Comparison of patients' TTP and prolotherapy costs

As shown in Table 6, the antagonist regimen in Group A had a significantly higher cost of promotion during ovulation than the PPOS regimen, and the antagonist regimen had a significantly lower TTP than the PPOS regimen (p < 0.001). The rate of embryo-free cycle transfer was not statistically different between the two regimens at very low AMH levels ($P > 0.05$), and the rate of embryo-free cycle transfer was higher for the PPOS regimen than for the antagonist regimen in Group B (19.2% *vs.* 2.1%, $P < 0.001$); the clinical pregnancy rate was higher for patients using the antagonist regimen than the PPOS regimen.

3.4 Analysis of perinatal outcomes

There were no statistical differences between groups in perinatal outcomes for neonatal outcomes and pregnancy complications in Groups A1, A2, B1, and B2 ($p > 0.05$) (Table 7).

4 Discussion

By competing with endogenous GnRH for pituitary cell GnRH receptors, GnRH antagonists regulate pituitary surface GnRH receptor levels, inhibit early LH peaks and endogenous FSH and LH levels, more closely resemble the development of follicles under normal physiological conditions, prevent early follicular luteinization or ovulation, and result in more high-quality eggs (12). PPOS (13) The European Society of Human

TABLE 5 Multi-factor logistic analysis of cumulative live birth rate.

Variables	*B*	Wald	*OR* value	95% CI	*P* value
Group A					
Age of woman	0.159	0.237	0.972	0.923~1.042	0.022
AFC	0.153	6.368	1.166	1.091~1.336	< 0.001
AMH	0.047	0.002	1.048	0.102~10.806	0.969
BMI	0.077	2.701	1.080	0.985~1.183	0.100
Number of good-quality embryos	0.976	33.894	2.654	1.911~3.687	<0.001
Ovulation Program					
Antagonists	-0.190	0.197	0.827	0.358~1.913	0.657
PPOS (control group)	–	–	–	–	–
Group B					
Age of woman	0.003	0.008	1.003	0.933~1.079	0.928
AFC	0.022	0.491	1.022	0.961~1.088	0.484
AMH	0.320	0.322	1.378	0.455~4.169	0.570
BMI	-0.018	0.337	0.982	0.924~1.044	0.562
Number of good-quality embryos	0.801	80.142	2.227	1.869~2.654	< 0.001
Ovulation Program#					
Antagonist (control group)	–	–	–	–	–
PPOS	-0.696	10.044	0.499	0.324~0.767	0.002

#Referenced by the antagonist protocol.

Reproduction and Embryology (ESHRE) guidelines for ovarian stimulation in IVF/ICSI state that antagonist regimens are routinely preferred ovulation regimens in the POR population (14). A recent study by Du et al. in our center showed that (15) for patients with a low prognosis diagnosed according to the POSEIDON criteria, the CLBR of the PPOS regimen was comparable to that of the GnRH antagonist regimen. In contrast with the PPOS regimen, which supports fresh embryo transfer, the PPOS regimen must be combined with total freezing and delayed embryo transfer; therefore, the antagonist regimen significantly reduces the time to clinical pregnancy, consistent with the data from this trial, and the reduction in TTP may reduce patient anxiety to some extent (16).

Many studies have compared pregnancy outcomes between the antagonist and PPOS regimens. Chen et al. (16) randomized 340 patients eligible for Bologna with POR to a PPOS regimen versus an antagonist regimen to

TABLE 6 Comparison of patient cost-effectiveness and TTP.

Projects	Antagonist regimen (1)	PPOS program (2)	*t/Z/x^2* values	*P* value
Group A				
Cycle TTP(d)*				
Fresh transplant cycle	41.11 ± 7.88	–		
Freeze-thaw transplant cycle	122.41 ± 36.42	177.94 ± 39.67	4.039	<0.001
Total TTP	116.56 ± 11.52	177.94 ± 39.67	-1.601	0.008
Embryo-free transfer cycle rate (%)	24.1 (13/54)	29.0 (62/214)	0.513	0.474
Cost of ovulation promotion treatment ($)	4903.81 (3444.84, 5905.44)	4311.91 (1101.75, 5905.44)	-2.275	0.023
Group B				
Cycle TTP(d)*				
Fresh transplant cycle	40.04 ± 7.74	–		
Freeze-thaw transplant cycle	108.18 ± 33.56	124.38 ± 41.51	1.023	0.311
Total TTP	52.34 ± 35.15	124.38 ± 41.51	-4.436	0.001
Embryo-free transfer cycle rate (%)	2.1 (4/191)	19.2 (65/339)	31.472	<0.001
Cost of ovulation promotion treatment ($)	3936.96 (2123.59, 4429.08)	3383.33 (702, 4921.20)	-1.058	0.290

*Fisher precision inspection.

TABLE 7 Analysis of perinatal outcomes.

Projects	A1	A2	B1	B2	P *value*
Number of live births	12	57	95	121	
Premature babies	25.00 (3/12)	26.32 (15/57)	15.79 (15/95)	12.40 (15/121)	0.111
Low birth weight babies	33.33 (4/12)	12.28 (7/57)	11.58 (11/95)	14.88 (18/121)	0.238
Very low birth weight babies*	0.00 (0/12)	7.02 (4/57)	6.32 (6/95)	2.48 (3/121)	0.359
Hypertension in pregnancy*	0.00 (0/54)	1.87 (4/214)	3.14 (6/191)	3.24 (11/339)	0.570
Gestational diabetes	1.85 (1/54)	3.27 (7/214)	5.24 (10/191)	4.42 (15/339)	0.707
Placenta praevia	1.85 (1/54)	0.80 (1/214)	1.05 (2/191)	0.30 (1/339)	0.280
Premature rupture of membranes	0.00 (0/54)	5.65 (7/214)	2.09 (4/191)	2.95 (10/339)	0.648

* Fisher precision inspection.

promote ovulation and found similar live birth rates for the PPOS and GnRH antagonist regimens. A recent meta-analysis showed that (17) the total number of Gn days, total number of eggs gained, clinical pregnancy rates after transplantation, persistent pregnancy rates, and miscarriage rates were similar in the PPOS and GnRH antagonist regimens. However, there are few studies on cumulative live birth rate. CLBR is a more comprehensive, rational, and objective method than traditional IVF pregnancy outcome statistics, based on pregnancies obtained after one controlled ovarian stimulation cycle of fresh embryos transferred and all subsequent frozen embryos allowed for transfer (18). The main observation of this study was the CLBR, and the subjects were women of reproductive age (< 35 years). The effects of antagonist and PPOS regimens on the CLBR and perinatal outcomes of young patients with different levels of low AMH were investigated. The results showed that higher AMH levels were associated with higher CLBR levels in younger patients with low AMH (AMH < 1. 2ng/mL) using the same ovulation induction regimen, no significant difference in the CLBR between the GnRH antagonist and PPOS regimens in young patients with very low AMH levels (AMH < 0. 5 ng/mL), and the GnRH antagonist regimen in young patients with low AMH (0. 5 ng/mL ≤ AMH < 1. 2 ng/mL)can increase the CLBR.

Since 2015, Kuang (19) et al. have proposed that progestins could be used as a low-cost alternative to GnRH analogs in the treatment of patients with POR, with MPA and dydrogesterone as the main progestins. Subsequently, Evans (20) et al. constructed a cost model for PPOS and GnRH antagonist regimens. If only fresh embryo transfer is considered, the antagonist progesterone cost is higher than that of PPOS, and is not economically advantageous. As the PPOS regimen has a frozen embryo policy only, assuming a certain frozen embryo cycle time and cost in the model setting, the PPOS regimen still has a higher economic benefit per live birth than the antagonist regimen. This result is consistent with the superior cost-effectiveness of the PPOS regimen in facilitating ovulation observed in this study. Due to the limited data on the experimental sample size, there were also confounding factors in the data screening and statistical calculations.

With the increasing maturity of IVF/ICSI technology, the rate of conceiving and deliveries using this technique is increasing, with more than 1 million babies born worldwide through assisted reproduction (21). However, the health of these children is a growing concern. To investigate the safety of the two protocols for offspring health, further analysis of perinatal outcomes and neonatal outcomes based on follow-up records was conducted, which showed no significant differences in perinatal outcomes among groups A1, A2, B1, and B2 ($p > 0.05$).

Conclusion

In summary, for young patients with AMH < 0.5 ng/mL levels, the CLBR outcome of the GnRH antagonist program was similar to that of the PPOS program, which could shorten the time to clinical pregnancy, and the PPOS program was more economical and beneficial than that of the GnRH antagonist program. For young patients with low AMH levels of 0.5 ng/mL ≤ AMH < 1.2 ng/mL, the GnRH antagonist regimen may be more suitable for improving CLBR than the PPOS regimen and the perinatal outcomes of the two regimens are similar.

Data availability statement

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

Ethics statement

Studies involving human participants were reviewed and approved by the Ethics Committee of the Third Affiliated Hospital of Zhengzhou University. Written informed consent

for participation was not required for this study, in accordance with national legislation and institutional requirements.

Author contributions

YG and ZL performed data extraction and analysis. KW, JZ, and BR reviewed the data. RJ and ZL drafted the manuscript. All authors contributed to the manuscript 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 potential conflicts of interest.

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Luteinizing hormone-based modified GnRH antagonist protocol in normal responders undergoing *in vitro* fertilization treatment: A multi-center randomized controlled trial

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Objective: To study the clinical efficacy and cost-effectiveness of a modified gonadotrophin-releasing hormone (GnRH) antagonist protocol based on luteinizing hormone (LH) levels through one complete assisted reproductive technology (ART) cycle in normal responders.

Design: Non-inferiority, multicenter randomized controlled trial.

Setting: University-based hospitals and an academic medical center.

Patients: A total of 372 patients fulfilled the inclusion criteria and were eligible to participate.

Intervention(s): Participants were randomized at a 1:1 ratio and stimulated with the conventional flexible GnRH antagonist protocol (control group) or LH-based modified GnRH antagonist protocol (study group).

Main Outcome Measures: The primary outcome was the cumulative ongoing pregnancy rate per aspiration. The secondary outcomes were number of oocytes retrieved, number of good quality embryos, cumulative positive βhCG rate, cumulative clinical pregnancy rate, pregnancy loss rate, moderate and severe ovarian hyperstimulation syndrome (OHSS), and financial expenditure.

Results: The cumulative ongoing pregnancy rate was 65.1% in the study group and 70.1% in the control group (odds ratio, 0.79; 95% confidence interval, 0.50–1.26; P = 0.33). The multivariate regression analyses results showed that the number of retrieved oocytes was positively associated with the odds for a

higher cumulative ongoing pregnancy rate (adjusted odds ratio, 1.11, 95% confidence interval, 1.06–1.17, $P < 0.001$). The treatment protocol, female age, and body mass index were not independent predictors. The incremental cost-effectiveness ratio for luteinizing hormone-based gonadotrophin releasing hormone antagonist protocol *versus* the conventional flexible gonadotrophin releasing hormone antagonist protocol was estimated at 3568.6 USD for each additional ongoing pregnancy.

Conclusion: The luteinizing hormone-based gonadotrophin releasing hormone antagonist protocol had clinical efficacy similar to the conventional flexible gonadotrophin releasing hormone antagonist protocol in normal responders undergoing *in vitro* fertilization treatment but was more cost-effective considering the cumulative ongoing pregnancy rate in the entire assisted reproductive technology cycle.

Clinical Trial Registration: www.chictr.org.cn, identifier: ChiCTR1800018077

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Date of first patient enrollment: 1 September 2018.

KEYWORDS

ovarian stimulation, gonadotrophin releasing hormone antagonist protocol, luteinizing hormone, reproductive outcome, *in vitro* fertilization

Introduction

Assisted reproductive technology (ART) is the most important method to treat infertility. Gonadotrophin releasing hormone (GnRH) antagonist protocol has been widely used in the past few decades. The conventional GnRH antagonist protocols include fixed and flexible protocols, with the antagonist administrated from day 5 or 6 onwards, or when the dominant follicle had reached 14 mm, respectively (1, 2). Since its introduction, many studies have sought to determine the best day to start GnRH antagonist administration and the optimal daily dosage (3, 4).

The GnRH antagonist administration aims to prevent an untimely luteinizing hormone (LH) surge and premature luteinization. Previous studies evaluating technical aspects of these protocols to guide antagonist administration mainly focused on the day of gonadotropin stimulation or the diameter of the follicles rather than the LH level. In recent years, increasing attention has been paid to the role of LH. The secretion and response of LH levels to the GnRH antagonists vary widely between individuals (5–8). Previous studies have indicated that an ultra-high or low LH level would cause harm to pregnancy outcomes (9–11). Although it has remained controversial, an LH stimulation threshold is required for adequate follicular development and oocyte maturation. This made us think whether LH could be used as an indicator for the timing of antagonist addition, and a modified protocol named "LH-based GnRH antagonist protocol" was proposed. Our previous proof-of-concept study showed that this protocol provided comparable results to the traditional flexible antagonist protocol (12). The results proved that LH levels may be used as an indicator for the time of antagonist addition. However, it had inherent limitations because of its nature of retrospective design. The inclusion criteria were kind of too general and needed to be further refined. On the other hand, assessment of the cost-effectiveness of treatment is essential before recommendations can be made. To further confirm the validity of our previous observations and evaluate its clinical efficacy, we performed this non-inferiority randomized controlled trial (RCT) in normal responders undergoing *in vitro* fertilization (IVF). Besides, cost-effectiveness analysis was also performed to provide reference for medical decision-making.

Materials and methods

Study design and patients

This trial was a non-blinded, multicenter RCT. It was approved by the Ethics Committee of Beijing Chao-Yang Hospital, Capital Medical University (number 2018-SCI-194), and the institutional review boards of all the participating centers. This trial was registered at the Chinese Clinical Trial Registry (number ChiCTR1800018077, registered in August 2018), and the protocol has previously been published (13). All participating couples provided written informed consent. Inclusion criteria were as follows: (1) aged 23–38 years; (2) a spontaneous menstrual cycle length of 21–35 days; (3) an antral follicle count (AFC) of 8–20; (4) a body mass index (BMI) of 18–28 kg/m^2. The exclusion criteria included the following: (1) recurrent spontaneous abortion; (2) a diagnosis of polycystic ovarian syndrome (PCOS); (3) uterine abnormalities such as submucosal myoma, adenomyosis, or intrauterine adhesion; (4) a chronic medical disease affecting pregnancy outcomes such as diabetes mellitus or uncontrolled hypertension.

Randomization and masking

Randomization was performed by a clinician on the initial day of ovarian stimulation. Patients were randomized at a 1:1 ratio using a web-based concealed randomization code generated by a central online database (www.medresman.org). After randomization, patients were assigned into two groups: a control group stimulated with the conventional flexible GnRH antagonist protocol or a study group stimulated with the LH-based GnRH antagonist protocol. Clinicians and patients were not masked for the assigned interventions.

Treatment

150-300 IU recombinant follicle-stimulating hormone (r-FSH, Gonal F, Merck Serono, Darmstadt, Germany) was administered daily for four to five days from Day 2-3 of the menstrual cycle. Hormone tests and ultrasonographic monitoring were performed 4-5 times at regular intervals during ovarian stimulation, as follows: 1) day 1 of stimulation (early follicular phase); 2) 4-5 days after stimulation initiation (mid-follicular phase); 3) 2 days later, i.e., 6-7 days after initiation (mid-late follicular phase); 4) the day of triggering (late follicular phase). Then gonadotropin dose might be adjusted according to the hormone levels and follicular development.

For patients in the study group, administration of the GnRH antagonist (Cetrotide, Merck Serono) was based on the LH level from day 6 of ovarian stimulation, as mentioned before (14). Briefly, no antagonist was administered if LH level was ≤4 IU/L; 0.125 mg antagonist was administered daily for two days, until the next blood test, if 4 < LH level ≤ 6 IU/L; 0.25 mg antagonist was administered daily for two days if 6 < LH level ≤ 10 IU/L; 0.375 mg antagonist was administered for one day if 10 < LH level ≤ 15 IU/L; 0.5 mg antagonist was administered for one day if LH level >15 IU/L. The decision to continue antagonist administration was based on whether the subsequent LH test result was > 4 IU/L. This procedure continued until trigger day. Patients in the control group were administered a flexible GnRH antagonist protocol. They received 0.25 mg antagonist daily until trigger day if at least one follicle had reached a diameter of 14 mm.

For both protocols, when more than two follicles were ≥18 mm in diameter, 0.2 mg triptorelin (Decapeptyl, Ipsen, Paris, France) and 2,000–3,000 IU human chorionic gonadotropin (hCG) were administered. Ovum pick up (OPU) was performed 36 h later, and the oocytes were fertilized by IVF or intracytoplasmic sperm injection (ICSI). The following treatment, including fresh and frozen embryo transfer, were underwent according to the clinical standards of the participating centers. The policies were described in detail elsewhere previously (14). Luteal-phase support was started from the day of oocyte retrieval with 90 mg daily of 8% vaginal progesterone gel (Crinone, Merck Serono) and 10 mg twice daily oral dydrogesterone (Duphaston, Abbott Biologicals B.V., Olst, the Netherlands). A maximum of two good-quality cleavage embryos were transferred on D3. All surplus embryos were cultured for two or three more days, and good-quality blastocysts were vitrified. If pregnancy was achieved, the luteal phase support was continued until 10 weeks' gestation after fresh embryo transfer. The endometrium was prepared for fresh and frozen embryo transfer (FET) cycles by either a natural or an artificial cycle regimen. Following a natural cycle regimen, luteal phase support with 10 mg dydrogesterone twice daily from ovulation day until 7 weeks' gestation was administered. For the artificial cycle regimen, 6 mg oral estradiol valerate (Progynova, Bayer AG, Leverkusen, Germany) daily was initiated from day 2–3 of the menstrual cycle for 10–14 days. If the endometrium was ≥7 mm, 90 mg vaginal progesterone gel daily and 10 mg dydrogesterone twice daily were added. Oral estradiol valerate was continued until 8 weeks' gestation, and vaginal progesterone gel and dydrogesterone were continued until 12 weeks' gestation.

Reproductive outcomes

End-of-study was the achievement of an ongoing pregnancy or transfer of all derived embryos through a complete ART cycle, including the fresh and FET cycles. The primary outcome was the cumulative ongoing pregnancy rate per aspiration. The

secondary outcomes were number of oocytes retrieved, number of good quality embryos, cumulative positive βhCG rate, cumulative clinical pregnancy rate, pregnancy loss rate, moderate and severe ovarian hyperstimulation syndrome (OHSS), and financial expenditure. Ongoing pregnancy was defined as visible fetal heart activity on ultrasonography from 12 weeks of gestation onwards. Positive βhCG was defined as plasma βhCG >10 IU/L. Clinical pregnancy was confirmed by observing a gestational sac by ultrasonography 2-3 weeks later.

Sample size

A retrospective assessment of our clinical database found a cumulative ongoing pregnancy rate per aspiration in normal responders of approximately 70%. Considering that a non-inferiority threshold should retain 80% of the conventional antagonist protocol clinical effect, a minimal clinical important difference of 14% was adopted. With a one-sided alpha of 2.5% and statistical power of 80%, 338 patients were needed. Considering a dropout rate of 10%, 372 patients were required.

Statistical analysis

A per-protocol comparison of the primary outcome was performed among those who adhered to their respective protocol. Intention-to-treat analysis was applied to the 367 eligible patients who did not withdraw their consent (Figure 1). Continuous data are expressed as means ± standard deviations. Between-group differences were tested by an independent samples *t*-test for normally distributed data and Wilcoxon rank-sum test for not normally distributed data. Categorical data are presented as frequency and percentage; differences were assessed by the chi-squared test or Fisher's exact test.

The cumulative ongoing pregnancy rate per aspiration was assessed crudely and using multivariate logistic regression analysis. The decision to add each potential confounding factor to the model was based on previous scientific evidence and the results in the unadjusted analyses. These factors included age, BMI, the number of oocytes retrieved, mean LH level during ovarian stimulation, and LH level on the triggering day. All analyses were done with the IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp., Armonk, NY, USA). A *P*-value < 0.05 was considered statistically significant.

Cost-effectiveness analysis

Economic evaluation in this study focused only on pharmacological compounds costs during ovarian stimulation till trigger day. The cost was equal to the unit cost of the drug multiplied by the total quantity administered. Costs were based on Beijing General Hospital prices and calculated in CNY and then converted to USD (1 CNY=0.1564 USD). We calculated the mean costs and effectiveness for both groups, with the conventional flexible GnRH antagonist protocol acting as the reference.

The incremental cost-effectiveness ratio (ICER) was based on the incremental cost per patient and cumulative ongoing pregnancy rate of the LH-based protocol compared to the flexible antagonist protocol. The incremental cost per patient reflected the additional cost per patient undergoing the LH-based antagonist protocol rather than the conventional protocol. The incremental cumulative ongoing pregnancy rate reflected

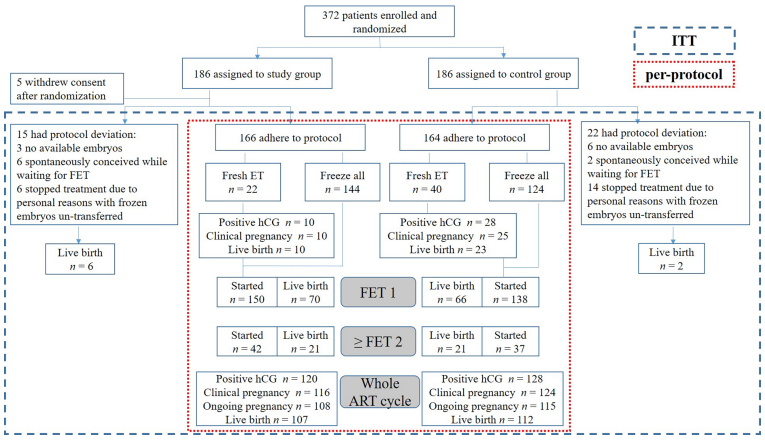

FIGURE 1
Trial flow chart. An overview of the study process and cumulative reproductive outcomes in the study and control groups. ITT, intention-to-treat; ET, embryo transfer; FET, frozen embryo transfer; hCG, human chorionic gonadotropin; ART, assisted reproductive technology.

the change in the cumulative ongoing pregnancy rate when the LH-based antagonist protocol was used instead of the conventional protocol. The ICER will be estimated as the ratio between difference in costs between protocols and the difference in cumulative ongoing pregnancy rates.

Results

Recruitment was done between September 2018 and June 2020. Patients were followed until all embryos obtained from an entire ART cycle were used or an ongoing pregnancy was achieved. Those who spontaneously conceived while waiting for FET and stopped treatment due to personal reasons with frozen embryos un-transferred for more than 12 months were exclude from the per-protocol analyses. Figure 1 displays the patient enrollment flowchart. The 372 enrolled patients were randomly assigned to either study or control group. Five patients in the study group withdrew their consent after randomization and were defined as dropped out cases. The baseline demographic and clinical characteristics in the two groups were compared, as shown in Table 1.

Stimulation outcomes

Patients in the study (n = 166) and control (n = 164) groups who adhered to their respective treatments were included in the following per-protocol analyses. As shown in Table 2, many ovarian stimulation characteristics were similar in the two groups; however, LH level on the trigger day was considerably higher in the study group (2.81 ± 1.89 vs. 2.14 ± 1.31 IU/L, P < 0.001). Hormone tests were performed regularly during ovarian stimulation, representing the early, mid, and late follicular phases. Then mean LH level could be figured out, which was much higher in the study group (3.64 ± 1.63 vs. 3.29 ± 1.27 IU/L, P = 0.032). The study group had a lower administered doses of GnRH antagonist (0.38 ± 0.27 vs. 1.01 ± 0.30 IU/L, P < 0.001) and less recombinant LH (rLH) (63.04 ± 44.63 vs. 124.39 ± 77.49 IU, P < 0.001) was used to maintain a reasonable LH level and normal follicular development. Similar ovarian response indexes, including follicle output rate (FORT), follicle-to-oocyte index (FOI), and ovarian sensitivity index (OSI), were noted in both groups, as were laboratory outcomes. Although similar total gonadotropin (Gn) dose was delivered in both groups, with lower administered doses of GnRH antagonist and rLH in the study group, it had considerably lower financial expenditure during ovarian stimulation (1,031.51 ± 281.59 vs. 1,209.94 ± 307.12 USD, P < 0.001). Ovarian stimulation and laboratory outcomes were also analyzed by intention-to-treat and showed similar results (data not shown).

Reproductive outcomes

The primary outcome, cumulative ongoing pregnancy rate per ART cycle, was 65.1% in the study group and 70.1% in the control group (odds ratio [OR], 0.79; 95% confidence interval [CI], 0.50–1.26; P = 0.33; Table 3), meeting the predefined non-inferiority objective. Fresh embryo transfer was performed in 22 patients in the study and 40 in the control group. Other patients were performed "freeze-all strategy" and underwent FET later.

TABLE 1 Baseline characteristics of the intention-to-treat population.

	Study group n = 181	Control group n = 186
Age (year)	31.40 ± 3.51	31.61 ± 3.08
BMI (kg/m^2)	22.38 ± 3.14	22.33 ± 3.12
Duration of infertility (year)	2.92 ± 1.90	2.71 ± 2.02
Diagnosis		
Primary infertility	121 (66.85%)	128 (68.82%)
Secondary infertility	60 (33.15%)	58 (31.18%)
Primary diagnosis of infertility		
Tubal factor	97 (53.59%)	135 (72.58%)
Anovulation	12 (6.63%)	7 (3.76%)
Endometriosis	6 (3.31%)	0 (0.00%)
Male factor	18 (9.94%)	10 (5.38%)
Combined factors	48 (26.52%)	34 (18.28%)
Basal FSH (IU/L)	6.85 ± 1.87	7.05 ± 2.15
Basal LH (IU/L)	4.57 ± 1.87	4.57 ± 1.97
Basal E_2 (pg/mL)	45.54 ± 15.49	45.67 ± 15.02
AFC	15.80 ± 5.67	15.67 ± 4.72

Values are represented as mean ± standard deviation, or n (%). BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone; AFC, antral follicle count; E_2, estradiol; Study group: LH-based GnRH antagonist protocol; Control group: conventional flexible GnRH antagonist protocol.

TABLE 2 The per-protocol population ovarian stimulation and laboratory outcomes.

Group	Study group	Control group	*P*-value
n	166	164	
Total Gn dose (IU)	1984.19 ± 582.33	2018.71 ± 536.63	0.58
Duration of Gn stimulation	9.58 ± 1.34	9.51 ± 1.09	0.59
LH on triggering day (IU/L)	2.81 ± 1.89	2.14 ± 1.31	<0.001
E_2 on triggering day (pg/mL)	3345.35 ± 1732.89	3211.84 ± 1448.31	0.74
P on triggering day (ng/mL)	0.83 ± 0.47	0.76 ± 0.34	0.50
Endometrial thickness on triggering day (mm)	10.69 ± 2.13	10.47 ± 2.25	0.37
Mean LH level during stimulation (IU/L)	3.64 ± 1.63	3.29 ± 1.27	0.03
GnRH antagonist dose (mg)	0.38 ± 0.27	1.01 ± 0.30	<0.001
rLH dose (IU)	63.04 ± 44.63	124.39 ± 77.49	<0.001
FORT (%)	0.81 ± 0.29	0.82 ± 0.33	0.87
FOI (%)	0.93 ± 0.41	0.98 ± 0.40	0.30
OSI	7.95 ± 4.68	7.90 ± 3.86	0.61
No. of oocytes retrieved	14.26 ± 6.27	14.68 ± 5.85	0.53
No. of 2PN	8.73 ± 5.36	8.84 ± 4.27	0.35
No. of high-quality embryos on D3	3.85 ± 3.14	4.03 ± 2.99	0.31
Financial expenditure (USD)	1,031.51 ± 281.59	1,209.94 ± 307.12	<0.001

Values are presented as mean ± standard deviation.
P-values were calculated using the chi-squared or Fisher's exact test for categorical data and the t-test for continuous data.
FORT, follicular output rate = No. of pre-ovulatory follicles/AFC; FOI, follicle-to-oocyte index = No. of oocytes retrieved/AFC; OSI, ovarian sensitivity index = number of retrieved oocytes/total Gn dose × 1,000; Gn, gonadotropin; GnRH, gonadotropin releasing hormone; LH, luteinizing hormone; rLH, recombinant luteinizing hormone; E_2, estradiol; P, progesterone; 2PN, two pronuclei; D3, Day 3.
Study group: LH-based GnRH antagonist protocol; Control group: conventional flexible GnRH antagonist protocol.

The secondary outcomes were comparable between the two groups for the fresh embryo transfer and first FET cycles in patients undergoing "freeze-all strategy". Cumulatively, the two groups had similar reproductive outcomes for the entire ART cycle, including the pregnancy loss rates. No severe treatment-emergent adverse events were reported, and no cycle was canceled due to excessive ovarian response or unexpected premature ovulation.

Table 4 summarizes the results of univariate and multivariate regression analyses for the cumulative ongoing pregnancy rate in the two groups. The results showed that the number of retrieved oocytes was associated with increased odds for a higher cumulative ongoing pregnancy (adjusted OR, 1.11; 95% CI, 1.06–1.17; $P < 0.001$). Treatment protocol, female age, and BMI were not independent predictors. The association between mean LH level during stimulation and cumulative ongoing pregnancy was statistically insignificant (OR, 1.07; 95% CI, 0.89–1.29; $P = 0.477$). Similar results were shown for LH level on trigger day.

Cost-effectiveness analysis

The mean pharmacological compounds cost per patient undergoing ovarian stimulation with the LH-based protocol was 1,031.51 USD, and was 1,209.94 USD for the control protocol. The cost per cumulative ongoing pregnancy was 1,584.50 USD for the study protocol *versus* 1,726.02 USD for the control protocol. This study estimated the ICER for the LH-based GnRH antagonist protocol *versus* the conventional flexible GnRH antagonist protocol at 3,568.6 USD for each additional ongoing pregnancy (Table 5).

Discussion

This study was the first RCT to investigate the clinical efficacy and cost-effectiveness of the LH-based modified GnRH antagonist protocol during ovarian stimulation in normal responders. We found that the reproductive outcomes were comparable, i.e., the LH-based protocol was not inferior in clinical efficacy. Moreover, it was more cost-effective considering the cumulative ongoing pregnancy rate in the entire ART cycle.

LH has a significant impact on morphological and functional changes of the oocyte and determines its meiotic status and ability to be fertilized (15). LH levels vary between individuals during ovarian stimulation. Recent evidence showed that LH glycosylation variants, genetic variants of LH and its receptor, and female age could negatively affect gonadotropin action and ovarian response (7, 8, 16). GnRH, estradiol, and anti-Mullerian hormone (AMH) are all potential factors implicated in the control of LH level (17, 18). Our previous proof-of-concept study demonstrated that patients

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TABLE 3 Reproductive outcome analysis.

	Study group	Control group	OR (95% CI)	*P*-value
First embryo transfer cycle				
n	166	164		
Positive ßhCG, *n* (%)	89 (53.6%)	104 (63.4%)	0.67 (0.43–1.04)	0.07
Clinical pregnancy, *n* (%)	81 (48.8%)	96 (58.5%)	0.67 (0.44–1.04)	0.08
Ongoing pregnancy, n (%)	76 (45.8%)	86 (52.4%)	0.77 (0.50–1.18)	0.23
Live birth, n (%)	75 (45.2%)	84 (51.2%)	0.78 (0.51–1.21)	0.27
Fresh ET cycle				
n	22	40		
Positive ßhCG, n (%)	10 (45.5%)	28 (70.0%)	0.36 (0.12–1.05)	0.10
Clinical pregnancy, *n* (%)	10 (45.5%)	25 (62.5%)	0.5 (0.17–1.44)	0.29
Ongoing pregnancy, n (%)	10 (45.5%)	23 (57.5%)	0.62 (0.22–1.76)	0.43
Live birth, n (%)	10 (45.5%)	23 (57.5%)	0.62 (0.22–1.76)	0.43
First FET cycle of the "freeze-all" patients				
n	144	124		
Positive ßhCG, n (%)	81 (56.3%)	77 (62.1%)	0.78 (0.48–1.28)	0.38
Clinical pregnancy, *n* (%)	73 (50.7%)	73 (58.9%)	0.72 (0.44–1.17)	0.22
Ongoing pregnancy, n (%)	67 (46.5%)	63 (50.8%)	0.84 (0.52–1.36)	0.54
Live birth, n (%)	66 (45.8%)	61 (49.2%)	0.87 (0.54–1.41)	0.62
Whole ART cycle				
n	166	164		
Cumulative positive ßhCG, *n* (%)	120 (72.3%)	128 (78.0%)	0.73 (0.44–1.21)	0.23
Cumulative clinical pregnancy, *n* (%)	116 (69.9%)	124 (75.6%)	0.75 (0.46–1.22)	0.24
Cumulative ongoing pregnancy, *n* (%)	108 (65.1%)	115 (70.1%)	0.79 (0.50–1.26)	0.33
Cumulative live birth, *n* (%)	107 (64.5%)	112 (68.3%)	0.84 (0.53–1.33)	0.46
Singleton	79	83		
Twins	28	29		
Pregnancy loss				
Biochemical miscarriage, *n* (%)	4 (3.5%)	4 (3.1%)	1.13 (0.28–4.61)	0.87
Clinical pregnancy loss				
First trimester pregnancy loss, *n* (%)	8 (7.3%)	9 (7.3%)	1.00 (0.37–2.69)	0.99
Second trimester pregnancy loss, *n* (%)	1 (1.0%)	3 (2.6%)	0.37 (0.04–3.61)	0.37
OHSS				
Moderate	0	1	–	–
Severe	0	0	–	–
Canceled cycles, *n*	0	0	–	–

For the first embryo transfer cycle, fresh embryos were transferred in 22 and 40 patients in the study group and control group, respectively. 144 patients in the study group and 124 in the control group were performed "freeze-all strategy" and underwent FET later. OR, odds ratio; CI, confidence interval; ßhCG, beta human chorionic gonadotropin; ET, embryo transfer; FET, frozen embryo transfer; OHSS: ovarian hyperstimulation syndrome.

with sustained low LH levels might not require antagonist administration during ovarian stimulation. If administered, the antagonist might further decrease the LH level and adversely affect reproductive outcomes (12). Besides, we found in another previous study that the cumulative live birth rate (CLBR) in the low LH group was significantly lower than in the high LH group. Patients with low LH levels had a lower live birth rate (LBR) after fresh embryo transfer but comparable LBR after the first FET in freeze-all cycles (19). On the other hand, high LH levels during the follicular phase were also associated with poor oocyte or embryo quality and impaired endometrial receptivity and, consequently, with a negative impact on reproductive outcomes (20, 21). Thus, we believed that the use of GnRH antagonists as LH level regulators should take the LH level during ovarian stimulation into consideration for a better individualized stimulation protocol. Regular hormone tests could conveniently monitor the LH level. The GnRH antagonist action is dose-dependent, so its flexible addition could maintain the LH within the desired range. The dosage of the antagonist was significantly reduced in our study group, with no cycle cancellation due to premature ovulation.

TABLE 4 Crude and adjusted OR for cumulative ongoing pregnancy rate of the per-protocol patients.

Exposure	OR	95% CI Univariate	*P*-value	adj. OR	95% CI Multivariate	*P*-value
Protocol						
Flexible GnRH antagonist protocol	1			1		
LH-based GnRH antagonist protocol	0.79	0.50–1.26	0.33	0.71	0.44–1.16	0.18
Age (years)						
<35	1			1		
≥35	0.67	0.38–1.17	0.16	0.80	0.44–1.45	0.46
BMI (kg/m^2)						
<24	1			1		
24-28	0.73	0.43–1.24	0.25	0.67	0.38–1.18	0.17
>28	0.57	0.23–1.43	0.23	0.62	0.23–1.64	0.33
No. of oocytes retrieved	1.12	1.07–1.17	<0.001	1.11	1.06–1.17	<0.001
Mean LH level during ovarian stimulation	1.04	0.89–1.22	0.61	1.07	0.89–1.29	0.48
LH on triggering day	0.87	0.76–0.99	0.04	0.93	0.79–1.09	0.34

OR, odds ratio; adj. OR: adjusted odds ratio; CI, confidence interval, GnRH, gonadotropin releasing hormone; LH, luteinizing hormone; BMI, body mass index.

A single serum LH measurement on a predefined day cannot reflect the average LH concentration during the follicular phase. This was one of the reasons for the controversial results reported in previous studies. Chen et al. measured LH level at a mean interval of 2.3 days across the follicular phase to demonstrate the low area under the curve for serum LH and obtain a cut-off value (22). In the present study, 4–5 hormone tests were performed at regular intervals for an average stimulation duration of 8–10 days. We believed these tests could optimally reflect the LH changes throughout the cycle, even though they were not measured daily. The mean LH levels during stimulation was compared between the two groups. Although the independent samples *t*-test indicated between-group differences, multivariate regression analyses did not demonstrate the influence on the cumulative ongoing pregnancy rates (Tables 2, 4). One possible explanation was that patients were randomly assigned to the study and control groups rather than grouped according to their LH levels. Although the mean LH level in the control group was lower, this did not mean that all patients in the control group had low LH. Besides, rLH was supplemented in both groups to benefit patients with LH deficiency. With a lower GnRH antagonist dosage and a relatively high mean LH level, the rLH dose in the study group was much lower than in the control group. Thus, the economical expenditure of the patients was reduced.

Many indicators have been raised to evaluate the ovarian response during stimulation, including FORT, FOI, and OSI. Each of them has advantages and disadvantages. FORT assesses only the number of follicles before and after stimulation, but does not consider the degree of ovarian stimulation, i.e., the Gn dosage, or assess the number of oocytes retrieved, which is closely related to live birth. FOI could be influenced by the initial Gn dosage, genetic or environmental factors, asynchronous follicular development, and technical issues (23). The OSI does not consider the Gn type (e.g., whether LH or LH analog was added) or the AFC (24). In the present study, we statistically analyzed all three indicators to provide a more comprehensive assessment of ovarian response. The results showed that the two groups were comparable, further suggesting that the LH-based GnRH antagonist protocol was not inferior to the conventional flexible GnRH protocol in terms of ovarian response.

Recent literature suggested that clinical efficacy should be accompanied by economic studies. Even when the primary outcome was similar (as was the case in this study), cost-effectiveness analysis should be performed to assess the effectiveness rather than the efficacy of the procedure (25). Both direct and indirect costs should be included when calculating the cost of IVF. Direct non-medical costs (e.g., travel and accommodation costs) and indirect costs (e.g., income lost) data were intangible and difficult to calculate. Furthermore, these costs had nothing to do with the difference in stimulation protocol. The differences between the groups in the present study were limited to the time and dose of the

TABLE 5 Cost-effectiveness analysis of the LH-based and conventional flexible GnRH antagonist protocols.

Protocol	Costs (USD)	Effectiveness	C/E	ICER (USD)
Conventional flexible GnRH antagonist protocol	1209.94	0.70	1726.02	—
LH-based GnRH antagonist protocol	1031.51	0.65	1584.50	3568.60

LH, luteinizing hormone; GnRH, gonadotropin releasing hormone; C/E, costs/effectiveness; ICER, incremental cost-effectiveness ratio.

antagonist administration. Other direct medical costs, such as the number of follow-up visits and examinations, were similar and, therefore, excluded from the analysis. There was no severe OHSS requiring treatment in either group. Taken together, the economic evaluation in this study focused only on drug costs during ovarian stimulation till trigger. The results demonstrated that the LH-based GnRH antagonist protocol was more cost-effective than the conventional protocol. It would be particularly important for patients in the developing countries without public health insurance coverage of treatments for infertility.

The present study has several limitations. First, we included a relatively selected population of females expected to show normal ovarian response. Hence, whether these results could be extrapolated to the general population requires further investigation. One more topic-related RCT (number ChiCTR1800018129) in which PCOS patients are enrolled is currently under way. Second, the LH cut-off values for the various GnRH antagonist doses were based on our previous study and clinical observations. However, we cannot affirm that they are the best discriminatory thresholds. Third, this study had a multi-center setting with subjects from the participating centers competing for inclusion. Most subjects were recruited from the Medical Center for Human Reproduction, Beijing Chao-Yang Hospital. Therefore, analyses considering differences among the participating centers were not performed. The representativeness of the study population was limited, affecting the ability to extrapolate the study findings. Fourth, the proportion of fresh embryo transfer cycles was relatively low in the present study, as clinicians tended to follow the freeze-all strategy. For future research, it would be worthwhile to collect more clinical data on fresh embryo transfer cycles to further demonstrate the mechanism of LH levels during ovarian stimulation on follicle development and endometria receptivity. And on this basis, it would provide more robust evidence in selection of the most appropriate cycle for fresh embryo transfer, and provide indications for individualized treatment and freeze-all strategy.

Conclusion

The LH-based GnRH antagonist protocol was not inferior to the conventional flexible GnRH antagonist protocol in clinical efficacy for normal responders. However, it was more cost-effective, considering the cumulative ongoing pregnancy rate in the entire ART cycle. Further large scale RCTs are needed to see if this protocol can be applied to the entire population undergoing IVF.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Beijing Chao-Yang Hospital, Capital Medical University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

SL: data collection and statistical analysis; drafting of the manuscript. YSL, ML, SH, XL, ZZ, WC, and AY: patient's treatment and revising of the manuscript. YL: supervision of the study concept and review of manuscript. All authors performed revision of intellectual content of the final version of the article.

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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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Effect of serum progesterone levels on hCG trigger day on pregnancy outcomes in GnRH antagonist cycles

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Objective: The present study analyzed the effect of hCG trigger day progesterone (P) levels on the live birth rate (LBR) in the gonadotropin-releasing hormone (GnRH) antagonist protocol.

Materials and methods: This study was a single-center retrospective study. *In vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI) cycles performed from January 2017 to December 2020 were included in the analysis. This study included people with a normal ovarian response to fresh embryo transfer of GnRH antagonist protocols. All cycles were divided into 2 groups by P level on the day of human chorionic gonadotropin (hCG) trigger, P<1.0 ng/ml and P≥1.0 ng/ml. The primary outcome measure was LBR.

Result: A total of 867 cycles with P<1.0 ng/ml and 362 cycles with P≥1.0 ng/ml were included in the analysis. The clinical pregnancy rate (CPR) was higher in the P<1.0 ng/ml group than the P≥1.0 ng/ml group (44.9% vs. 37.6%, P=0.02). The early spontaneous abortion rate was comparable between the groups (14.4% vs. 14.7%, P=0.93). For live birth, the rate for the P<1.0 ng/ml group was 35.3%, which was significantly higher than the 29.0% in the P≥1.0 ng/ml group (P=0.03). After binary logistic regression analysis, the P level on the hCG trigger day (adjusted odds ratio=0.74, 95% CI=0.55-0.99, P=0.04) was an independent risk factor for LBR. For the P level on the hCG trigger day, the LBR was lower in the P≥1.0 ng/ml group compared to the P<1.0 ng/ml group.

Conclusion: For normal ovarian response patients using the GnRH antagonist protocol, serum P≥1.0 ng/ml on the hCG trigger day resulted in a lower LBR than the P<1.0 ng/ml group. When P≥1.0 ng/ml, whole embryo freezing may be considered.

KEYWORDS

progesterone, GnRH antagonist, live birth rate, clinical pregnancy rate, *in vitro* fertilization

Introduction

Ovarian stimulation (OS) is a critical step for intracytoplasmic sperm injection (ICSI)/*in vitro* fertilization (IVF) (1). The rationale for OS is to achieve more follicle development using exogenous follicle-stimulating hormone (FSH), which stimulates the growth of multiple follicles in a single cycle (2, 3). However, the increase in estrogen (E2) caused by the development of multiple follicles increases luteinizing hormone (LH) levels before the follicles mature, which leads to earlier ovulation. Therefore, the key to OS is to prevent premature luteinization in advance. The most commonly used controls for endogenous LH peaks are gonadotropin-releasing hormone (GnRH) analogs, including GnRH agonists and GnRH antagonists (4, 5). A GnRH agonist protocol has been used in assisted reproductive technology since 1984 (6), and it is one of the most widely used OS protocols. GnRH agonists effectively inhibit the LH level and the occurrence of an early-onset LH surge, which improve the uniformity of follicle development. However, prolonged stimulation increases the gonadotropin (Gn) dose, which increases the risk of ovarian hyperstimulation syndrome (OHSS). GnRH antagonist protocols have been gradually used in the clinic since 2001. These protocols use a relatively short duration of stimulation with a lower Gn dose, which reduces the risk of OHSS (7, 8). Controversy exists in the use of GnRH agonists and GnRH antagonists (4, 9, 10). Based on the safety of OS, the GnRH antagonist protocol is more recommended for normal or high ovarian responders (1). However, the GnRH agonist protocol was more advantageous based on the live birth rate (LBR) of fresh embryo transfer, but there was no difference in the cumulative LBR between the two protocols (11).

Optimization of the LBR of fresh embryo transfer with GnRH antagonist protocols is the focus of much research. Serum progesterone (P) level is an important indicator in the pregnancy rate of the fresh cycle, and elevated P levels on the day of human chorionic gonadotropin (hCG) administration negatively influence clinical outcomes (12–14). Many studies examined the effect of serum P on clinical outcomes by measuring serum P levels on the day of the hCG trigger. The main reason for the controversy is that the threshold value of the serum P level is different between studies and ranges from 0.8 to 2.0 ng/ml, and there are differences in the determination methods (12, 15–17). The mechanisms of elevated P primarily include increased doses of gonadotropins (Gn), higher FSH levels, higher oocyte retrieval numbers and higher E2 levels on the trigger day (18). Therefore, the effect of elevated P levels on clinical outcomes may vary in different ovarian responders. Due to differences in populations, races, protocols, etc., the currently reported elevated P values are not uniform (13, 19, 20).

People with a normal ovarian response have a low risk of OHSS and a relatively stable number of oocytes retrieved and available embryos are the main population for fresh embryo transfer. Therefore, the present study analyzed the effect of P levels on hCG trigger day on LBR in a population with a normal ovarian response in a GnRH antagonist protocol.

Methods

This study was a single-center, retrospective, observational, cohort study. This study was performed in the Reproductive Center of the Third Affiliated Hospital of Zhengzhou University. Ethical approval was obtained from the Ethics Committee of Third Affiliated Hospital of Zhengzhou University. IVF/ICSI cycles performed from January 2017 to December 2020 were included in the analysis.

Population

A total of 1229 cycles of the GnRH antagonist protocol were included in the study analysis, all of which underwent the first IVF/ICSI cycle with fresh embryo transfer. This study included people with a normal ovarian response (age: 20-40 years old, baseline FSH<10 IU/L, anti-Mullerian hormone (AMH)≥1.1 ng/ml, antral follicle count (AFC)≥6). Patients with polycystic ovary syndrome were excluded from the analysis. Women with a history of uterine malformation (e.g., bicornuate uterus, unicornuate uterus or septate uterus), hydrosalpinx, history of ovarian surgery, adenomyosis or intrauterine adhesion were excluded from the analysis. Patients with recurrent spontaneous abortion were also excluded. All of the couples were screened *via* karyotyping, and couples with an abnormal karyotype were excluded.

GnRH antagonist protocol and IVF/ICSI-embryo transfer

A routine flexible GnRH antagonist protocol was performed in our reproductive center as described in previous studies (21). OS was initiated on the second or third day of the menstrual cycle, and the appropriate Gn starting dose (100-300 IU) was chosen based on maternal age, weight, body mass index (BMI) and AMH. Vaginal ultrasonography was performed and serum LH and E2 levels were determined 3-5 days later. A GnRH antagonist (0.25 mg/day) was added once the diameter of the dominant follicle reached 12-14 mm and was continued up to the trigger day. The GnRH antagonist was injected at approximately 5 pm each day. If the LH peak occurred during the process of ovarian stimulation, the GnRH antagonist was also injected in time. When there were 3 follicles > 17 mm or 2 follicles > 18 mm, and patients were

undergoing fresh embryo transfer, 250 μg recombinant hCG was applied for follicle maturation. Oocyte retrieval was performed 36 hours later. Based on sperm quality, conventional IVF or ICSI was performed, as appropriate. Luteal support was started on the day of oocyte retrieval using oral dydrogesterone (DYG; 10 mg, 2 times daily) (Abbott Co. America). Intravaginal progesterone sustained-release vaginal gel (90 mg, Merck Co. Germany) was given. One or two cleavage stage embryos were transferred 3 days after oocyte retrieval, or 1 blastocyst was transferred 5 days after oocyte retrieval. If pregnancy occurred, corpus luteum support was continued for at least 55 days after embryo transfer.

Serum hormone level measurement and grouping

Serum hormone levels, including FSH, LH, E2 and P, were analyzed using the Roche Cobas immunoassay (Roche Diagnostics, Germany). The preparation, setup, dilution, adjustment, assay and quality control procedures were performed according to the manufacturer's instructions, and the intra-assay and inter-assay coefficients of variation were less than 10%. On the hCG trigger day, whole blood was collected between 7:00 and 9 a.m. We routinely measured serum LH, E2 and P levels. Fresh embryo transfer was cancelled when the serum P level was >2 ng/ml, and whole embryo freezing was performed. We divided all cycles into 2 groups by the P level on the day of hCG trigger, P<1.0 ng/ml and P≥1.0 ng/ml. This grouping was primarily based on data distribution characteristics and reference to current related research (13, 22, 23).

Outcome measures and definition

The primary outcome measure was LBR after fresh embryo transfer. Live birth was defined as any viable neonate ≥ 28 gestational weeks. The secondary outcome measure was clinical pregnancy rate (CPR), which was defined as a pregnancy diagnosed *via* ultrasonographic visualization of one or more gestational sacs and included intrauterine pregnancy and a clinically documented ectopic pregnancy (24). Early spontaneous abortion was defined as a loss of clinical pregnancy before 12 gestational weeks and was included as an outcome measure of this study.

Statistical analysis

All data were obtained from retrospective review of our reproductive center's medical records. All statistical management and analyses were performed using SPSS software, version 22.0.

For continuous variables, the one-sample Kolmogorov–Smirnov test was performed to check for normality. Continuous variables with abnormal distributions are expressed as medians (P25, P75), and the Wilcoxon rank sum test was used to assess between-group differences. Categorical variables are represented as the number of cases (n) and percentage (%). The between-group differences were assessed using chi-squared analyses with Fisher's exact test when necessary. Binary logistic regression was performed to adjust for potential confounding factors for the main outcome, LBR. Adjusted odds ratios (AORs) with 95% confidence intervals (CIs) were calculated. Statistical significance was set at p value < 0.05.

Results

Study population

A total of 867 cycles with P<1.0 ng/ml and 362 cycles with P≥1.0 ng/ml were included for analysis. There were no statistically significant differences in maternal age, paternal age, duration of infertility, gravidity, type of infertility, infertility diagnosis, AMH, basal AFC or method of assisted reproductive technology (ART) (all p>0.05) between groups. The BMI in the P<1.0 ng/ml group was 23.8 (21.8, 26.0), which was significantly different than the P≥1.0 ng/ml group at 23.4 (21.4, 25.4) (p<0.01). Basal FSH was higher in the P<1.0 ng/ml group than the P≥1.0 ng/ml group (p<0.01). The detailed characteristics of the participants at baseline between the two groups are described in Table 1.

Characteristics of controlled ovarian hyperstimulation cycles

The starting dose of Gn, endometrial thickness on the hCG trigger day and type of transferred embryos (cleavage embryo/blastocyst) were comparable between the two groups (all p>0.05). There were statistically significant differences in the number of days of ovarian stimulation, total dose of Gn, estradiol level on hCG trigger day, number of follicles ≥14 mm, 16 mm and 18 mm on hCG trigger day, oocytes retrieved, two distinct pronuclei (2PN), available embryos on day 3 and available embryo rate between the groups. The number of transferred embryos was higher in the P<1.0 ng/ml group than the P≥1.0 ng/ml group (p=0.04). The detailed characteristics of the cycles between the two groups are described in Table 2.

Clinical outcomes

The CPR was higher in the P<1.0 ng/ml group than the P≥1.0 ng/ml group (44.9% vs. 37.6%, p=0.02). The early spontaneous

TABLE 1 Characteristics of the participants at baseline.

Characteristic	P<1.0 ng/ml	P≥1.0 ng/ml	p value
Number of cases	867	362	
Maternal age (year)	32.0 (29.0, 38.0)	33.0 (29.0, 38.0)	0.43
Paternal age (year)	33.0 (29.0, 38.0)	33.0 (30.0, 39.0)	0.41
Body mass index (kg/m2)	23.8 (21.8, 26.0)	23.4 (21.4, 25.4)	<0.01
Duration of infertility (year)	3.0 (2.0, 5.0)	3.0 (1.0, 5.0)	0.08
Gravidity	1 (0, 2)	1 (0, 2)	0.26
Type of infertility (%)			0.43
Primary infertility	38.2 (331/867)	40.6 (147/362)	
Secondary infertility	61.8 (536/867)	59.4 (215/362)	
Infertility diagnosis (%)			0.09
Tubal factor	33.8 (293/867)	32.6 (118/362)	
Male factor	14.9 (129/867)	20.7 (75/362)	
Male+female factors	22.6 (196/867)	19.9 (72/362)	
Others	28.7 (249/867)	26.8 (97/362)	
Basal FSH (IU/L)	7.1 (5.9, 8.8)	6.7 (5.6, 8.2)	<0.01
AMH (ng/ml)	1.9 (1.0, 4.5)	2.3 (1.3, 4.1)	0.10
Basal antral follicle count	12 (7, 16)	11 (8, 16)	0.95
Method of ART (%)			0.07
IVF	69.9 (606/867)	64.6 (234/362)	
ICSI	30.1 (261/867)	35.4 (128/362)	

Data are presented as medians (P25, P75) for continuous variables and % (n/N) for categorical variables.

abortion rate was comparable between the groups (14.4% vs. 14.7%, p=0.93). The LBR of the P<1.0 ng/ml group was 35.3%, which was significantly higher than the 29.0% in the P≥1.0 ng/ml group (p=0.03). A binary logistic regression model was performed to adjust the influence of confounding factors, including maternal age (continuous variable), maternal BMI (continuous variable), type of infertility (primary/secondary), infertility diagnosis (tubal/male/male+female/others), basal AFC (continuous variable), basal FSH

TABLE 2 Characteristics of controlled ovarian hyperstimulation cycles.

Characteristic	P<1.0 ng/ml	P≥1.0 ng/ml	p value
Number of cases	867	362	
Starting dose of Gn (IU)	225.0 (187.5, 300.0)	225.0 (200.0, 300.0)	0.45
Number of days of ovarian stimulation	9 (8, 10)	10 (9, 11)	<0.01
Total dose of Gn (IU)	2400.0 (1800.0, 3000.0)	2625.0 (2000.0, 3000.0)	<0.01
Estradiol level on hCG trigger day (pg/ml)	1817.4 (1208.0, 2658.9)	2546.8 (1826.8, 3703.1)	<0.01
Number of follicles ≥ 14 mm on hCG trigger day	6 (4, 9)	8 (6, 11)	<0.01
Number of follicles ≥ 16 mm on hCG trigger day	5 (3, 6)	6 (4, 8)	<0.01
Number of follicles ≥ 18 mm on hCG trigger day	3 (2, 4)	3 (2, 5)	<0.01
Number of oocytes retrieved	7 (5, 11)	10 (7, 13)	<0.01
Number of 2PN	4 (3, 8)	6 (4, 9)	<0.01
Number of available embryos on day 3	4 (2, 6)	4 (2, 7)	<0.01
Available embryo rate (%)	50.0 (37.5, 70.0)	50.0 (33.3, 64.5)	<0.01
Endometrial thickness on the hCG trigger day (mm)	10.0 (8.8, 11.7)	10.0 (8.4, 12.0)	0.65
No. of transferred embryos			0.04
1	33.3 (289/867)	27.3 (99/362)	
2	66.7 (578/867)	72.7 (263/362)	
Type of transferred embryos			0.14
Cleavage embryo	86.2 (747/867)	82.9 (300/362)	
Blastocyst	13.8 (120/867)	17.1 (62/362)	

Data are presented as medians (P25, P75) for continuous variable and % (n/N) for categorical variables.

(continuous variable), method of ART (IVF/ICSI), total dose of Gn, endometrial thickness on the hCG trigger day (continuous variable), number of oocytes retrieved (continuous variable), number of transferred embryos (1/2), type of transferred embryos (cleavage embryo/blastocyst) and P level on hCG trigger day (<1.0 ng/ml/≥1.0 ng/ml). Binary logistic regression analysis revealed that maternal age (AOR=0.91, 95% CI=0.88-0.93, p<0.01), endometrial thickness on the hCG trigger day (AOR=1.13, 95% CI=1.06-1.20, p<0.01), P level on hCG trigger day (AOR=0.74, 95% CI=0.55-0.99, p=0.04), number of transferred embryos (AOR=2.01, 95% CI=1.37-2.95, p<0.01) and type of transferred embryos (AOR=2.20, 95% CI=1.28-3.79, p<0.01) were independent risk factors for LBR. For the P level on the hCG trigger day, the LBR was lower in the P≥1.0 ng/ml group compared to the P<1 ng/ml group. The specific data are described in Tables 3, 4.

Discussion

Our single-center, retrospective cohort study involving normal ovarian response patients with the GnRH antagonist protocol found that the LBR for patients with P≥1.0 ng/ml was lower than patients with P<1.0 ng/ml. The risk of early spontaneous abortion did not differ significantly between the two groups.

There are many studies on the effects of P level on pregnancy. The synergistic effect of P and E2 is a necessary factor for embryo implantation in the natural state. Under the physiological state, when the dominant follicle is close to maturity and before ovulation, the follicle slightly increases the secretion of P to coordinate the positive feedback effect of E2 and induce the appearance of the peak of FSH and LH during ovulation. A large amount of P is secreted after ovulation (25). The increase in P level completely changes the endometrial state and endometrial receptivity (26). With the application of GnRH agonists and antagonists in OS cycles, the occurrence of endogenous LH peaks may be effectively prevented, but some patients still exhibit elevated serum P levels in the late follicular development stage (27). Schoolcraft et al. (28) first reported the phenomenon of elevated serum P levels on the day of hCG injection in some populations during the IVF treatment cycle with GnRH agonist protocols.

Subsequent reports of elevated P gradually appeared, with the overall incidence ranging from 5% to 38%. The incidence in GnRH agonist protocols ranged from 5% to 35%, and the incidence in GnRH antagonist protocols ranged from 9% to 38% (29, 30). Due to differences in study populations, laboratory testing methods, and groupings, the relationship between elevated serum P levels and IVF pregnancy outcomes remains controversial, and there are differences in the definition of elevated P. A large retrospective cohort study by Xu et al. (12) included populations with different ovarian responses and defined different serum P levels on the hCG trigger day. The defined values of serum P in patients with low ovarian response, normal response and high response were 1.5 ng/ml, 1.75 ng/ml and 2.25 ng/ml, respectively. Bosch et al. (31) and Van Vaerenbergh et al. (32) set the limit of the hCG daily P level to 1.5 ng/ml, which is widely used in clinical practice. However, studies show that when P>1 ng/ml, the CPR or LBR decreases (13, 19, 23). Only people with normal ovarian response with GnRH antagonist protocols were included for analysis in our study, and P>1 ng/ml affected the LBR of fresh embryo transfer. Fresh embryo transfer should be canceled, and whole embryo freezing should be performed when P is high.

Although many clinical studies showed that elevated serum P levels had a negative impact on CPR and LBR, the specific endocrine mechanisms are not clear. Major mechanistic studies focused on the effects of elevated serum P on endometrial receptivity and oocyte and embryo quality. Elevated serum P levels reduced CPR in fresh embryo transfer cycles but did not affect clinical outcomes in frozen-thawed embryo transfer cycles (33). Chen et al. (34) showed that high serum P does not affect embryo quality, and most studies believe that elevated serum P levels had no effect on oocyte quality, fertilization rate, or embryo quality. Santos-Ribeiro et al. (35) reported that IVF fertilization rates were similar between different P levels (≤0.50 ng/ml, 0.5–1.5 ng/ml and >1.5 ng/ml), which confirmed that serum P levels did not affect IVF fertilization rates. A recent study using the 90th percentile of the distribution of serum P levels as a basis for grouping also showed that P levels had no negative effects on oocyte or embryo quality (36). Embryo implantation theory suggests a specific implantation window for embryo implantation. When the endometrium and embryonic development are out of sync for more than 3 days, the pregnancy rate is extremely low (37). Premature elevation of serum P levels affects endometrial receptivity by altering the expression of endometrial-specific genes and promoting endometrial transition from early secretory to late secretory

TABLE 3 Clinical outcomes between the two groups.

	P<1.0 ng/ml	P≥1.0 ng/ml	p value
Clinical pregnancy rate (%)	44.9 (389/867)	37.6 (136/362)	0.02
Early spontaneous abortion rate (%)	14.4 (56/389)	14.7 (20/136)	0.93
Live birth rate (%)	35.3 (306/867)	29.0 (105/362)	0.03

Data are presented as % (n/N) for categorical variables.

TABLE 4 Binary logistic regression analysis to account for confounding variables of live birth rate.

	AOR	95%CI	p value
Maternal age (year)	0.91	0.88-0.93	<0.01
Body mass index (kg/m2)	0.99	0.95-1.04	0.77
Type of infertility (primary/secondary)	1.15	0.86-1.52	0.34
Infertility diagnosis (tubal/male/male+female/others)	0.96	0.86-1.06	0.42
Basal antral follicle count	1.01	0.99-1.04	0.23
Basal FSH (IU/L)	0.98	0.93-1.03	0.34
Method of ART (IVF/ICSI)	0.90	0.69-1.19	0.47
Total dose of Gn (IU)	1.00	1.00-1.00	0.15
Endometrial thickness on the hCG trigger day (mm)	1.13	1.06-1.20	<0.01
Number of oocytes retrieved	0.97	0.94-1.01	0.10
Progesterone level on hCG trigger day (<1/≥1.0 ng/ml)	0.74	0.55-0.99	0.04
Number of transferred embryos (1/2)	2.01	1.37-2.95	<0.01
Type of transferred embryos (cleavage embryo/blastocyst)	2.20	1.28-3.79	<0.01

AOR, adjusted odds ratio; CI, confidence interval.

(38). The increase in serum P levels has a specific effect on the gene expression profile of the endometrium (32). Different serum P levels induce different gene expression in the endometrium, and the expression of specific genes is related to embryo adhesion, the implantation process, and the immune system, which affect CPR in fresh cycles (39).

According to the previous data of the center and other studies, fresh embryo transfer was cancelled when the serum P level on hCG day >2 ng/ml, and whole embryo freezing was performed in our reproductive center. However, current data at our center show that the LBR of fresh embryo transfer with GnRH antagonist remains lower than a GnRH agonist. Therefore, the present study investigated whether slightly elevated P levels also affected LBR. First, this study only included GnRH antagonist regimens with normal ovarian response to minimize the influence of confounding factors. Second, the observational endpoint of this study was the LBR, which is more clinically valuable than comparisons of only the CPR. However, the current study is limited by its retrospective cohort nature. Second, this study did not further analyze the impact of the quality of different embryos or blastocysts transferred on clinical outcomes, and there may be confounding factors. A retrospective cohort study revealed that a slight increase in P levels (0.85 ng/mL) affected the CPR of cleavage-stage embryo transfers, but it did not affect clinical outcomes after blastocyst transfer (40). Therefore, for patients with mildly elevated P, whether blastocyst transfer improves clinical outcomes is a direction of further research. The current study only included people with normal ovarian response, and further research is needed in populations with high and low ovarian responses.

Conclusion

For normal ovarian response patients with the GnRH antagonist protocol, serum P≥1.0 ng/ml on the hCG trigger day resulted in a lower LBR compared to P<1.0 ng/ml. When serum P≥1.0, whole embryo freezing may be considered, followed by frozen-thawed embryo transfer.

Data availability statement

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

Ethics statement

This study was approved by the Ethics Committee of the Third Affiliated Hospital of Zhengzhou University. Study reference number: 2022-198-01. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

JZ, MD and YG designed the study and selected the population to be included and excluded. YW and ZW were involved in the data extraction and analyses. MD reviewed the data. JZ was involved in drafting this article. All authors

approved the final version of the manuscript. JZ and MD contributed equally to this article. 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.

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Androgens improve ovarian follicle function impaired by glucocorticoids through an androgen-IGF1-FSH synergistic effect

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High concentrations of glucocorticoids caused by chronic stress are known to affect ovarian function and cause diminished ovarian reserve. Androgens are essential for early-stage ovarian follicle development, but the effects and mechanisms of androgens on follicle development under chronic stress remain unclear. In this study, we aim to investigate the effects of high concentrations of glucocorticoids on the function of *in vitro* cultured ovarian cells and mouse early-stage ovarian follicles and to validate the hypothesis that androgen–insulin-like growth factor 1 (IGF1)–follicle-stimulating hormone (FSH) synergistic signaling helps to ameliorate the damage caused by high concentrations of glucocorticoids. KGN cells (human granulosa cell line) and mouse primary cells were treated with different concentrations of glucocorticoids, and the cell proliferation, apoptosis, and sex hormone secretion were detected. The effects of glucocorticoid and androgens on IGF1 receptor (IGF1R) and FSH receptor (FSHR) expression in KGN cells were detected by Western blot. Steroidogenic synthase expressions under androgens and androgen-IGF1-FSH combination treatment were examined by qPCR after manipulation using low and high concentrations of glucocorticoids. The mechanism of androgen regulation of IGF1R and FSHR was explored by small interfering RNA (siRNA) and chromatin immunoprecipitation (ChIP)-qPCR. Damage of glucocorticoids and the treatment effects of androgens were further validated in mouse ovarian follicles cultured *in vitro*. The results demonstrated that prolonged treatment with high-dose glucocorticoids reduced cell viability of granulosa cells, inhibited their sex hormone secretion, and impaired their sensitivity to IGF1 and FSH signaling by affecting IGF1R and FSHR functions. Androgens at an appropriate dose range improved early-stage follicle development and their hormone secretion under high-dose glucocorticoid treatment, which was related to increased transcription of *Igf1r* and *Fshr*. This work showed that

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excessive glucocorticoids impaired ovarian function and validated that balanced concentrations of androgens synergized with IGF1 and FSH to improve the function of early-stage ovarian follicles under conditions of chronic stress.

KEYWORDS

glucocorticoids, androgen, IGF1, FSH, ovarian follicle

Introduction

Diminished ovarian reserve (DOR) is defined as a decrease in ovarian follicle number or oocyte quality and plays an important role in the etiology of female infertility (1). A recent study reports that DOR prevalence in infertile patients from *in vitro* fertilization centers in the United States increased from 19% to 26% from 2004 to 2011 (2). DOR is characterized by a decreased antral follicle count, elevation of serum follicle-stimulating hormone (FSH) concentration, and decreased serum estradiol (E2) and anti-Müllerian hormone (AMH) concentrations. Along with many other causes of DOR including genetic factors, environmental pollution, and infection, chronic stress is emerging in importance in modern society (3). Stress is commonly defined as a state of real or perceived threat to homeostasis that may challenge an organism's wellbeing (4). The hypothalamic-pituitary-adrenal (HPA) axis, composed of the hypothalamus, pituitary gland, and adrenal glands, regulates the body's response to stress (5). As the end product of the HPA axis (6), aberrant glucocorticoid release [i.e., cortisol (CORT) in humans, corticosterone (CORTN) in rodents (7)] can be damaging to the reproductive system owing to extreme or chronic stress exposure (8). However, studies reporting the adverse effect and underlying mechanism of elevated glucocorticoids on ovarian follicle development are still limited.

Insulin-like growth factor 1 (IGF1) and FSH signals are two important growth factors for folliculogenesis and increase with follicle maturation (9, 10). Studies in pigs have shown that direct exposure to high concentrations of glucocorticoids decreases ovarian IGF1 production both *in vitro* (11) and *in vivo* (12). In female mice, injection of glucocorticoids significantly decreases IGF1 transcription in granulosa cells and decreases E2 concentrations in both the serum and ovary (13). Our previous work revealed that protein expression of FSH receptors (FSHRs) is significantly decreased in a chronic unpredictable stress-induced DOR mouse model (14, 15). Such studies indicate that IGF1 and FSHR synthesis in ovaries can potentially be impaired by excess glucocorticoids and influence follicle maturation.

In recent decades, it has been recognized that a balanced concentration of androgens is essential for early-stage follicle development (16–18). Clinical studies have found that DOR patients possess a lower serum androgen concentration compared to those with a normal ovarian reserve (19). Our previous work found that serum androgen concentrations were also significantly reduced in DOR mice induced by chronic unpredictable stress (14). In addition, studies have found an inverse correlation between serum testosterone (T) and serum CORT concentrations in women (20). Decreased serum T levels affect the function of the hypothalamic neuroendocrine system and promote activation of the HPA axis, leading to abnormal stress responses and stress-related disorders (21). Lower levels of sex hormones are also related to lower negative feedback of glucocorticoids on the HPA axis (22). Therefore, a "vicious cycle" of high glucocorticoid and low androgen levels under conditions of chronic stress may exacerbate the impairment of early ovarian follicle development, leading to DOR.

Clinical studies suggest that supplementation with androgens or their precursor dehydroepiandrosterone (DHEA) in patients with low androgen levels can significantly preserve the ovarian reserve and improve egg retrieval rate and pregnancy outcome in DOR patients, but the specific mechanism remains to be elucidated (23, 24). In mouse ovarian follicles cultured *in vitro*, transcription of FSHR was significantly improved after T or dihydrotestosterone (DHT) treatment (25). The action of androgens is also suggested to be related to the expression of IGF1R in human granulosa cells from primordial and primary follicles (26). Moreover, it has been demonstrated in rodent studies that the actions of FSH on granulosa cell and follicle development depend on the presence of IGF1 and active IGF1R (27). These results suggest that androgen supplementation may ameliorate glucocorticoid-induced damage to ovarian follicle development through IGF1 and FSH signaling.

Collectively, the above findings suggest that excess glucocorticoids and low levels of androgens under chronic stress may potentially impair early-stage ovarian follicle development. Androgen supplementation may improve ovarian function under chronic stress by acting synergistically with IGF1 and FSH signaling. To test the hypothesis, the effects

of glucocorticoids and androgens on a human granulosa-like tumor cell line (KGN cells), mouse granulosa cells (mGCs), mouse theca/interstitial cells (TICs), and mouse preantral follicles were observed. The underlying mechanism was verified by comparing the changes of IGF1R, FSHR, and steroidogenesis hormone synthase after androgen application and androgen-IGF1-FSH combined application.

Materials and methods

Experimental design

The study was composed of three phases. To first explore the effect of glucocorticoids on ovarian steroidogenesis cells, especially granulosa cells, KGN cells were cultured and treated with different concentrations of CORT for 24, 48, and 72 h. Cell viability, apoptosis, and hormone secretion of E2 and IGF1 were detected in different groups. To double the confirmation of the results, mGC and TIC were isolated and treated with CORTN over different time periods and E2 and T secretion were detected, respectively. To validate the mechanism of the reduced E2 secretion after high concentrations of glucocorticoid treatment, the expression of androgen receptor (AR), IGF1R, p-IGF1R, and FSHR was detected in KGN cells. The expression of IGF1R in mGC was observed after treatment with different concentrations of CORTN. Furthermore, according to a recent report, except for the canonical action through their nuclear receptor to regulate protein synthesis, glucocorticoids can exert a direct impact on some cell signals (28). Therefore, we also detected the phosphorylation activation levels of the steroidogenesis signaling molecules Protein kinase B (AKT) and extracellular signal-regulated kinase (ERK). In order to clarify the mechanism by which long-time exposure to high-dose glucocorticoid treatment inhibits the autophosphorylation of IGF1R in KGN cells, low-dose (0.1 μM) CORT and high-dose (1 μM) CORT were applied to KGN cells respectively for 48 and 72 h. The cells were collected for co-immunoprecipitation (co-IP) assay to detect the protein–protein interaction between glucocorticoid receptors (GRs) and IGF1R.

In the second phase, to validate our hypothesis of androgen-IGF1-FSH synergistic effect in ameliorating the impairment of high concentrations of glucocorticoids, we first verified the bidirectional regulation effect of androgens at 0–10-nM concentrations on the protein expression of IGF1R and FSHR in KGN cells after 12- and 24-h treatments. Based on the results, we adopted 10 nM as the "low dose" and 50 nM as the "high dose" and reconfirmed the bidirectional effect of androgens by treating KGN cells with FSH combined with low/high concentrations of androgens for 24 h. The messenger RNA (mRNA) expression difference of steroid hormone synthase in KGN cells was detected by qPCR. The synergistic effect of androgen-FSH-IGF1 signaling was verified by treating KGN cells with 0.1 μM CORT or 1 μM CORT for 24 h, followed by treatment with a combination of the three factors or treatment with one of the three factors for 48 h. The qPCR results of steroid hormone synthase expression were evaluated in the different groups. To further explore the mechanism of the synergistic effects of androgens with IGF1 and FSH, we transfected KGN cells with small interfering RNA (siRNA) to knock down the expression of ARs. The expression of ARs after transfection was validated by qPCR and Western blot (WB). Based on our further hypothesis that ARs may regulate the gene expression of *Igf1r* and *Fshr* as a transcription factor, the chromatin immunoprecipitation (ChIP)-qPCR technique was applied after treating KGN cells with androgens at 10 nM concentration for 1 h.

In the last phase of the study, we observed the growth rate and E2 secretion of *in vitro* cultured mouse preantral follicles with different factors added to their culture media, including low and high concentrations of CORTN, androgens, androgen-high concentration of CORTN combined treatment, and androgen–IGF1–high-concentration CORTN combined treatment. Through analysis of changes in follicle diameter and the enzyme-linked immunosorbent assay (ELISA) results of the hormone levels in different groups after 3 days of culture, we aimed to further verify our hypothesis in an *ex vivo* model.

Drugs and reagents

The following drugs and reagents were used in the study: L-15 media, McCoy's 5a, M199 media, α-MEM media, and DMEM/F12 media without phenol red (Gibco), fetal bovine serum (FBS; Sciencell), fetuin (MCE), insulin-transferrin-selenium solution (Sigma), type IV collagenase (Absin, abs47048003), DNase I (Sangon Biotech), bovine serum albumin (BSA; BioFroxx), T (Shanghai Standard Technology Co. Ltd.), DHT (Selleck, S4757), CORT (Selleck, S1696), CORTN (Selleck, S4752), RNA isolation kit, 4× reverse transcription Mix and Color SYBR Green qPCR Master Mix (EZBioscience, A0012), Lipofectamine 3000 and opti-MEM (Invitrogen), shAR vectors and negative control shRNA vectors (GeneChem), protein A/G magnetic beads (Bimake, B23201), normal rabbit IgG (CST, 2729), ELISA kits (E2, Labor Diagnostika Nord, Cat# FR E-2000, RRID : AB_2916329; T, Labor Diagnostika Nord, Cat# AR E-8000, RRID : AB_2916330; IGF1, R and D Systems Cat# DG100B, RRID : AB_2915951), cell counting kit-8 (CCK-8) reagent (DOJINDO), Annexin V fluorescein isothiocyanate/propidium iodide (FITC/PI) apoptosis detection kit (BD), GR antibody (Cell Signaling Technology Cat# 12041, RRID : AB_2631286), IGF1R antibody (Cell Signaling Technology Cat# 3027, RRID : AB_2122378), p-IGF1R antibody (Cell Signaling Technology Cat# 3024, RRID : AB-331253), FSHR antibody (Bioss Cat# bs-20658R, RRID : AB_2916328), AKT

antibody (Cell Signaling Technology Cat# 9272, RRID : AB_329827), p-AKT antibody (Cell Signaling Technology Cat# 4051, RRID : AB_331158), ERK1/2 antibody (Cell Signaling Technology Cat# 4695, RRID : AB_390779), p-ERK1/2 antibody (Cell Signaling Technology Cat# 4370, RRID : AB_2315112), AR antibody (Santa Cruz Biotechnology Cat# sc-7305, RRID : AB_626671), CYP11A1 antibody (Proteintech Cat# 13363-1-AP, RRID : AB_2088552), AMH antibody (Abcam Cat# ab103233, RRID : AB_10711946), anti-Rabbit IgG antibody (Cell Signaling Technology Cat# 4414, RRID : AB_10693544), and anti-Mouse IgG antibody (Jackson ImmunoResearch Labs Cat# 115-005-008, RRID : AB_2338449). All first antibodies were diluted at 1:1,000.

Animals

Six-week-old C57BL/6 male and female mice were purchased from Shanghai SLAC Laboratory Animal Ltd. After adaptive feeding for 1 week in the specific pathogen-free (SPF) facility, they were used to breed next-generation mice. Female C57 mice, aged 14–16 days, were bred for preantral follicle isolation. Female C57 mice aged 21 days were used to extract mGC and TIC. All experimental procedures followed the Criteria of the Medical Laboratory. Animal studies were reviewed and approved by the Animal Experimental Ethical Committee of Fudan University.

Isolation and culture of mouse granulosa cells and theca/interstitial cells

Twenty-one-day-old female C57 mice were sacrificed by cervical dislocation. The bilateral ovaries were collected and kept in ice-cold L-15 media supplemented with 100 U/ml penicillin-G and 100 μg/ml streptomycin (LSP). The ovaries were washed with LSP twice and were punctured using 25G needles in a 35-mm dish on ice. The ovaries were extensively punctured to ensure that granulosa cells were released from follicles. The tissue-cell mixture was filtered through 100-μm and 40-μm Falcon mesh to screen out large oocytes and tissue remnants from granulosa cells. The suspensions were centrifuged at 1,000 rpm for 5 min and resuspended in McCoy's 5a culture media containing 5% FBS and seeded to 12-well plates. The remaining tissue was washed with LSP twice and resuspended with 0.2 ml M199 media containing 2 mg/ml type IV collagenase and 1 mg/ml DNase I per ovary. The mixture was digested at 37°C for 1 h, during which the tissue mixture was pipetted >20 times every 15 min. The digestion was aborted by adding 5% FBS McCoy's 5a media, and the dispersed cells were centrifuged at 1,000 rpm for 5 min. Cell pellets were resuspended with TIC culture media and seeded on 12-well plates. All cells were cultured under conditions of 37°C and 5% CO_2. Cells were starved for 12 h before drug treatment. The upper range of CORTN concentration chosen in this study referred to the serum CORTN concentration results observed in mice under chronic unpredictable stress in previous studies (7, 14).

KGN cell culture and treatment

KGN cells (human granulosa-like tumor cell line) were purchased from Guangzhou Saiku Biotechnology Co., Ltd., and identified using the short tandem repeat (STR) technique. KGN cells were cultured in Dulbecco's Modified Eagle Medium/Ham's F-12 Nutrient Mixture (DMEM/F12) media without phenol red containing 10% FBS. Culture medium was changed every 2 days. For drug treatments, cells were digested and seeded on six-well plates. After cell adhesion and achievement of a suitable density, the culture medium was replaced with DMEM/F12 and starved for 12 h before drug treatment. The upper range of CORT concentration chosen in this study referred to the serum CORT concentration results observed in previous clinical studies (29, 30). Androgen concentrations were chosen with the upper range above the diagnosis criteria of polycystic ovarian syndrome (31) and with the lower range below the serum androgen concentrations of DOR patients (19).

Preantral follicle isolation and three-dimensional culture

The method of mouse preantral follicle isolation and three-dimensional (3D) culture was modified based on previous reports (32–35). Female mice aged 14–16 days were sacrificed by cervical dislocation and placed in 75% alcohol for sterilization for 10 min. The bilateral ovaries were collected in a sterile manner and placed in L-15 media containing 1 mg/ml BSA. The redundant fallopian tubes and fat around the ovaries were carefully removed under a stereomicroscope. The ovaries were transferred to a new 35-mm dish containing 1 ml α-MEM media supplemented with 2 mg/ml collagenase and 1 mg/ml DNase. Dishes were placed at 37°C for 10 min. After being washed with L-15 media twice, the ovaries were placed in a new 35-mm dish containing 1 ml L-15 media supplemented with 1 mg/ml BSA. Two insulin needles were used to carefully dissect secondary follicles from the ovaries under a stereomicroscope. The follicles were selected according to the following criteria: 100–130 μm diameter; intact basal layer with some theca cells attached; and a clear oocyte in the middle of the follicle. Selected follicles were transferred to α-MEM media containing 1% BSA for 1 h at 37°C.

Alginate solution [1% (m/v)] was prewarmed to 37°C, and 7.5 μl drops were pipetted onto a glass slide wrapped with

parafilm and a 3-mm spacer on each side. Each follicle was transferred into an alginate drop with the minimum volume of media. A solution containing 40 mM $CaCl_2$ 150 mM NaCl was dropped onto the alginate drops, quickly followed by another parafilm-wrapped glass slide to cover the drops. The two slices were placed upside down, and left for 2 min to allow the gel to solidify. The follicle-containing alginate beads were washed once in follicle culture media (α-MEM supplied with 3 mg/ml BSA, 1 mg/ml fetuin, 5 μg/ml insulin-transferrin-selenium solution, 100 U/ml penicillin-G, 100 μg/ml streptomycin, and 100 ng/ml FSH). Each bead was transferred into one well of a 96-well plate containing 100 μl of culture media. The follicles were cultured in an incubator at 37°C and 5% CO_2. The cultured follicles were examined daily under a light microscope, and diameters were measured using Image-Pro Plus software. After a 3-day culture, the culture medium was collected and an ELISA kit was used to detect E2 concentrations. All follicle culture experiments were repeated three times with at least 10 follicles contained in each group. Supernatants from 3–4 follicles were pooled together as one ELISA sample in each group. A brief flowchart of the process is shown in Figure 1. The concentration of DHT chosen for this study was based on a previous study in which the effect of a wide dose range of different types of androgens was observed on mouse follicles cultured *in vitro* (36).

Cell transfection

Lipofectamine 3000 was used. AR plasmid vector (2.5 μg) or control vector was mixed with 125 μl Opti-MEM medium. Lipofectamine 3000 reagent (7.5 μl) was diluted with 125 μl Opti-MEM medium. The two mixtures were combined and incubated for 10–15 min at room temperature (RT). Each mixture was used to transfect KGN cells for 6 h followed by normal culture media treatment for 48 h. Subsequently, cells were collected for RNA or protein analysis.

Determination of estradiol, testosterone, and insulin-like growth factor 1 concentrations

Cell culture supernatant was collected after a 72-h intervention and was centrifuged at 4°C and 3,000 g for 15 min. The cell supernatant was detected for E2, T, and IGF1 using ELISA kits following the manufacturer's instructions. Briefly, the culture supernatant was centrifuged to remove all unsolvable pellets. Serially diluted standard reagents and diluted samples were prepared as instructed. Standard reagents and samples were added to 96-well plates, followed by enzyme conjugate being added to each well. The plate was incubated in a specific temperature environment and washed several times with wash buffer. After addition of the substrate solution, the plate was incubated in the dark and the reaction was stopped with stop solution. The absorbance of each well was read at 450 nm by a plate reader (Biotek Multisken MK3). The concentrations were calculated using a standard curve. The analytic sensitivity for E2 was 10.6 pg/ml with an intra-assay and inter-assay of variation of <9.2% and 14.9%, respectively. The lowest analytical detectable concentration of T was 0.066 ng/ml with an intra-assay and inter-assay variability of <11.3%. The minimum detectable concentration of IGF1 ranged from 0.004 to 0.022 ng/ml with an intra-assay and inter-assay precision of <6.2%.

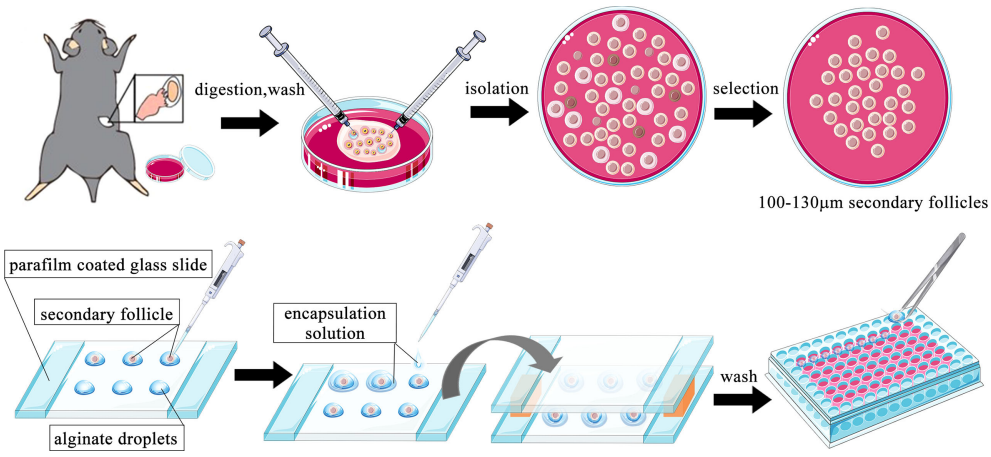

FIGURE 1
Schematic diagram of the follicle isolation and three-dimensional (3D) culture steps.

Cellular RNA extraction and real-time PCR

Cells were washed in twice. RNA was extracted using an RNA isolation kit. The RNA concentration was determined by NanoDrop (Thermo Scientific). RNA (1 μg) was mixed with reverse transcription mix and diethyl pyrocarbonate (DEPC) water and reverse transcribed into cDNA under the following conditions: one cycle at 42°C for 15 min and one cycle at 95°C for 30 s. cDNA was diluted and mixed with primers, diethyl pyrocarbonate (DEPC) water, and SYBR Green Mix. RT-PCR reactions were performed under the following conditions: one cycle at 50°C for 2 min, one cycle at 95°C for 5 min, followed by 40 cycles at 95°C for 10 s and 60°C for 30 s. Data were normalized by the B2m level and calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences are listed in Table 1.

Annexin V fluorescein isothiocyanate/propidium iodide (FITC/PI) flow cytometry

Cell apoptosis was analyzed by flow cytometry using the Annexin-V/PI staining method. After treatment with different doses of glucocorticoids, KGN cells were harvested, washed twice with cold PBS, resuspended in 100 μl binding buffer, and stained with 5 μl Annexin V-FITC and 5 μl PI. The cells were incubated for 15 min in the dark and at RT before being analyzed by a flow cytometer (Beckman Coulter).

Western blot analysis

Cells in six-well plates were washed in PBS twice and were lysed on ice with 80 μl radioimmunoprecipitation assay (RIPA) buffer containing PMSF and protease inhibitor cocktail for 10 min. Cells were scraped into 1.5 ml Eppendorf tubes and kept on ice for 30 min, then centrifuged at 4°C and 12,000 rpm for 20 min. The supernatant was transferred to a new tube. The protein concentration was determined by a bicinchoninic acid (BCA) kit. The protein solution was combined with Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) loading buffer and boiled for 5 min at 95°C. Polyacrylamide gel (10%) was used to run the SDS-PAGE. Equal amounts of 25 μg protein samples were loaded onto the gel and transferred to polyvinylidene fluoride membranes by electrophoresis. The membrane was blocked in skim milk at RT for 1 h and then incubated in first antibody solutions at 4°C overnight. After being washed three times with tris

TABLE 1 Primer sequences of target genes.

Species	Gene	Direction	Sequence
Human	*B2m*	Forward	GCTGGCGGGCATTCCTGAAG
		Reverse	AGAGCGGGAGGGTAGGAGAGAC
Human	*Igf1r*	Forward	GTTGGTGATTATGCTGTACGTC
		Reverse	TCCTTCATAGACCATCCCAAAC
Human	*Fshr*	Forward	GCATTCAATGGAACCCAACTAG
		Reverse	CGTGGAAAACATCATTAGGCAA
Human	*Star*	Forward	CATGGAGAGGCTCTATGAAGAG
		Reverse	GGACCTTGATCTCCTTGACATT
Human	*Cyp11a1*	Forward	TTTGAGTCCATCACTAACGTCA
		Reverse	GGTAGATGGCATCAATGAATCG
Human	*Cyp19a1*	Forward	GACTTTGCCACTGAGTTGATTT
		Reverse	CGATCAGCATTTCCAATATGCA
Mouse	*B2m*	Forward	TTCTGGTGCTTGTCTCACTGA
		Reverse	CAGTATGTTCGGCTTCCCATTC
Mouse	*Cyp11a1*	Forward	AGGTCCTTCAATGAGATCCCTT
		Reverse	TCCCTGTAAATGGGGCCATAC
Mouse	*Cyp19a1*	Forward	ATGTTCTTGGAAATGCTGAACCC
		Reverse	AGGACCTGGTATTGAAGACGAG
Mouse	*Lhr*	Forward	AATGAGTCCATCACGCTGAAAC
		Reverse	CCTGCAATTTGGTGGAAGAGA
Mouse	*Fshr*	Forward	CCTTGCTCCTGGTCTCCTTG
		Reverse	CTCGGTCACCTTGCTATCTTG

B2m, beta 2 microglobin; Igf1r, insulin-like growth factor 1 receptor; Fshr, follicle-stimulating hormone receptor; Star, steroidogenic acute regulatory protein; Cyp11a1, cytochrome P450 family 11 subfamily A member 1; Cyp19a1, cytochrome P450 family 19 subfamily A member 1; Lhr, luteinizing hormone receptor.

buffered saline with Tween-20 (TBST) for 10 min for each replicate, the membranes were incubated in second antibody solutions at RT for 1 h. Excess antibodies were washed off three times with TBST for 10 min for each replicate. An enhanced chemiluminescent substrate kit was used to detect the immunoreactive bands using an ImageQuant LAS 4000 mini system. Relative quantitative protein expression was determined using ImageJ, version 1.51k (NIH, USA), by normalizing to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) internal control.

Co-immunoprecipitation

KGN cells were lysed with RIPA buffer added with PMSF and protease inhibitor cocktail on ice for 20 min. GR antibody (1 µg) was added to the lysate sample and incubated on a rotating platform overnight at 4°C. Protein A/G magnetic beads (30 µl) were added to 1 ml of antibody–antigen lysate. The mixture was incubated on a rotating platform for 1 h at 4°C. The tubes were placed on a magnetic separation rack and rested for 1 min for the solution to be clear. The supernatant was discarded, and the beads were washed with 500 µl lysis buffer and placed on a magnetic rack for separation again. The wash step was repeated five times. The samples were kept on ice throughout the procedure. The final wash aimed to remove all trace substances from the beads. The beads were resuspended in 1× SDS-PAGE loading buffer and placed in an iron bath for 5 min at 95°C with a 300-rpm rotation. The denatured protein complex was released from the beads and was separated by a magnetic rack. The supernatant was collected for further WB analysis.

Chromatin immunoprecipitation

KGN cells were cultured in a 14-cm dish (80% confluent) in 20 ml of DMEM + 10% FBS. DHT (10 nM) was added to the culture media for 1 h before the ChIP experiment. Formaldehyde (540 µl of 37%) was added to the media and incubated for 10 min at RT. Cross-linking was stopped by adding 2 ml of 10× glycine followed by incubation for 5 min at RT. The cells were washed with ice-cold PBS + protease inhibitor cocktail twice and scraped into a 1.5-ml tube. After centrifuging at 1,000 g for 5 min at 4°C, the cell pellet was resuspended with cell lysis buffer containing the protease inhibitor cocktail. After a 15-min incubation on ice, the tube was centrifuged again and the pellet was resuspended with nuclear cell lysis buffer containing the protease inhibitor cocktail. The cell lysate was then sonicated under high power, 30 s On 30 s Off, 10 cycles for three repeats. Sheared chromatin was centrifuged at 10,000 g at 4°C for 10 min, and the clear supernatant was collected for IP. Two aliquots of 50 µl of chromatin solution, namely, "AR" and "IgG," were supplemented with 450 µl dilution buffer containing a protease inhibitor cocktail. Aliquots (5 µl) of the supernatant were saved at 4°C as "input" for further use. AR antibody (5 µg) was added to the "AR" sample, and 1 µg of mouse normal IgG was added to the "IgG" sample. Both tubes were also supplemented with 20 µl protein A/G magnetic beads and incubated overnight at 4°C with rotation. The beads were pelleted with a magnetic separator and washed with low-salt wash buffer, high-salt wash buffer, lithium chloride wash buffer, and Tris-EDTA buffer once, serially. The protein/DNA complexes were released from the beads by adding ChIP elution buffer and incubated at 62°C for 2 h with shaking, followed by being incubated at 95°C for 10 min. Immunoprecipitated chromatin was separated from the beads in a magnetic separation rack, and DNA was purified using spin columns. Immunoprecipitation of AR-associated DNA fragments was verified by qPCR using primers directed against *Igf1r* and *Fshr*. The primer sequences used for ChIP-qPCR are listed in Table 2. The values from the immunoprecipitated samples were normalized to that from the input DNA.

Statistical analysis

Concentrations are expressed as mean ± SEM. Statistical analysis was carried out with SPSS 22.0 software. t-tests were used to compare differences between two groups with equal variance. One-way ANOVA was used to compare differences between more than two groups. Two-way ANOVA was used to compare the effect of different concentrations of CORTN and treatment time on the secretion of T in TIC culture supernatant. The comparison of ovarian follicle diameter changes over 3 days

TABLE 2 Primer sequences for ChIP-qPCR.

Species	Gene	Direction	Sequence
Human	*Igf1r*	Forward	GGAACATCCAAAACTGAAACTCTT
		Reverse	TTCATATGATGGGATGGTTTTG
Human	*Fshr*	Forward	GAAGGAGGATCCAGGGAAAG
		Reverse	ACAGGAGGGCAGAGGAAAAT

between groups was made using repeated-measures ANOVA. $P < 0.05$ was taken to indicate a significant difference.

Results

Cortisol affected the viability and estradiol secretion of KGN cells in a time- and dose-dependent manner

As Figure 2A shows, cell viability decreased with increasing CORT dose. When the dose of CORT reached >500 nM and KGN cells were treated for 24 h, cell viability decreased significantly compared to that of the control group. Cell viability decreased more obviously when the treatment time was increased to 48 and 72 h. KGN cells were treated with CORT at different doses for 72 h followed by examination of apoptosis. As shown in Figure 2B, there was no significant difference in cell apoptosis among the groups. The cell culture supernatant of KGN cells after treatment with CORT at different doses after 48 and 72 h was collected and detected for E2 and IGF1 concentrations. Figure 2C shows no significant difference in IGF1 concentrations between different groups, but the E2 concentrations significantly decreased in the high-dose CORT groups (≥100 nM) compared to the solvent groups after 48 and 72 h (Figure 2D). The degree of decrease in E2 concentration increased with treatment time.

Long-time exposure to high doses of corticosterone decreased estradiol secretion of mouse granulosa cells and testosterone secretion of theca/interstitial cells

We first identified the two cell types by morphology and the expression of specific markers using qPCR and WB. As shown in Figure 3A, mGC exhibited a polygonal and paving stone-like shape under the microscope, while TIC showed a spindle shape

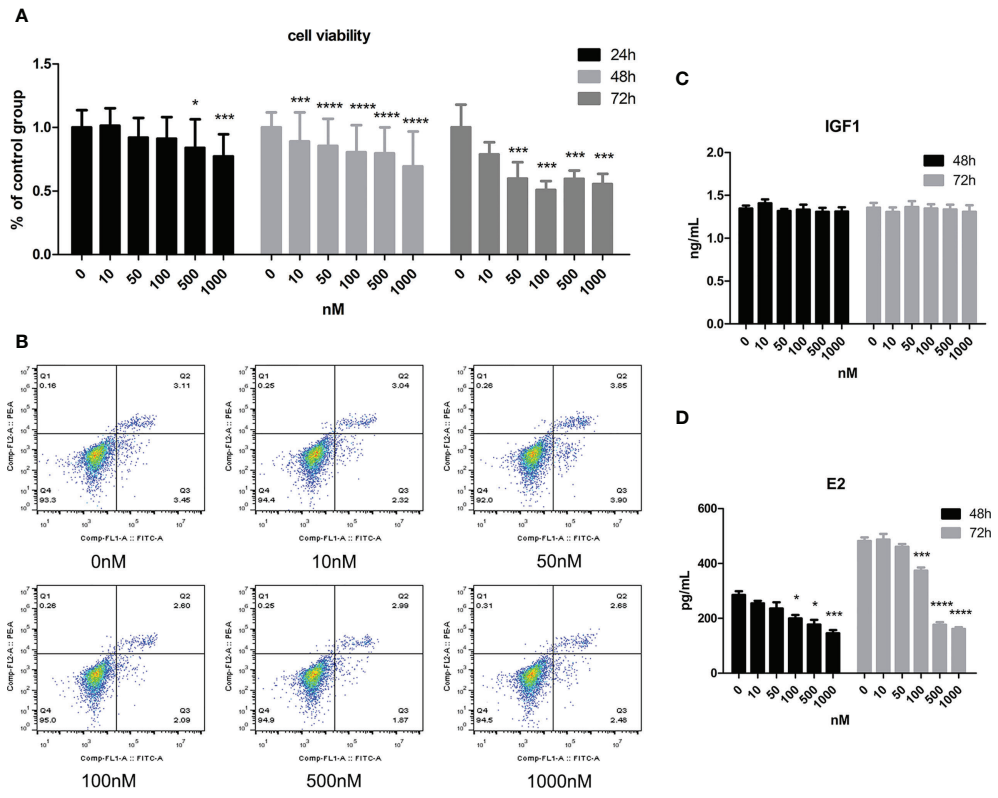

FIGURE 2
The impact of CORT at different doses on cell viability, apoptosis, E2 secretion, and IGF1 secretion. KGN cells were treated with solvent or 10, 50, 100, 500, and 1,000 nM CORT, respectively. Cell viability was assessed at 24, 48, and 72 h by the CCK-8 method. n ≥ 17 in each group at each time point; one-way ANOVA. **(A)** Cell apoptosis was determined at 72 h by flow cytometry. n = 3 with two repeats in each assay; one-way ANOVA. **(B)** IGF1 **(C)** and E2 **(D)** concentrations were determined by ELISA after treatment for 48 and 72 h n = 3 with one repeat in each assay; one-way ANOVA. *P < 0.05, ***P < 0.001, ****P < 0.0001 compared to group "0."

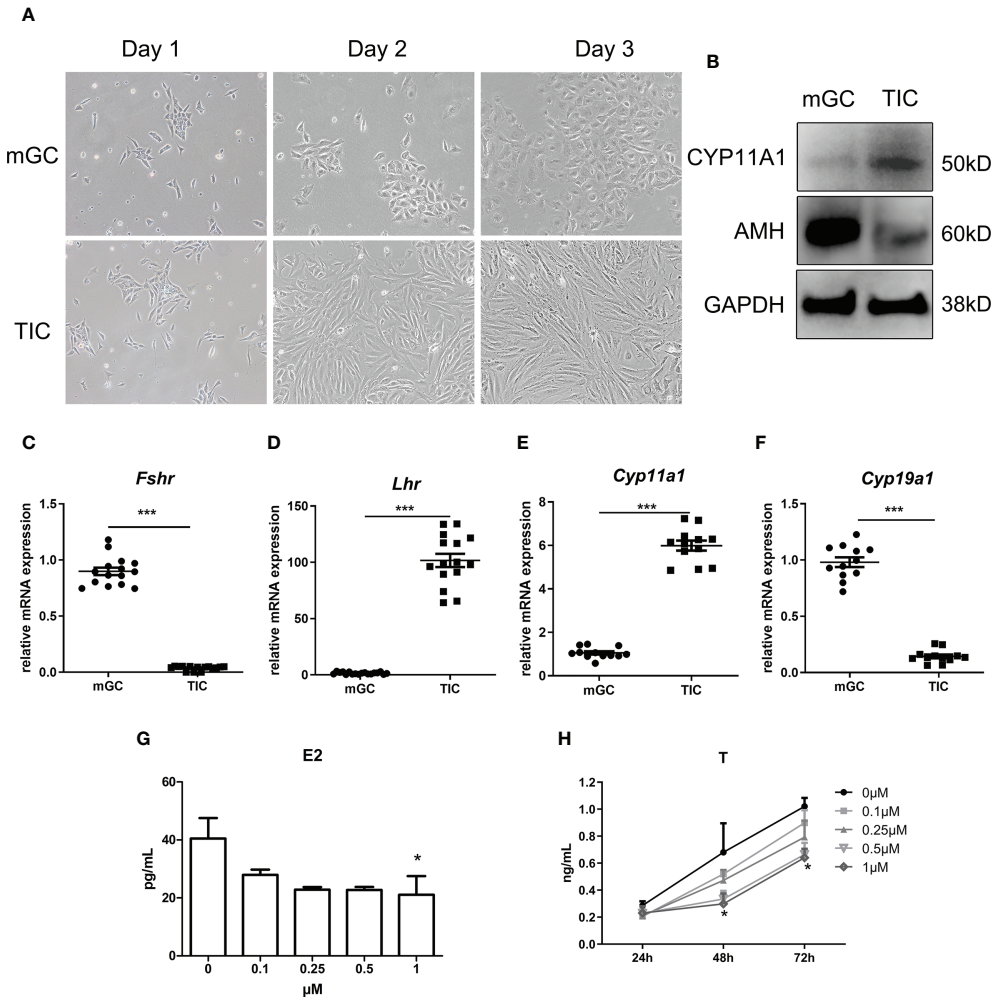

FIGURE 3
Identification of mGC and TIC and the influence of CORTN on sex hormone secretion of the two cell types. Morphology of the primary cell was recorded for 3 days after isolation using an inverted microscope. **(A)** Protein and RNA were extracted from the cells. CYP11A1 and AMH protein expression of the two cell types were examined by Western blot. n = 3; t-test. **(B)** Relative mRNA expression of *Fshr*, *Lhr*, *Cyp11a1*, and *Cyp19a1* was examined by qPCR. n = 15; t-test. **(C–F)** mGC and TIC were treated with solvent or 0.1, 0.25, 0.5, and 1 μM CORTN, respectively. Cell culture supernatant of mGC after 72 h was collected to determine the concentration of E2 by ELISA. n = 3 with one repeat in each assay; one-way ANOVA. **(G)** Cell culture supernatant of T after 24, 48, and 72 h was collected to determine the concentration of T by ELISA. **(H)** *P < 0.05, ***P < 0.001 compared to group "0." n = 3 in each group at each time point with one repeat in each assay; two-way ANOVA.

and cord-like shape; these findings were consistent with the morphological manifestations of epithelial cells and mesenchymal cells. Figure 3B shows that the protein expression of AMH and Cytochrome P450 Family 11 Subfamily A Member 1 (CYP11A1) in mGC was significantly higher than those in TIC. Furthermore, Figures 3C–F shows that the mRNA expression of *Fshr* and *Cytochrome P450 Family 19 Subfamily A Member 1 (Cyp19a1)* in GCs was significantly higher than that in TIC. *Cyp11a1* and *luteinizing hormone receptor (Lhr)* mRNA expression was significantly higher in TIC than that in mGC, which agrees with the expression of markers of the two cell types. CORTN at different doses was applied to mGC and TIC for 72 h. As shown in Figure 3G, E2 secretion decreased with the increase of CORTN concentration. The secretion of E2 after the 1-μM CORT treatment for 72 h was significantly lower than that of the control group. T secretion of TIC also decreased with the increase of CORTN concentration (Figure 3H). T level in the 1-μM CORTN group was significantly lower than that in the control group after 48 and 72 h of treatment.

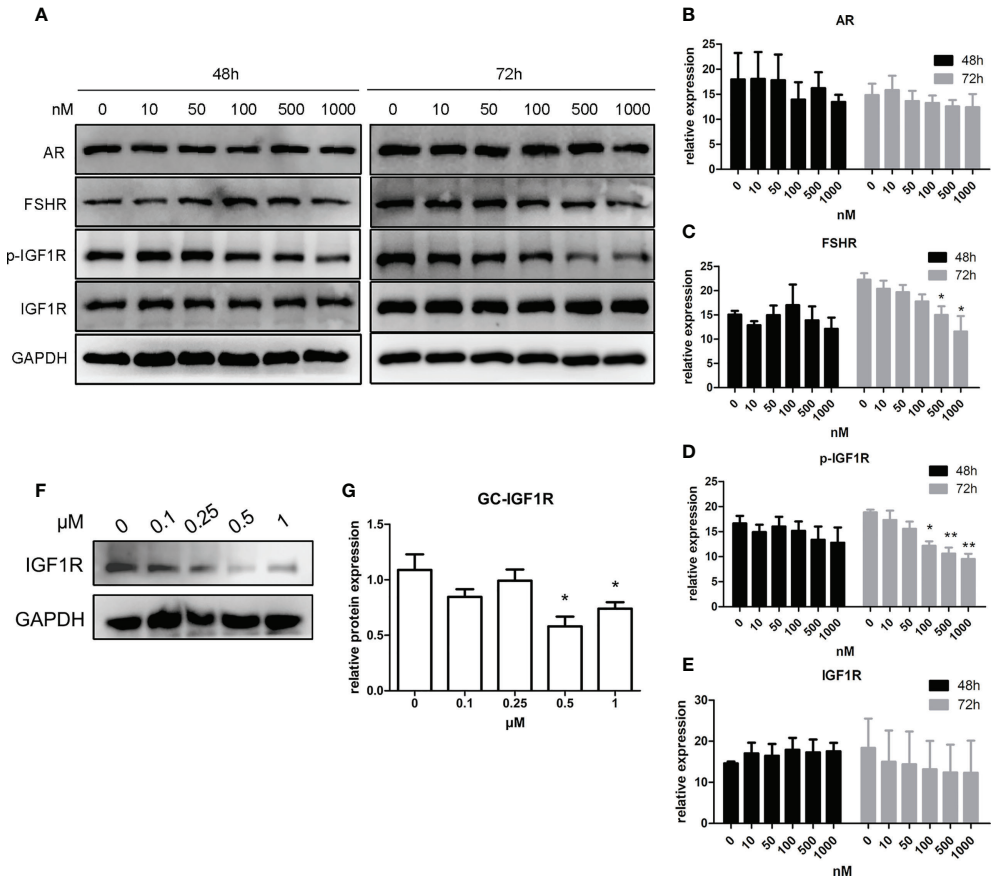

FIGURE 4
Impact of glucocorticoids at different doses on the protein expression of hormone receptors of KGN cells and mGC. KGN cells were treated with solvent or 10, 50, 100, 500, and 1,000 nM CORT, respectively. Protein levels of AR, FSHR, IGF1R, and p-IGF1R were determined by Western blot after 48 and 72 h **(A)** Semiquantitative analysis was performed after three repetitions. n = 3 in each time point and each group; one-way ANOVA. **(B–E)** mGCs were treated with solvent or 0.1, 0.25, 0.5, and 1 μM CORTN, respectively, for 72 h Cells were harvested for Western blot analysis of IGF1R **(F)** and semiquantitative analysis. n = 3; one-way ANOVA. **(G)** *P < 0.05, **P < 0.01 compared to group "0."

Long-time exposure to high-dose glucocorticoids decreased the responsiveness of granulosa cells to follicle-stimulating hormone and insulin-like growth factor 1 signals by decreasing their receptor number

As shown in Figures 4A–E, after a 48-h manipulation of CORT, the protein expressions of FSHR, p-IGF1R, and IGF1R were not significantly changed in KGN cells. However, when the treatment time was extended to 72 h, FSHR expression significantly decreased in the 500-nM CORT group and 1,000-nM CORT group compared to that of the control group. The protein expression of IGF1R remained unchanged, but p-IGF1R protein expression decreased significantly in 100-nM–1,000-nM CORT groups compared to that of the control group. mGC was cultured and treated with CORTN at different doses for 72 h. As shown in Figures 4F, G, IGF1R protein expression was significantly decreased in 0.5-μM and 1-μM CORTN groups compared to that of the control group.

Long-time exposure to high-dose glucocorticoids hindered the interaction between glucocorticoid receptor and insulin-like growth factor 1 receptor in KGN cells

Figures 5A–C show that the phosphorylation level of AKT and ERK after treatment with CORT at various concentrations was almost the same. Figures 5D, E show that at 48 h, there was a positive interaction between GR and IGF1R, and there

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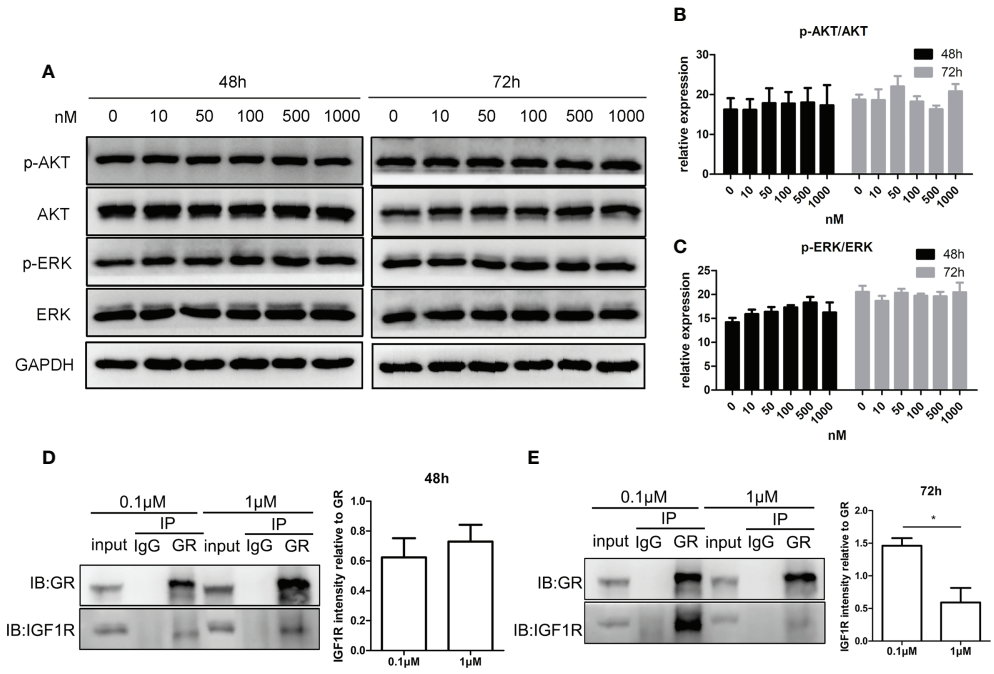

FIGURE 5
Impact of glucocorticoids on cell signals of steroidogenesis and the protein interaction between GR and IGF1R in KGN cells. KGN cells were treated by different doses of CORT for 48 and 72 h, respectively, and harvested for Western blot analysis of AKT, p-AKT, ERK, and p-ERK. **(A)** Phosphorylation levels of AKT **(B)** and ERK **(C)** were determined to assess the direct influence of glucocorticoids on the steroidogenesis signal. n = 3 in each time point and each group; one-way ANOVA. KGN cells were treated with low-dose (0.1 μM) and high-dose (1 μM) CORT, respectively. Protein–protein interaction between GR and IGF1R was detected by Co-IP after 48 h **(D)** and 72 h **(E)**. n = 3, t-test. *P < 0.05.

was no significant difference between the two CORT dose groups. However, after 72 h of treatment, the interaction between GR and IGF1R decreased significantly in the high-dose CORT group compared to that in the low-dose CORT group.

An appropriate dose range of androgens increased the expression of insulin-like growth factor 1 receptor and follicle-stimulating hormone receptor in granulosa cells

As shown in Figures 6A–C, IGF1R expression of KGN cells increased with androgen concentration in the range of 0–10 nM, while IGF1R expression decreased with androgen concentration in the range of 10–100 nM. The trend of FSHR expression was similar to that of IGF1R after 12 and 24 h of treatment (Figures 6D, E). IGF1R and FSHR both reached the highest expression with 10-nM DHT treatment and were significantly higher than those in the control groups after 24 h of treatment. The expression of IGF1R and FSHR was lowest in the 100-nM groups, which was significantly lower than that in the 10-nM groups.

Androgens enhanced the effect of insulin-like growth factor 1 and follicle-stimulating hormone in promoting the expression of steroid hormone synthase in the presence of high-dose glucocorticoids

As shown in Figures 7A–C, all of the gene expressions in cells treated with low levels of DHT and FSH at various doses were significantly higher compared to those in cells treated with FSH alone, while gene expressions in cells treated with high levels of DHT and FSH were significantly lower than those in cells treated with FSH alone.

Figures 7D, E show the expressions of steroid hormone synthase after treatment with low/high concentrations of CORT followed by either one of the three factors or the combination of the three factors. The expression of Cyp19a1 and Star in cells treated with the combination of DHT-IGF1-FSH was significantly higher than that of cells treated without the three factors or cells, treated with one factor regardless of the dose of CORT. For the expression of other steroid hormone synthases, the results in the combined treatment groups were also significantly higher than those in the cells treated without factors or treated with androgens or IGF1 alone.

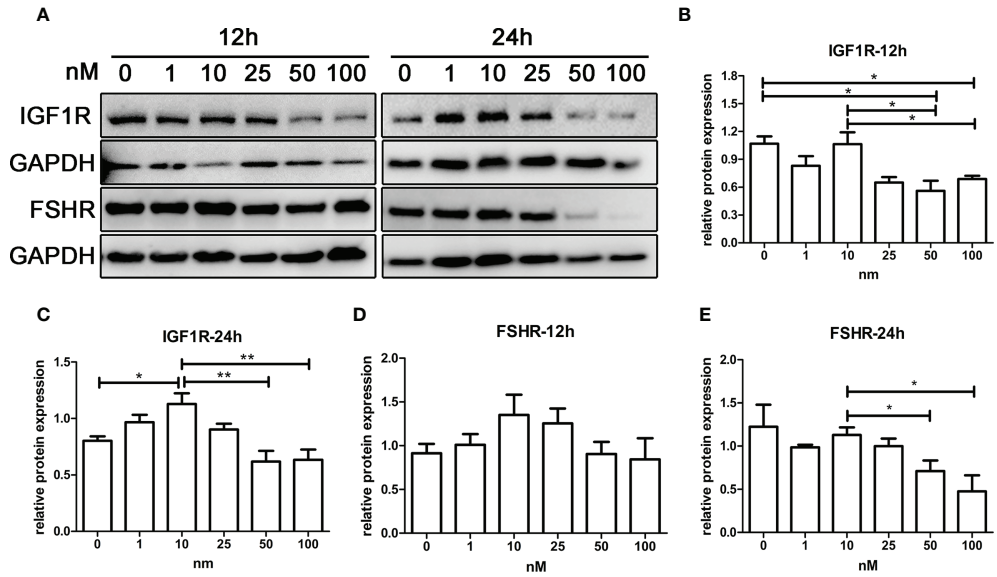

FIGURE 6
The effect of androgens on the expression of FSHR and IGF1R of KGN cells. KGN cells were treated with solvent or 1, 10, 25, 50, and 100 nM DHT. The cells were collected for Western blot analysis **(A)** to determine the IGF1R **(B, C)** and FSHR **(D, E)** protein levels after 12 and 24 h. n = 3 in each time point and each group; one-way ANOVA. *P < 0.05, **P < 0.01.

Androgens improved insulin-like growth factor 1 receptor and follicle-stimulating hormone receptor expression of granulosa cells by directly binding to the promoters of *Igr1r* and *Fshr*

As shown in Figures 8A, B, the efficiency of three siRNAs was tested using qPCR and WB. The expression of AR in the shAR2 group showed good efficiency in both qPCR and WB results. Therefore, shAR2 was applied in subsequent experiments. The gene and protein expressions of IGF1R and FSHR were significantly downregulated in AR-knockdown KGN cells compared to those of the control group (Figures 8C, D). ChIP-qPCR results (Figure 8E) showed that the relative enrichment degree of two sequences was significantly higher than those in the IgG negative control, thus confirming the combination of ARs to the promoter regions of *Igf1r* and *Fshr*.

High-dose glucocorticoids inhibited the growth of mouse ovarian follicles cultured *in vitro*, which was improved by androgens at appropriate doses

As shown in Figures 9A, B, the follicles supplemented with low-dose CORTN (LC) or high-dose CORTN (HC) grew slower than those in the control groups (N). Follicles treated with both 10-nM DHT and high-dose CORTN (HC+DHT) grew significantly faster than those in the HC group. DHT combined with IGF1 treatment (HC+DHT+IGF1) also significantly improved follicle growth, and the growth trend of the follicles was superior to that of the HC+DHT group. E2 concentration was detected by ELISA after a 3-day *in vitro* culture. As shown in Figure 9C, E2 concentration in the LC group was not significantly changed compared to that of the N group, but the E2 concentration in the HC group was significantly decreased over that in the N group. E2 concentration in the HC+DHT group was significantly improved compared to that of the HC group, and E2 concentration in the HC+DHT+IGF1 group was significantly higher than those of any other group.

Discussion

DOR is an important pathological factor affecting female reproductive health and pregnancy rate. An important contributor to DOR, chronic stress adversely affects ovarian reserve mainly through high concentrations of glucocorticoids released after HPA axis activation. In this study, we first investigated the effects of glucocorticoids on apoptosis, cell viability, and E2 secretion in human granulosa cell lines, KGN cells, and mouse primary granulosa cells. We found that high concentrations of glucocorticoids reduced granulosa cell viability and steroid hormone secretion, and the adverse effects increased with glucocorticoid concentrations and duration of

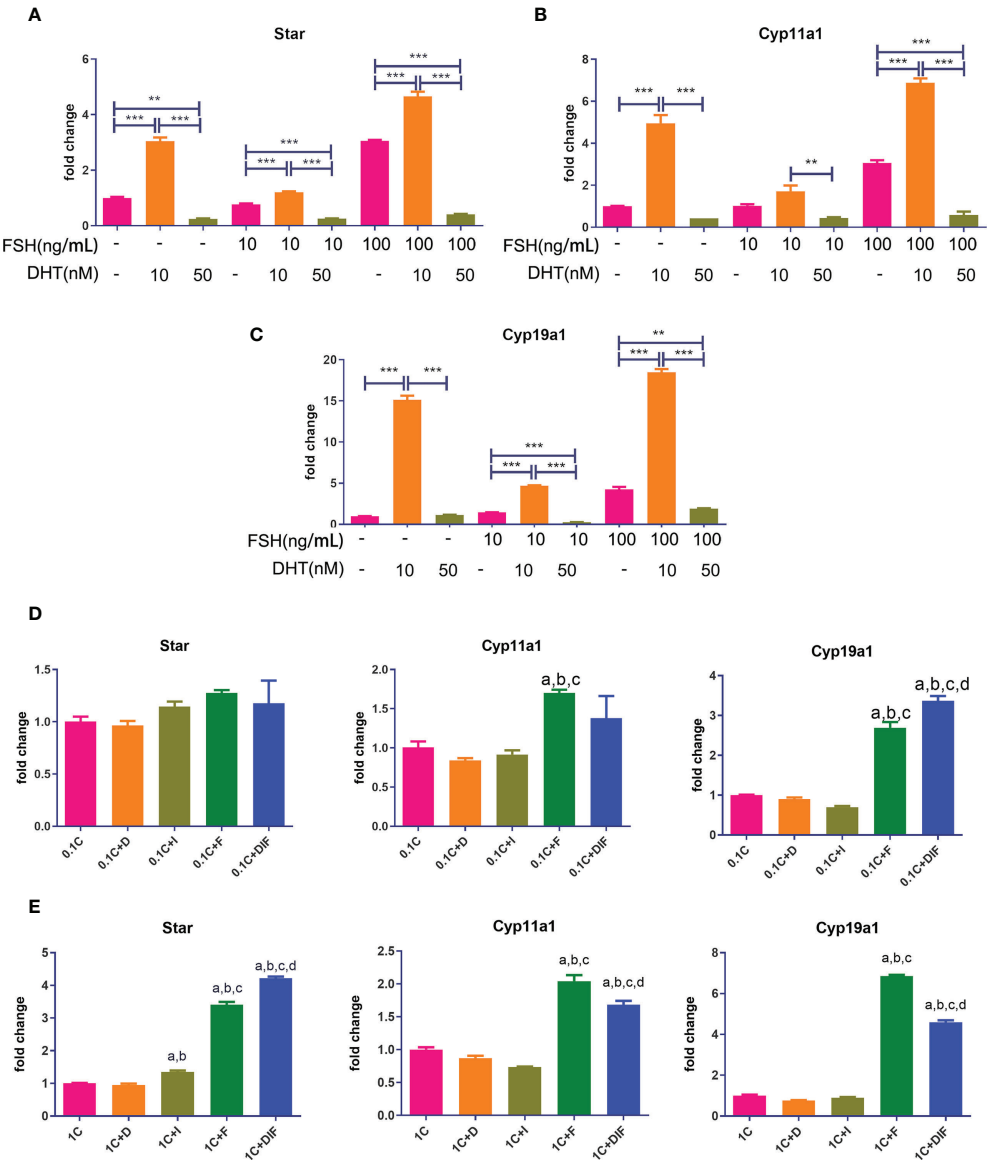

FIGURE 7
Androgen-IGF1-FSH synergistic effect on promoting the expression of steroid hormone synthase and the influence of glucocorticoids on this effect. KGN cells were treated with solvent or low-dose (10 nM) and high-dose (50 nM) DHT in the presence of different doses of FSH for 24 h mRNA expressions of *Star* **(A)**, *Cyp11a1* **(B)**, and *Cyp19a1* **(C)** were determined by qPCR. n = 3; one-way ANOVA. KGN cells were treated with low-dose **(D)** and high-dose **(E)** CORT for 24 h and then treated with and without DHT, IGF1, or FSH alone and combined DHT-IGF1-FSH, respectively, for 48 h mRNA expressions of *Star*, *Cyp11a1*, and *Cyp19a1* were determined by qPCR. n = 3; one-way ANOVA. 1C: 1 μM CORT; 1C +D: 1 μM CORT + 10 nM DHT; 1C+I: 1 μM CORT + 100 ng/ml IGF1; 1C+F: 1 μM CORT + 20 ng/ml FSH; a: P < 0.05 compared to "1C" group; b: P < 0.05 compared to "1C+D" group; c: P < 0.05 compared to "1C+I" group; d: P < 0.05 compared to "1C+F" group. **P<0.01;***P<0.001.

intervention. Similarly, in a previous *in vivo* mouse study, Gao et al. (37) found that the oocyte developmental potential of growing follicles decreased with the increase of chronic stress time and intensity. These results suggest that attention be paid to the impairment of long-term chronic stress on ovarian function. When interpreting clinical studies on stress that have conflicting results, special attention should be given to distinguishing between different effects caused by acute and chronic stress, which may lead to different trends in patient serum E2 levels (38, 39). In the results of cell apoptosis, we found that glucocorticoids did not directly induce apoptosis in granulosa cells, which was different from a previous study in mice that found that chronic stress promotes of cumulus cells and granulosa cells in mice (40). This difference in results suggests that chronic stress states may not directly damage granulosa cells but indirectly damage follicle development through certain changes in the ovarian

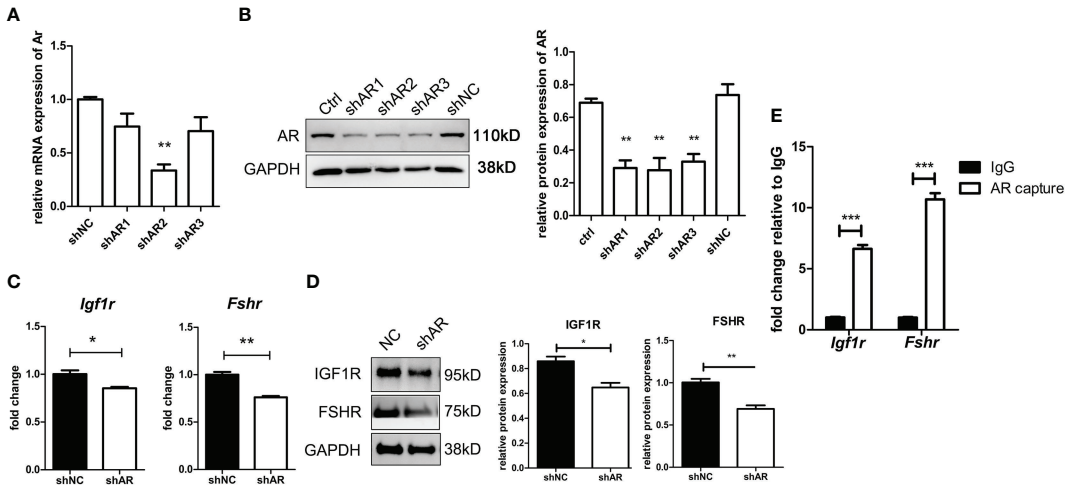

FIGURE 8
AR directly stimulates IGF1R and FSHR expression by binding to the promoter regions of IGF1R and FSHR. KGN cells were transfected by three shAR vectors. The efficiency of knockdown was tested by qPCR **(A)** and Western blot **(B)**. n = 3; t-test comparison between the control group and other groups. shAR2 was used for further experiments, and the transfected cells were collected to determine the mRNA expression **(C)** and protein expression **(D)** of IGF1R and FSHR. n = 3; t-test. KGN cells were treated with DHT for 1 h followed by the ChIP assay. AR antibody-enriched DNA fragments were detected for promoter sequences of IGF1R and FSHR by qPCR. n = 3; t-test. **(E)** *P < 0.05; **P < 0.01; ***P < 0.001.

microenvironment. Notably, we observed a significant decrease in T secretion following chronic administration of high-dose glucocorticoids in mouse TIC. To our knowledge, this is the first evidence that chronic stress may affect steroid hormone synthesis of theca cells. Because the binding signal of androgens to ARs is an important factor supporting early follicular development, high levels of glucocorticoids caused by chronic stress can significantly reduce the supporting effect of androgens on early follicular development; concurrently, the precursors of E2 are also reduced, which indirectly leads to ovarian follicle dysfunction. However, the specific mechanism through which glucocorticoids reduce androgen production requires further study.

The expression level of FSHR in granulosa cells determines the sensitivity of follicles to FSH signaling. Although early follicles are gonadotropin-independent, certain concentrations of FSH are required (35). In a mechanistic exploration, we examined the inhibition of FSHR protein expression in KGN cells by prolonged exposure to high-dose glucocorticoids. This result is consistent with our previous reports in a mouse model of unpredictable chronic stress-induced DOR (14, 15). We exposed mice to randomly imposed stressors such as restraint, day-night reversal, single-cage feeding, and tail suspension daily for 8 weeks. We found that the follicular reserve of mice was significantly reduced, so was the protein expression of FSHR in ovarian follicles. In a recent study of primary cultured rat granulosa cells, Kashino et al. (41) found that dexamethasone treatment dose-dependently decreased E2 production induced by FSH and cAMP synthesis induced by FSH. This decreased FSHR signaling may suggest a decreased FSHR expression. Another study in weaned sows found that long-term repeated intravenous administration of adrenocorticotropin hormone, the hormone promoting glucocorticoid secretion, affects E2 secretion and reduced LHR mRNA expression in the corpus luteum (42). Unfortunately, the FSHR expression in follicles remains unknown.

IGF1 signaling is another important growth signal for follicular development, which can regulate E2 production independently or through synergistic effects with FSH (43). Although IGF1R signaling is important for maintaining follicle development and hormone secretion, there are very few previous reports on the effects of excess glucocorticoids or chronic stress on IGF1 and IGF1R expression. An old study in bovine reported that cortisol at physiological levels had little or no effect on the number of IGF1R in granulosa cells from small follicles (44). In this study, we assessed the secretion of IGF1 after glucocorticoid treatment and found no significant differences between groups. However, we found that the autophosphorylation level of IGF1R protein in KGN cells decreased significantly after prolonged exposure to high concentrations of CORT, while the expression of IGF1R did not change significantly. We did not observe identical changes as in KGN cells after treatment of mGC with CORTN. The results showed directly decreased IGF1R protein expression after high concentrations of CORTN intervention. Although there existed this difference in human and mouse cells, the results suggest that chronic stress-induced high concentrations of glucocorticoids impair granulosa cell sensitivity to IGF1 signaling.

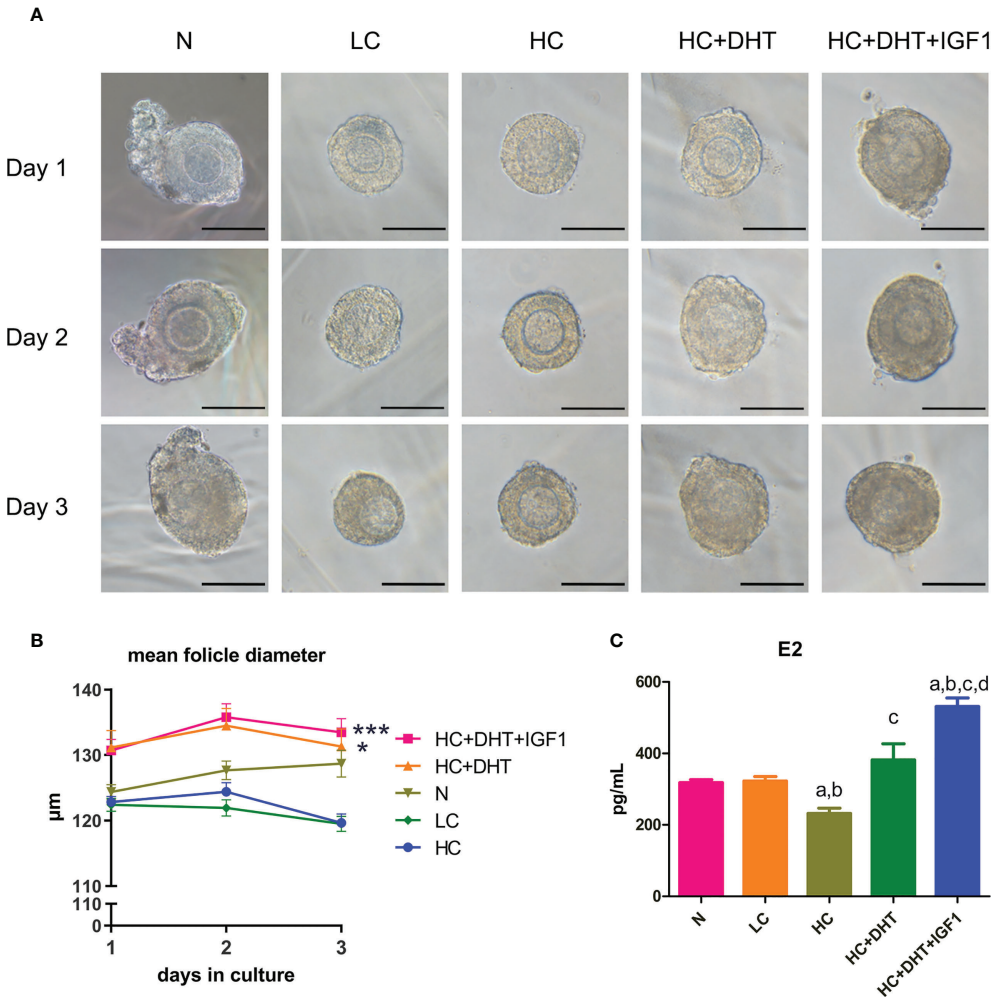

FIGURE 9
The effect of glucocorticoids on the development of 3D cultured mouse ovarian follicles and the therapeutic effect of androgens. Mouse ovarian follicles were isolated and 3D-cultured in alginate beads for 3 days. The follicles were divided into normal (N), 0.1 μM CORTN (LC), 1 μM CORTN (HC), HC + 10 nM DHT (HC+DHT), and HC + 10 nM DHT + 100 ng/ml IGF1 (HC+DHT+IGF1) groups. The culture medium was supplied with and without listed drugs, respectively. The morphology **(A)** and average diameter **(B)** of follicles in each group were monitored for 3 days. n ≥ 30 in each group; repeated-measures ANOVA. After 3 days of culture, culture medium supernatant was collected for ELISA assay to determine the E2 concentration. n ≥ 7 in each group; one-way ANOVA. **(C)** *P < 0.05 compared to group "N"; ***P < 0.001 compared to group "N"; a: P < 0.05 compared to group "N"; b: P < 0.05 compared to group "LC"; c: P < 0.05 compared to group "HC"; d: P < 0.05 compared to group "HC+DHT.".

Because IGF1 mainly regulates hormone secretion by binding to IGF1R to activate downstream AKT and ERK signaling (10, 45, 46), we also tested whether glucocorticoids themselves can affect the phosphorylation levels of AKT and ERK signaling in KGN cells; results found no significant difference, indicating that high concentrations of glucocorticoids reduced IGF1 signaling sensitivity and E2 secretion by inhibiting IGF1R autophosphorylation in KGN cells. Autophosphorylation of IGF1R occurs on the cell membrane (47); therefore, we hypothesized that when additional ligands bind to GRs under chronic stress, GRs may interact with IGF1R on the cell membrane and thus affect the exposure of IGF1R phosphorylation sites. The co-IP results demonstrate for the first time that GRs and IGF1R interact in KGN cells. This interaction may sustain a certain level of autophosphorylation of IGF1R. Long-time manipulation of high-dose glucocorticoids could weaken the interaction of the two receptors and thus interfere with the autophosphorylation of IGF1R in KGN cells, which leads to the low responsiveness to IGF1 signals. However, the specific protein conformational and phosphorylation site changes during this process remain to be verified.

Treatment for DOR includes various ovarian stimulation regimens and adjuvant sequential hormone therapy. Androgen

supplementation is the first treatment option that promises to increase the pool of recruitable follicles (48). Several clinical findings suggest that supplementation with T or DHEA significantly improves ovarian reserve and increases oocyte retrieval and pregnancy rates in DOR patients (24). However, some clinical studies have concluded that androgen supplementation is ineffective (49), which may be attributable to differences in androgen type, dose, and duration of treatment used in different studies. T and DHT are the only two androgens that bind directly to ARs (50). We used DHT in our cellular experiments for this study to exclude T from converting to estrogen and acting through estrogen receptors. We first observed the effect of different doses of DHT on the expression of IGF1R and FSHR on KGN cells. The results showed that androgens had opposite effects at different doses, and only a relatively low dose range (10–25 nM) of androgens could promote the protein expression of IGF1R in KGN cells. This result reemphasizes that maintaining the balance of androgen concentrations is critical for ovarian follicle development (17). A recent study on AR metabolism in granulosa cells showed that ligand binding of ARs significantly prolongs AR half-life by maintaining its nuclear localization and protecting it from degradation in the cytoplasm (51). This positive feedback effect may ensure androgen function at low secretion levels.

We treated KGN cells with DHT and FSH to observe the regulatory effect of different concentrations of androgens on FSH signaling. By examining the mRNA expression of steroid hormone synthase in KGN cells, we found that the effect of androgens on FSH signaling is also bidirectional. Only a low dose range of DHT enhanced the effect of FSH in promoting steroid hormone synthase transcription. Furthermore, both in the context of low- and high-concentration glucocorticoids, androgens in the low-dose range synergized with IGF1 and FSH to promote steroid hormone synthase transcription. These results suggest that appropriate levels of androgens are expected to enhance E2 secretion in granulosa cells under chronic stress conditions through synergistic signaling with IGF1 and FSH.

In the ChIP-qPCR results, we verified that ARs act as transcription factors that directly bind to the *Igf1r* and *Fshr* promoters in KGN cells, thereby regulating the transactivation of the two receptor genes. A recent ChIP sequencing (ChIP-seq) study found that ARs can regulate gene expression by altering the methylation of histones near the gene promoters or enhancers (52). Whether ARs regulate the transcription of *Igf1r* and *Fshr* genes directly or indirectly through the methylation regulation in KGN cells requires future study. However, in mGC, Sen et al. (53) report a different regulatory mechanism of DHT. They demonstrate that 25 nM of DHT promotes FSHR protein expression, but not *Fshr* transcription. They further validated that DHT regulates FSHR through paxillin-activated ERK1/2 signaling, thereby regulating FSHR protein synthesis. This reflects another difference between humans and mice in the mechanism by which androgens regulate the sensitivity of granulosa cells to FSH signaling.

For a more intuitive observation, mouse secondary follicles were isolated and cultured in alginate beads *in vitro*. We observed the effects of glucocorticoids on early follicular development and steroidogenesis and the efficacy of androgens alone and androgens in combination with IGF1. The results confirmed that high concentrations of glucocorticoids inhibited early-stage follicle growth and E2 secretion of ovarian follicles. Androgens at an appropriate dose range can synergize with IGF1 to ameliorate the damage caused by glucocorticoids. To our knowledge, this is the first direct evidence that chronic stress impairs early-stage follicle growth and function in mice. Previous studies have reviewed the effect of different doses of different types of androgens on ovarian follicles cultured *in vitro* (36, 54). They demonstrate that different types of androgens work best at improving mouse early-stage ovarian follicle development at nearly the same 10-nM concentration. This dose is consistent with the optimal dose of androgens we observed in KGN cell experiments. Therefore, we used this dose of androgens in follicle culture to verify their therapeutic effects. Furthermore, we observed that when IGF1 was added to the culture system, there was a better effect not only on follicle diameter changes but also on E2 secretion. It is worth mentioning that in our pilot study, it was observed that follicle growth was not ideal if FSH was not added to the medium, so the follicle media we used in this study all contained FSH. Based on this fact, we only examined differences between androgen therapy and androgen-IGF1 combination therapy. Our results further validated the synergistic effect of androgens and IGF1.

In conclusion, the present study demonstrates that high-dose glucocorticoids impair E2 secretion in granulosa cells and early-stage ovarian follicles by inhibiting FSHR and IGF1R expression or indirectly by inhibiting autophosphorylation of IGF1R. Appropriate concentrations of androgens can alleviate the damage of high-dose glucocorticoids on follicular growth and steroidogenesis. Androgens can increase ovarian follicle E2 secretion by activating the transcription of IGF1R and FSHR.

Data availability statement

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

Ethics statement

This study was reviewed and approved by the Animal Experimental Ethical Committee of Fudan University.

Author contributions

WW and LG contributed to the conception and design of the study. LG and HG performed the experiments. LG performed the statistical analysis and drafted the manuscript. WW assisted in manuscript modification. All authors contributed to manuscript revision, read, 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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Serum kisspeptin levels mainly depend on ovarian expression of *Kiss1* mRNA in female rats

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The hypothalamic kisspeptin/KISS1 receptor system is essential for puberty onset and reproductive development. Although serum kisspeptin might be associated with puberty, its levels, according to developmental stage, and its origin still remain unclear. This study evaluated the changes in serum kisspeptin levels during puberty and the corresponding *Kiss1* mRNA and protein expression in various organs of female rats to identify the source of serum kisspeptin. Tissues from several organs, including the ovaries and anteroventral periventricular nucleus (AVPV) and arcuate nucleus (ARC) in the hypothalamus, were obtained for assessing *Kiss1* mRNA and protein expressions. Serum kisspeptin levels progressively increased with developmental stages until the peripubertal stage. The ovaries showed the highest *Kiss1* expression among the organs examined. Next, we explored the changes in serum kisspeptin levels and hypothalamic *Kiss1* expression in ovariectomized and estradiol-treated ovariectomized rats. Serum kisspeptin levels decreased regardless of estradiol treatment; *Kiss1* expression was enhanced by ovariectomy and estradiol treatment in the ARC, while it was decreased by ovariectomy and enhanced by estradiol in the AVPV, suggesting that serum kisspeptin may be associated with pubertal development and mainly depended on ovarian *Kiss1* expression. Thus, serum kisspeptin levels are associated with puberty and may serve as a downstream marker of ovarian reproductive function.

KEYWORDS

kisspeptins, Kiss1, ovary, hypothalamus, puberty, female, rats

1 Introduction

Puberty is a highly orchestrated and regulated process that occurs by activating the hypothalamic–pituitary–gonadal (HPG) axis. The HPG axis is a complex biological system that is not yet fully characterized. Kisspeptin, produced by *KISS1*, acts as a gatekeeper for puberty onset *via* its cognate receptor GPR54 (also known as the KISS1 receptor [KISS1R]) (Ohtaki et al., 2001; Seminara et al., 2003). The kisspeptin/KISS1R system has been established as a regulator of puberty based on studies showing that inactivating and activating mutations in either *KISS1* or *KISS1R* were associated with

hypogonadotropic hypogonadism and precocious puberty (de Roux et al., 2003; Seminara et al., 2003; Topaloglu et al., 2012; Novaira et al., 2014). In addition, *KISS1* is expressed within the hypothalamus, especially in the anteroventral periventricular nucleus (AVPV) and arcuate nucleus (ARC) (Smith et al., 2005; Clarkson and Herbison, 2006), and its expression increases as puberty progresses (Clarkson and Herbison, 2006; Semaan and Kauffman, 2015). In animal studies, central infusion of a kisspeptin antagonist suppresses gonadotropin-releasing hormone (GnRH) pulses and reduces luteinizing hormone (LH) pulse frequency (Adachi et al., 2007; Li et al., 2009; Roseweir et al., 2009), while central administration of kisspeptin induces precocious activation of the HPG axis and results in precocious puberty (Navarro et al., 2004). Therefore, the kisspeptin/KISS1R system is an upstream regulator of GnRH release and has been proposed to play an important role in the onset of puberty.

Since hypothalamic kisspeptin affects puberty onset, serum kisspeptin has been suggested as an attractive marker for puberty onset. Evaluating the serum kisspeptin level, according to pubertal stage, may help determine whether it can be used as a biomarker for puberty. To date, several studies (Vries et al., 2009; Pita et al., 2011; Rhie et al., 2011; Jayasena et al., 2014; Xue et al., 2020) have shown conflicting results regarding serum kisspeptin levels during puberty. Serum kisspeptin levels increase with age and peak around puberty (Jayasena et al., 2014); they are significantly higher in girls with central precocious puberty than pre-pubertal girls of the same age (Vries et al., 2009; Rhie et al., 2011). In contrast, there were no differences in serum kisspeptin levels between pre-pubertal and pubertal groups (Pita et al., 2011) or between a central precocious pubertal and normal group (Xue et al., 2020). Therefore, a detailed evaluation of the change in serum kisspeptin levels according to developmental stage is urgently needed.

Identifying the origin of serum kisspeptin might elucidate the association between serum kisspeptin levels and puberty. *KISS1* is widely distributed not only in the hypothalamus but also other organs, such as the pituitary gland, placenta, kidneys, pancreas, adrenal gland, adipose tissue, testes, and ovaries (Lee et al., 1996; Terao et al., 2004; Gaytán et al., 2009). Thus, it is not clear whether changes in *Kiss1* expression in the hypothalamus reflect changes in serum kisspeptin, or whether these changes are brought about by some other organ. Emerging evidence has indicated potential physiological roles of extra-hypothalamic kisspeptins in modulating puberty and the reproductive system (Laoharatchatathanin et al., 2015; Uenoyama et al., 2016). In particular, ovaries express *Kiss1* (Terao et al., 2004; Castellano et al., 2006; Laoharatchatathanin et al., 2015), which seems to be controlled by LH (Castellano et al., 2006). Furthermore, local administration of a high dose kisspeptin antagonist to an ovary exerts a negative influence on puberty onset (Ricu et al., 2012). Therefore, the ovary is also suspected to be a candidate organ for the main source of serum kisspeptin. Investigating the association between serum kisspeptin levels and changes in *Kiss1* expression in various organs, including the hypothalamus and ovary at different developmental stages, will help determine which organ expressing *Kiss1* is the main source of serum kisspeptin during puberty. This analysis might help in understanding the biological significance of serum kisspeptin levels during puberty.

In this study, we explored changes in serum kisspeptin levels in female rats from the neonatal stage to puberty. We also evaluated *Kiss1* mRNA and protein expression in several organs during development to explore the source of serum kisspeptin. In addition, we investigated serum kisspeptin levels and hypothalamic *Kiss1* mRNA and protein expression in ovariectomized (OVX) rats, with and without estradiol replacement, to explore the role of the ovaries and hypothalamus in regulating serum kisspeptin levels.

2 Materials and methods

2.1 Animals and study design

The experimental procedures used in this study were approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine (approval number 2016-0046) and conducted in accordance with the Guide for the Care and Use of Laboratory Animals (1996 [7th ed.] Washington, DC: National Research Council, National Academies Press). To ensure that all the female rats used were of the same conditions, timed-pregnant Sprague–Dawley rats (17th day of pregnancy, n = 8) were purchased from Japan SLC, Inc., (Shizuoka, Japan). Each pregnant rat bore six to eight female littermates, and a total of 56 littermates were used in this study. The current study was designed to minimize the number of animals used. Female littermate rats born before 1,000 h were considered to be 1 day old. The animals were maintained under standard conditions of a 12:12 light: dark cycle (lights on at 0800 h) and a temperature of 22°C. The rats were weaned on day 21 and housed, three per cage, with free access to pellet food and tap water. Body weights were checked every morning at 1,000 h. To confirm the completion of puberty, vaginal opening (VO), which was defined by the vagina being pink, wrinkled, and completely canalized [25], was also checked every morning at 1,000 h. The developmental stages of the female rats were defined as follows: neonate (days 1–7), infant (days 8–21), peripubertal (days 22–31), and pubertal completion (day of VO) (Ojeda and Urbanski, 1988). To evaluate serum kisspeptin patterns at differential development stages, serum samples were obtained at onset and half-way through each developmental stage; that is, on day 4 (P4, middle of neonate stage), day 8 (P8, onset of infancy), day 14 (P14, mid-infancy), day 23 (P23, onset of

peripuberty), day 27 (P27, mid-peripuberty), and the day of VO (completion of puberty). Each day, samples were taken from four to eight female rats. To evaluate *Kiss1* mRNA and protein expression in the hypothalamus, pituitary gland, ovaries, uterus, adrenal glands, and pancreatic tissues, tissue samples from these organs were obtained at the same time points. The rats were euthanized by decapitation between 1,000 h and 1,100 h. The hypothalamus was removed according to the rat brain atlas (Khazipov et al., 2015), using a micro knife to make a 2 mm-deep horizontal cut that began 1 mm away (in the anterior direction) from the optic chiasm and continued to the posterior borders of the mammillary bodies and the hypothalamic fissures. The AVPV and ARC were compartmentalized according to the rat brain atlas (Khazipov et al., 2015). The anterior and posterior ends of the AVPV tissue sections were approximately 0.84 mm and 0.60 mm posterior to the bregma, respectively. The anterior and posterior ends of the ARC tissues were approximately 1.80 and 4.08 mm posterior to the bregma, respectively.

Next, to evaluate the role of ovarian *Kiss1* expression on serum kisspeptin levels, we measured serum kisspeptin levels and hypothalamic *Kiss1* mRNA and protein expression in OVX rats. Rats were subjected to ovariectomies at P14, and serum samples and hypothalamic tissues were obtained on P23, P27, and P34. Because OVX rats had a delayed pubertal onset, samples were obtained at P34 (mean day of VO in intact female rats), instead of the day of VO. We also measured serum kisspeptin levels and *Kiss1* mRNA and protein expression in the hypothalamus in OVX rats after estradiol replacement to explore the regulatory effect of estradiol on serum kisspeptin levels. We subcutaneously administered estradiol (E-8875, Sigma Chemical Co., St. Louis, MO) at a dose of 25 μg/kg/day from P14 to the day of VO. Serum samples and hypothalamic tissues were obtained on P23, P27, and the day of VO.

2.2 Measuring serum kisspeptin levels

Serum samples were collected by immediate centrifugation for 15 min at 1,000 × g at 4°C and stored at −70°C before determining serum kisspeptin levels. Serum kisspeptin levels were measured using a highly sensitive enzyme-linked immunosorbent assay kit (E-EL-R2530, Elabscience Biotech, Wuhan, China) with a detection range of 78.13–5,000 pg/ml. Serum kisspeptin concentration was measured by diluting the blood of rats to one-fiftieth.

2.3 RNA extraction and reverse transcription polymerase chain reaction (RT-PCR) analysis

To determine *Kiss1* mRNA expression in tissues at each developmental stage, RNA was extracted and analyzed by RT-PCR. Tissues from several rat organs were removed immediately following decapitation, frozen in liquid nitrogen, and stored at −80°C until being processed for mRNA analyses. Total RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA, United States). A total of 2 μg RNA was synthesized using LaboPass cDNA kit (Cosmo Gentech, Seoul, South Korea) according to the manufacturer's instructions. Primer sequences used for RT-PCR were obtained from the published sequence of *Kiss1* (GenBank accession number AY196983.1; Table 1), and *β-actin* was used as the reference gene. PCR was carried out at an initial denaturation cycle at 95°C for 5 min, followed by a variable number of amplification cycles defined by denaturation at 94°C for 40 s, annealing at 55°C for 40 s, and 30 cycles of extension at 72°C for 50 s. A final extension cycle of 72°C for 10 min (Takara, Japan) was also included. PCR products were separated on 1.5% agarose gels and visualized by ethidium bromide staining.

2.4 mRNA analyses by reverse transcription quantitative real-time PCR (RT-qPCR)

The *Kiss1* mRNA levels in representative samples from female rats in different developmental stages were quantified by RT-qPCR. RT-qPCR was performed with 20 μl of PCR amplification reaction mixture containing 900 ng of complementary DNA, hydrolysis probe (*Kiss1*: Rn00710914_m1, *β-actin*: Rn00667869_m1), and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, United States). Amplification was performed in duplicate with the following thermocycling profile: 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min. A StepOnePlus instrument (Applied Biosystems) was used for the reactions. The relative expression of mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method (Rao et al., 2013). The reference samples were arbitrarily taken from each organ from 4-day-old female rats. RT-qPCR analyses were performed in triplicate.

2.5 Western immunoblotting analysis

To determine the protein expression levels of Kiss1, we performed western immunoblotting analysis. Frozen hypothalamus, ovary, pituitary gland, adrenal gland, and uterus tissues were homogenized and lysed on ice in RIPA lysis buffer (WSE-7420, ATTO, Tokyo, Japan.) Protein extracts were centrifuged at 17,000 × *g* for 30 min. Then, the protein concentrations were determined using the BCA protein assay (Applygen Technologies Inc., Beijing, China). Equal amounts of proteins (150 μg) were boiled for 5 min at 100°C with loading buffer (containing 8% *β*-mercaptoethanol) for denaturation. The amount of the protein used as a reference

TABLE 1 RT-PCR primer pair sequences.

Gene	Forward primer	Reverse primer
β-actin	5′-TGTCACCAACTGGGACGATA-3′	5′-TCTCAGCTGTGGTGGTGAAG-3′
Kiss1	5′-ACTCGTTAATGCCTGGCAAA-3′	5′-AGGCCAAAGGAGTTCCAGTT-3′

gene was 20 μg, and the experiment was repeated. The denatured proteins were loaded on a 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel for 1 h. The proteins were separated by SDS-PAGE and transferred to a 0.25-μm polyvinylidene fluoride membrane for 30 min. The membranes were blocked with phosphate-buffered saline (PBS) containing 5% skim milk for 30 min at room temperature (as around 20–22°C). Then, the membranes were incubated separately with primary antibodies against kisspeptin (1:500, catalog number GTX130503, GeneTex, Irvine, California, USA) and *β*-actin (1:3000, catalog number #4970, Cell Signaling Technology, Danvers, Massachusetts, United States) at 4°C overnight. The polyclonal kisspeptin antibody is of IgG isotype and reacts with human, mouse, and rat kisspeptin. After washing thrice with PBS containing 0.2% Tween 20 (PBS-T) for 10 min, the membranes were incubated with a horseradish peroxidase-conjugated secondary goat anti-rabbit antibody (1: 5000) for 30 min at room temperature, followed by four washes in PBS-T for 10 min. Then, the membranes were exposed to enhanced chemiluminescence. Blots were exposed to medical X-ray film.

2.6 Statistical analyses

Quantitative RNA data are presented as the mean ± standard error of the mean. One-way ANOVA followed by Tukey's test was performed to compare changes in serum kisspeptin levels at different developmental stages. To compare changes in serum kisspeptin levels in OVX and estradiol-treated OVX female rats at different developmental stages, two-way (age and OVX/estradiol-treated OVX) ANOVA was performed. In addition, if age-by-OVX/estradiol-treated OVX interaction effects were significant with p-value < 0.05, then an independent t-test was performed to confirm the treatment effect on each group by post-hoc analysis. The Bonferroni multiple comparison test was used for multiple comparisons. All analyses were performed using SAS software (version 9.2; SAS Inc., Cary, NC, United States). p values < 0.05 were considered to reflect statistically significant differences.

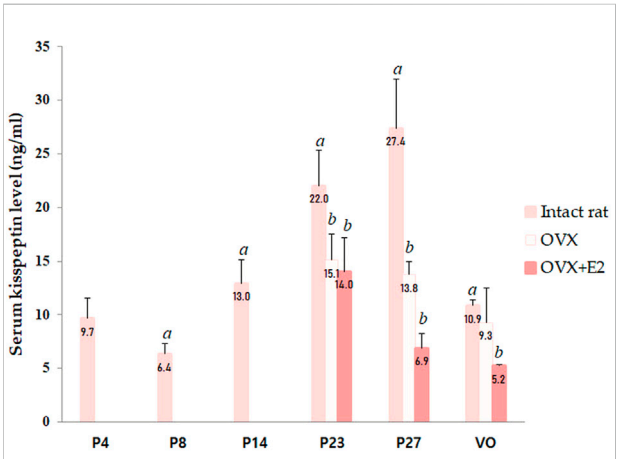

FIGURE 1
Changes in serum kisspeptin levels at different developmental stages. Serum kisspeptin levels in intact female rats; changes in serum kisspeptin levels in ovariectomized (OVX) and estradiol-treated OVX female rats (OVX + E2) were measured. Specimens were collected on days 4, 8, 14, 23, and 27, and on the day of vaginal opening (VO) in intact female rats. Ovariectomy was conducted on day 14; specimens were collected on days 23, 27, and on the day of vaginal opening (VO) or day 34 in OVX and OVX-estradiol treated rats. Each group had four to eight animals. Data are presented as the mean ± standard error of mean. [a]Significant difference ($p < 0.05$) in treatments just before each stage; [b]Significant difference ($p < 0.05$) in levels compared to those in intact female rats.

3 Results

3.1 Developmental profile of serum kisspeptin levels in intact female rats

Serum kisspeptin levels were analyzed at different developmental stages, and each group contained four to eight animals. Serum kisspeptin levels were moderate during the neonatal period, slightly decreased in early infancy, and significantly increased during successive developmental stages (compared to just before stages, all $p < 0.05$), peaking at peripuberty (P27) (Figure 1). However, the levels began to decrease at the completion of puberty ($p < 0.001$) (Figure 1).

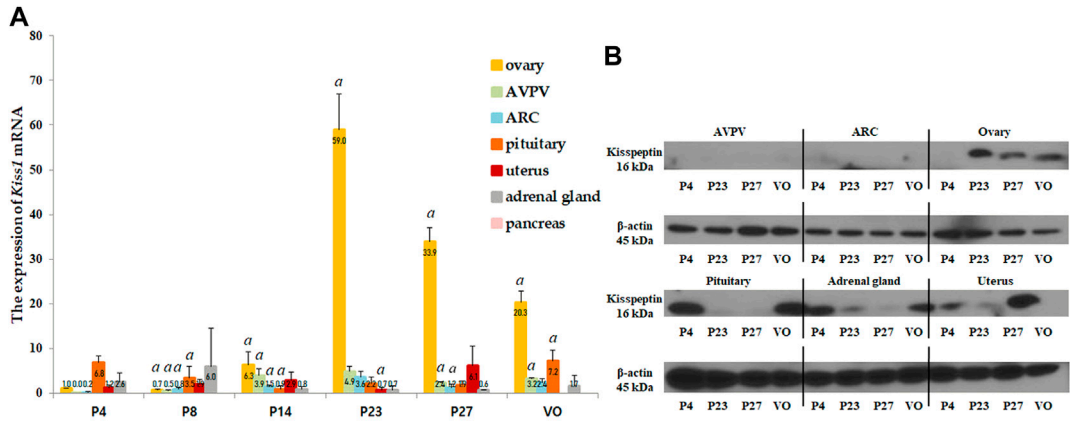

FIGURE 2
(A) Developmental profiles of *Kiss1* mRNA expression in several organs, such as the anteroventral periventricular nucleus (AVPV) and arcuate nucleus (ARC) in the hypothalamus, and the pituitary gland, ovaries, uterus, adrenal glands, and pancreas. Specimens were collected on days 4, 8, 14, 23, and 27, and on the day of vaginal opening (VO). *Kiss1* mRNA expression were quantified by RT-qPCR in triplicate. Each value was quantified on the basis of the value of *Kiss1* mRNA-expression in the ovary on day 4. **(B)** Protein expression levels of Kiss1 were quantified by western immunoblotting in triplicate. Each group had four to eight animals. Data are presented as the mean ± standard error of mean. [a]Significant difference ($p < 0.05$) in treatments just before each stage.

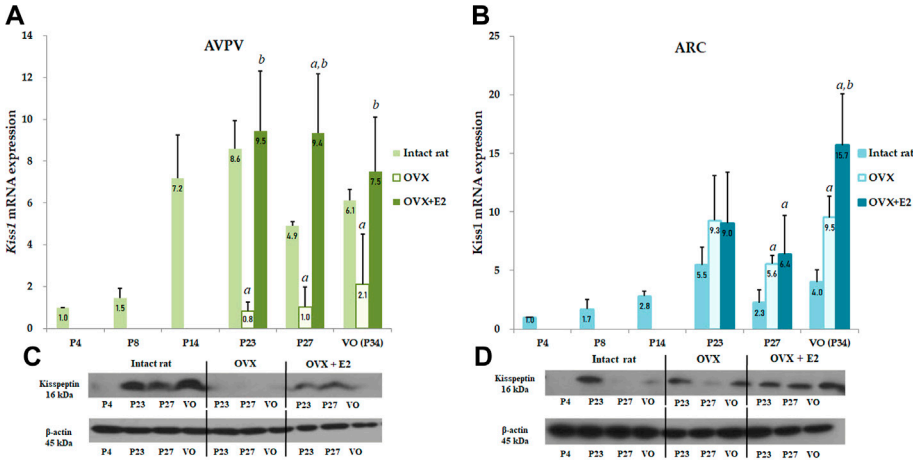

FIGURE 3
Developmental profiles of *Kiss1* mRNA and protein expression in the anteroventral periventricular nucleus (AVPV) and arcuate nucleus (ARC) in intact female, ovariectomized (OVX), and estradiol-treated OVX rats (OVX + E2) from the neonatal stage to the onset of puberty. Ovariectomy was conducted on day 14, and specimens were collected on days 23, 27, and on the day of vaginal opening (VO) or day 34. Each group contained four to eight animals **(A)** *Kiss1* mRNA expression of the AVPV was quantified by RT-qPCR. Each value was quantified on the basis of the value of *Kiss1* mRNA expression in the AVPV on day 4. **(B)** *Kiss1* mRNA expression of the ARC was quantified by RT-qPCR. Each value was quantified on the basis of the value of *Kiss1* mRNA expression in the ARC on day 4. **(C)** Kiss1 protein levels in the AVPV were quantified by western immunoblotting. **(D)** Kiss1 protein levels in the ARC were quantified by western immunoblotting. Each group contained four to eight animals. RT-qPCR analyses and western immunoblotting analyses were performed in triplicate. Data are presented as the mean ± standard error of the mean. [a]Significant difference ($p < 0.05$) in levels compared to those in intact female rats; [b]Significant difference ($p < 0.05$) in levels compared to those in OVX rats.

3.2 Kiss1 mRNA and protein expressions in various organs in intact female rats according to the developmental stage

Kiss1 mRNA and protein expression were analyzed in several organs, including the AVPV and ARC in the hypothalamus, pituitary gland, ovaries, uterus, adrenal glands, and pancreas. *Kiss1* mRNA in each organ was expressed relative to the *Kiss1* mRNA in the ovaries of 4-day-old female rats. The ovaries expressed the highest levels of *Kiss1* mRNA near the onset of puberty, and the pituitary gland expressed the second highest levels (Figure 2A). *Kiss1* mRNA was barely expressed in the

pancreas (Figure 2A). In the ovaries, the *Kiss1* mRNA levels were lowest from the neonatal to infant periods but significantly rapidly increased at the peripubertal stage ($p < 0.001$), peaking on P23, and then gradually decreasing until puberty completed (Figure 2A). Changes in *Kiss1* mRNA expression in the ovaries corresponded to changes in serum kisspeptin levels, although they were one developmental step ahead of the corresponding serum kisspeptin levels. That is, changes in mRNA expression corresponding to the developmental stage were observed prior to the changes observed in serum levels. *Kiss1* mRNA expression in the AVPV was low from the neonatal period until early infancy, then increased during the peripubertal stages and peaked at P23 (compared to just before the stage at P14, $p < 0.05$, Figures 2A, 3A). Similarly, *Kiss1* mRNA levels in the ARC were moderate during the neonatal period, gradually increased during developmental stages until P23, and slightly decreased at P27 and VO (compared to just before the stage at P14, $p < 0.05$, Figures 2A, 3B). Although *Kiss1* mRNA expression in the pituitary gland was persistent and increased at VO (compared to just before the stage, both $p < 0.01$), no specific pattern was observed during developmental stages (Figure 2A).

We then performed western immunoblotting analysis to determine changes in the protein expression of Kiss1 in various organs at P4, P23, P27, and VO. Similar to the *Kiss1* mRNA expression pattern, the ovaries expressed the highest levels of Kiss1 protein at P23 and P27 (Figure 2B).

3.3 Changes in serum kisspeptin levels in OVX and estradiol-treated OVX (OVX + E2) female rats at different developmental stages

We performed ovariectomy and measured the serum kisspeptin level in OVX female rats to elucidate the effects of the ovaries on changes in serum kisspeptin levels. In addition, the serum kisspeptin levels in OVX female rats were compared with those in estradiol-treated OVX female rats to determine whether the change in serum kisspeptin level was due to the ovaries or estradiol. Since there was a significant interaction effect between developmental stage and OVX/estradiol-treated OVX for serum kisspeptin ($p < 0.001$), we confirmed the change in serum kisspeptin according to OVX or estradiol-treated OVX at each developmental stage. The serum kisspeptin levels in OVX female rats were significantly lower than those in intact female rats at P23 ($p < 0.01$) and P27 ($p < 0.001$); after adjusting the age, the serum kisspeptin levels in intact female rats were higher than those in OVX female rats ($\beta = -8.595$, SE = 2.175, $p < 0.001$). The serum kisspeptin levels in OVX female rats did not recover after estradiol treatment (compared with those in intact female rats; P23, $p < 0.01$; P27 and VO, $p < 0.001$); after adjusting the age, the serum kisspeptin levels in estradiol-treated OVX rats were significantly lower than those in intact female rats ($\beta = -6.339$, SE = 1.235, $p < 0.001$) (Figure 1).

3.4 Changes in hypothalamic Kiss1 mRNA and protein expression in OVX and estradiol-treated OVX female rats

To determine the effect of *Kiss1* expression in the AVPV and ARC on serum kisspeptin levels, *Kiss1* mRNA and protein expression in the AVPV and ARC were measured in both OVX and estradiol-treated OVX rats and compared to the serum kisspeptin pattern. In the AVPV, there was a significant interaction effect between developmental stage and OVX/estradiol-treated OVX ($p < 0.05$). We confirmed the change in *Kiss1* mRNA and protein expression in the AVPV according to OVX/estradiol-treated OVX at developmental stages. *Kiss1* mRNA expression in the AVPV was significantly lower in OVX female rats than in intact rats at all developmental stages (P23 and P27, $p < 0.001$; P34, $p < 0.01$), even after adjusting the age ($\beta = -5.384$, SE = 1.207, $p < 0.001$) (Figure 3A). However, these levels normalized after estradiol treatment, implying that *Kiss1* mRNA expression was increased by estradiol (compared with those in OVX rats, P23, $p < 0.01$; P27 and VO, $p < 0.001$; VO) (Figure 3A). After adjusting the age, *Kiss1* mRNA expression in AVPV was recovered and increased after estradiol treatment in OVX female rats ($\beta = 2.482$, SE = 0.749, $p = 0.002$). In contrast, *Kiss1* mRNA expression in the ARC was increased in OVX rats compared to that in intact female rats (P27 and VO, $p < 0.01$) (Figure 3B), and was also statistically significant after adjusting the age ($\beta = 4.215$, SE = 0.809, $p < 0.001$). After estradiol administration, as well as after adjusting the age ($\beta = 2.991$, SE = 0.630, $p < 0.001$), *Kiss1* mRNA expression also increased more than that in intact female rats (P27, $p < 0.05$; VO, $p < 0.01$). Furthermore, *Kiss1* mRNA expression in estradiol-treated OVX rats increased even more than that in OVX rats at VO ($p < 0.05$); however, it was not statistically significant after adjusting the age ($\beta = 2.258$, SE = 1.676, $p = 0.192$) (Figure 3B). Kiss1 protein expression measured by western immunoblotting analysis showed a pattern similar to *Kiss1* mRNA expression in the AVPV and ARC (Figures 3C,D).

4 Discussion

This study evaluated patterns in serum kisspeptin levels in different developmental stages and investigated the main source of serum kisspeptin in female rats. Serum kisspeptin level increased progressively in accordance with the developmental stage until the peripuberty stage, suggesting that it may be an associated marker of puberty. In addition, we evaluated *Kiss1* expression in various organs at similar stages and demonstrated that the pattern of *Kiss1* expression in the ovary, AVPV, and ARC was similar to that of serum kisspeptin. Especially, the ovaries expressed the highest levels of *Kiss1* mRNA and Kiss1 protein near the onset of puberty. Because the highest expression of *Kiss1* mRNA and Kiss1 protein during development was observed in

the ovary, we evaluated the changes in serum kisspeptin level, and the *Kiss1* mRNA and protein expression in the AVPV and ARC in OVX and estradiol-treated OVX rats. Serum kisspeptin level decreased in OVX rats independent of estradiol treatment, unlike the changes in *Kiss1* mRNA expression in the AVPV and ARC. These results suggest that the ovaries are the main source of serum kisspeptin, and an increase in serum kisspeptin levels along with the progression of puberty is not related to *Kiss1* expression in the AVPV and ARC.

Just as kisspeptin in the AVPV and ARC in the hypothalamus control pubertal development (Ohtaki et al., 2001; Seminara et al., 2003), peripheral serum kisspeptin might also have significant implications in pubertal development (Vries et al., 2009; Rhie et al., 2011; Young et al., 2013; Jayasena et al., 2014; Decourt et al., 2016; Xue et al., 2020). However, due to conflicting results (Pita et al., 2011; Xue et al., 2020), studies on the patterns of serum kisspeptin levels in successive developmental stages are required to clarify the pattern of serum kisspeptin. To the best of our knowledge, this is the first study to evaluate the patterns of serum kisspeptin levels at different developmental stages in female rats. We found that serum kisspeptin levels increased progressively during the sequential developmental stages until the peri-pubertal stage, suggesting that serum kisspeptin may reflect puberty or serve as a marker associated with puberty.

However, it remains unclear whether the increase in serum kisspeptin levels during successive developmental stages reflects the changes in hypothalamic *Kiss1* expression that occur at puberty onset. A recent report showed that serum kisspeptin levels were elevated in girls compared to boys, suggesting that the higher levels may be due to greater hypothalamic kisspeptin signaling in girls than in boys (Jayasena et al., 2014). However, since kisspeptin is expressed in several other organs (Lee et al., 1996; Terao et al., 2004; Gaytán et al., 2009), these organs may also be candidates that control the serum kisspeptin levels during developmental stages. Kanasaki et al. (2013) suggested that only local kisspeptin, which is produced within the hypothalamic neuron, may exert its stimulatory effect on GnRH *via* a paracrine mechanism and that circulating kisspeptin may originate from other peripheral organs. Little effort has been directed toward determining the source of serum kisspeptin, and only a limited number of studies have been performed to analyze the significance of *Kiss1* expression in various organs according to the developmental stage. Therefore, to better identify the origin of serum kisspeptin, we evaluated changes in *Kiss1* mRNA and protein expression in several organs known to express *Kiss1* according to the developmental stage in intact female rats. In this study, while the other organs sparsely expressed *Kiss1* mRNA, *Kiss1* mRNA and/or did not show significant patterns depending on the development stage, expression prominently increased with the progression of puberty in the ovary and the AVPV and ARC, similar to the changes in serum kisspeptin levels.

To test this possibility more specifically, we investigated serum kisspeptin levels and *Kiss1* expression in the AVPV and ARC in both OVX and estradiol-treated OVX rats. Serum kisspeptin levels decreased significantly in OVX rats, suggesting that the ovaries are the main organs that contribute to serum kisspeptin or that estrogen exclusion, due to ovariectomy, decreased *Kiss1* expression in the AVPV, thereby decreasing serum kisspeptin levels. Previous studies (Roa et al., 2006; Smith et al., 2006; Kauffman et al., 2007; Takase et al., 2009) have shown that *Kiss1* expression decreased after ovariectomy and increased with estradiol treatment in the AVPV, whereas an opposing trend was observed in the ARC. These results led to the hypothesis that estradiol may play a positive-feedback role in the AVPV in regards to kisspeptin, whereas in the ARC, it may serve a negative-feedback role (Smith et al., 2005; Roa et al., 2006). In this study, changes in *Kiss1* mRNA and protein expression in the AVPV in OVX and estradiol-treated OVX rats were consistent with the results of previous studies (Smith et al., 2005; Kauffman et al., 2007; Takase et al., 2009). However, the decrease in the serum kisspeptin level in OVX rats, which was similar to the reduced *Kiss1* expression in the AVPV, was not restored even after estradiol treatment, which was different from *Kiss1* expression in the AVPV in estradiol-treated OVX rats. In addition, although the change in *Kiss1* expression in the ARC in OVX and estradiol-treated OVX rats was not consistent with the finding of previous studies, *Kiss1* expression increased in OVX and estradiol-treated OVX rats. These findings suggest that *Kiss1* expression in the ovaries, rather than in the hypothalamus mainly regulates serum kisspeptin level. Furthermore, the serum kisspeptin level in OVX rats was not restored to the level observed in intact female rats even after estradiol treatment. In a previous study on goose ovaries, the serum kisspeptin level was negatively correlated with serum estradiol level (Hua et al., 2014). Therefore, the findings of this study confirmed that the serum kisspeptin level is not regulated by ovarian estradiol.

In the present study, although there was no characteristic change according to the developmental stage, *Kiss1* mRNA expression was high in the pituitary gland. In addition to the expression in hypothalamus, expression of both *Kiss1* and *Kiss1r* in the pituitary has been reported in several species (Kauffman et al., 2007; Richard et al., 2008; Witham et al., 2013; Ikeda et al., 2017). Ikeda et al. (2017) examined the expression profile of kisspeptin in the mouse pituitary at all stages, and *Kiss1* mRNA was detected in the rostroventral portion and the dorsocaudal portion in the anterior pituitary from embryonic period to adulthood. In addition, the expression of kisspeptin was detected in rat LHβ cells (Richard et al., 2008) and coexpression percentage of LHβ and kisspeptin cells increased during development (Ikeda et al., 2017). Therefore, the pituitary may also be a candidate for one of main organs of serum kisspeptin origin. However, *Kiss1* expression in pituitary is mainly regulated in a gonad-independent manner, as kisspeptin expression in pituitary was not found to be

different from that in agonadal mice (Ikeda et al., 2017). Although the present study did not evaluate in the expression pattern of pituitary *Kiss1* in OVX and estradiol-treated OVX rats, it may suggest serum kisspeptin is primarily sourced from ovaries, not pituitary. However, *Kiss1* mRNA levels in female mice pituitary increased when puberty began and further increased during postpubertal and adulthood ages (Ikeda et al., 2017). In addition, although the action of kisspeptin on gonadotropin release is primarily mediated *via* hypothalamic GnRH (Shahab et al., 2005), administration of kisspeptin to cultured pituitary cells induced gonadotropin gene expression (Witham et al., 2013) and LH secretion (Gutiérrez-Pascual et al., 2007; Suzuki et al., 2008). These results suggest a special role of pituitary kisspeptin in regulating reproductive function, especially, ovarian differentiation and gonadotrope function during adulthood (Ikeda et al., 2017).

Terao et al. (2004) was the first to report that *Kiss1* was expressed in rat ovaries, which was then confirmed in several studies (Castellano et al., 2006; Laoharatchatathanin et al., 2015; Fernandois et al., 2016). Although different expression patterns were observed among these studies because of age discrepancies and samples obtained having been at different points in the estrous cycle, ovarian *Kiss1* expression has been associated with follicle growth, oocyte maturation, and ovulation (Castellano et al., 2006; Laoharatchatathanin et al., 2015; Fernandois et al., 2016). In addition, ovarian *Kiss1* expression is directly stimulated by LH surge through the LH receptor (Laoharatchatathanin et al., 2015) or by the injection of human chorionic gonadotropin (Castellano et al., 2006), which is blocked by prevention of the preovulatory gonadotropin surge (Castellano et al., 2006). These suggest that ovarian *Kiss1* expression might be regulated by LH (Castellano et al., 2006; Peng et al., 2013; Laoharatchatathanin et al., 2015). To summarize, ovarian kisspeptin might play a role in follicular development and ovulation, which is associated with LH secretion and not hypothalamic kisspeptin. However, hypothalamic *Kiss1* expression increases with puberty, leading to HPG axis activation and increased LH levels. Although whether LH treatment directly affects ovarian *Kiss1* expression has not been elucidated yet, the increase in LH levels is thought to increase ovarian kisspeptin expression. As a result, serum kisspeptin levels increase according to pubertal development. Therefore, although hypothalamic *Kiss1* expression does not directly affect serum kisspeptin, it indirectly affects its pattern during puberty. Thus, although serum kisspeptin could not be a direct biomarker for the onset of puberty or the activation of HPG axis, it can be an indirect indicator for puberty by judging the maturation of reproductive function according to puberty development. Further studies are needed to provide evidence on the association between LH levels and ovarian *Kiss1* expression pre-, peri-, and post-puberty.

In the present study, we evaluated patterns in serum kisspeptin levels as well as *Kiss1* mRNA and protein expression levels in accordance with different developmental stages in various organs. Serum kisspeptin levels increased as puberty progressed, and among the organs studied, the ovaries expressed the highest level of *Kiss1* in a pattern similar to that of serum kisspeptin levels. In addition, serum kisspeptin levels decreased in OVX rats independent of estradiol treatment. Our findings support the conclusion that serum kisspeptin levels mainly depend on *Kiss1* expression in the ovaries, thereby suggesting that the ovaries are the main source of serum kisspeptin; however, these findings need to be verified in future studies with ovarian-specific *Kiss1* knockout rats.

One limitation of our study was that only intracellular expression of *Kiss1* was shown, but the actual amount of ovarian kisspeptin being secreted from the ovaries was not determined. In order to clearly identify the role of serum kisspeptin according to developmental stage and the main source of serum kisspeptin during puberty, studies using *in vitro* ovarian culture will be needed. Furthermore, the exact distinction between AVPV and ARC in the hypothalamus was not confirmed through immunofluorescence staining. Although this study was conducted with anatomical compartments according to the methods of previously published studies, we did not demonstrate that AVPV and ARC were accurately separated. Third, we indicate that serum kisspeptin may be a downstream marker for ovarian activity but does not represent the degree of activation of the HPG axis during puberty; however, since LH stimulates *Kiss1* expression in the ovaries, the serum kisspeptin level might serve as an indirect marker of puberty. Nevertheless, further studies are needed to evaluate the roles of serum kisspeptin and the mechanism of the Kiss1/Kisspeptin system in the development of the reproductive system.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine (approval number 2016-0046).

Author contributions

Conceptualization, AK and H-SK. Methodology, AK, JE, and WL. Validation, HSC, KS, SJ, HWC, and WL. Formal analysis, JE. Investigation, AK and JE. Data curation, AK and JE. Writing—original draft preparation, AK. Writing—review and

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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

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Clinical outcome analysis of frozen-thawed embryo transfer on Day 7

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Objective: To investigate the clinical outcomes of Day 7 (D7) frozen-thawed embryo transfer (FET) and to provide a reference value for clinical work.

Methods: This was a retrospective cohort study. Patients undergoing FET cycles in the Reproductive Medicine Center of the Third Affiliated Hospital of Zhengzhou University between December 2015 and January 2021 were included. According to the developmental stage of the embryos at transfer, the embryos were divided into three groups: Day (D) 5, D6 and D7 blastocysts. Group D7 was compared with Groups D5 and D6. Simultaneously, the preimplantation genetic testing (PGT) and non-PGT cycles in Group D7 were analyzed and compared. The main outcomes were the clinical pregnancy, live birth and miscarriage rates. The secondary outcomes were the implantation and euploidy rates.

Results: In total, 5945, 4094 and 137 FET cycles were included in the D5, D6 and D7 groups, respectively. The clinical pregnancy rate was significantly lower in Group D7 than in Groups D5 (13.9% vs 62.9%, P <0.001) and D6 (13.9% vs 51.4%, P <0.001). Additionally, the live birth rate was significantly lower in Group D7 than in Groups D5 (7.3% vs 50.7%, P <0.001) and D6 (7.3% vs 40.5%, P <0.001). However, the miscarriage rate was significantly higher in Group D7 than in Groups D5 (47.4% vs 18.2%, P =0.001) and D6 (47.4% vs 20.6%, P =0.004). The clinical pregnancy and live birth rates for D7 blastocysts were significantly higher in the PGT group than in the non-PGT group (41.7% vs 13.9%, P=0.012; 33.3% vs 7.3%, P =0.003).

Conclusions: D7 blastocyst transfer can yield a live birth rate that is lower than that for D5 and D6 blastocysts but has value for transfer. PGT for D7 blastocysts may reduce the number of ineffective transfers and improve the outcome of D7 blastocyst transfer, which can be performed according to a patient's situation.

KEYWORDS

D7, FET, clinical pregnancy, live birth, abortion, euploid

Introduction

With continuous improvements in embryo culture systems, to improve the implantation rate of infertile couples (1), an increasing number of embryos are cultured to the blastocyst stage and then transferred. The number of days of blastocyst development represents the developmental potential of blastocysts (2) and affects the outcome of blastocyst transfer (3). The developmental potential of Day (D) 5, D6, and D7 blastocysts decreases gradually with the extension of culture time. Therefore, the conventional practice in the laboratory is to select blastocysts for transfer, biopsy or cryopreservation at D5 or D6 of embryo culture. This traditional standard was challenged in 2008 with the first report of successful pregnancy after vitrification cryopreservation of D7 blastocysts in humans (4).

At present, studies on D7 blastocysts are very limited and controversial. M.J. Gorrill et al. (5) argued that prolonging blastocyst culture to D7 did not increase the number of available embryos, and that the implantation rate of D7 blastocysts was very low, which would easily lead to adverse outcomes. Therefore, they did not advocate prolonging blastocyst culture to D7. However, recent studies (6, 7) have suggested that D7 blastocyst transfer has important clinical value, and approximately 3% of patients have usable blastocysts formed only on D7. Tiegs et al. (7) performed preimplantation genetic testing for aneuploidy (PGT-A) for 532 D7 blastocysts, screened out 229 D7 euploid blastocysts for frozen-thawed embryo transfer (FET), and found that the rate of continuous embryo implantation after D7 euploid blastocyst transfer is as high as 52.6%, which is similar to that for D5 or D6 euploid blastocyst transfer.

Clinically, among some patients, blastocysts fail to form at D5/D6 due to advanced age, poor ovarian function, poor embryo quality or other reasons, or blastocysts do form but only at D7, with fewer blastocysts formed at D5/D6. Therefore, the use of slow-developing D7 blastocysts will be of great significance for these patients. This article aimed to study the clinical outcomes of D7 blastocyst transfer and provide a factual basis for clinical practice.

Materials and methods

Study design

This retrospective cohort study was conducted at the Reproductive Medical Center of the Third Affiliated Hospital of Zhengzhou University. We enrolled all patients who underwent FET between December 2015 and January 2021.

Part I: The inclusion criteria were as follows: D5, D6, and D7 FET cycles. The exclusion criteria were as follows (1): a donor egg-assisted pregnancy cycle (2); repeated implantation failure (3); FET cycles on Day 2 (D2), Day 3 (D3) or Day 4 (D4) (4); FET cycles with sequential transfer (5); FET cycles with mixed embryo transfer with only one gestational sac implantation (6); preimplantation genetic testing (PGT) cycles; or (7) the absence of a delivery record or follow-up data (e.g., lost to follow-up). The embryos were divided into three groups according to the developmental day of the embryo at transfer: D5 blastocysts (D5 group), D6 blastocysts (D6 group) and D7 blastocysts (D7 group).

Part II: All D7 FET cycles were included, and D7 blastocysts were divided into preimplantation genetic testing (PGT) cycles and non-PGT cycles according to whether they originated from PGT cycles.

This study was performed in accordance with the basic principles of the Declaration of Helsinki. This study was approved by the Institutional Review Board of the Third Affiliated Hospital of Zhengzhou University.

Controlled ovarian hyperstimulation

The conventional gonadotrophin-releasing hormone agonist (GnRH-a) (Diphereline, Ipsen, France) long protocols of our center were used for ovulation stimulation (8), and eggs were collected 36-38 h after human chorionic gonadotropin (hCG) injection.

Endometrial preparation protocols

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

Insemination, embryo culture and embryo observation

IVF/ICSI insemination was performed 38-40 h after hCG injection, depending on the maturity of the egg and the

processing time of the semen sample. An embryologist observed the fertilization of oocytes at 16-20 h after insemination. Sequential culture media were used for embryo culture (Vitrolife, Sweden; COOK, USA). After insemination, embryos were cultured in cleavage culture medium. On D3 after oocyte retrieval, the embryos were observed and scored. According to the patient's own condition, 1-2 embryos with a cleavage stage grade of III or above were selected for fresh cycle transfer or cryopreserved. The remaining embryos were cultured with blastocysts, and then fresh-cycle blastocysts were transferred or cryopreserved according to the situation. The embryo was switched to blastocyst culture medium on D3 of culture, and the culture medium was not be changed again. On D5, D6, and D7, the development of blastocysts was observed and scored according to Gardner blastocyst classification standards (10). The blastocysts at stage 3 or above with an inner cell mass score ≥B were rated as blastocysts available for clinical transfer or freezing. Blastocysts with scores of 4BB and above were defined as high-quality blastocysts.

Vitrified-warmed blastocysts

In order to avoid blastocyst hatching, start from D5 of the embryo, the development of blastocyst observation not only performance in the morning, but also in the afternoon. When stage 4 blastocysts are available, the laser is used to collapse and about 10 minutes later they are frozen. If the workload is heavy, the number of freezing personnel will be increased to ensure that the freezing process is carried out in strict accordance with the operation Standard Operation Procedure (SOP).

Vitrified-warmed reagents and carriers (Kitazato, Japan) were used. The reagents were removed from the refrigerator and equilibrated at room temperature for at least 30 min. The blastocysts were transferred to equilibrium solution (ES) for 10 min at room temperature and then transferred to vitrification solution (VS) for equilibrium. The blastocysts were placed on the labeled refrigerating carrier within 60 s. Then, the blastocysts were rapidly injected with liquid nitrogen and loaded into the cannula. At 37°C, the carrier cannula was removed, and the blastocyst ends were immediately immersed in thawing solution (TS) at 37°C for 1 min. Then, the blastocyst ends were transferred into diluent solution (DS) and then washing solutions 1 and 2 (WS1 and WS2) for 3 min. Finally, the cells were transferred into blastocyst culture medium (Vitrolife, Sweden) and placed in an incubator containing 6% CO_2 at 37° C (tabletop incubator, Cook Company, USA) for transfer.

Follow-up

Serum β-HCG levels were measured on the 14th day after transfer. For patients with positive serum β-HCG levels (≥50 IU/L), ultrasonographic assessment was performed on the 35th day after transfer. If the gestational sac was visible in the intrauterine cavity, clinical pregnancy was determined. The termination of pregnancy at less than 28 weeks of gestation with a fetal weight of less than 1000 g was considered a miscarriage. A live birth was considered if the pregnancy reached 28 weeks of gestation and a live neonate was delivered.

Calculation of outcome measures

The outcome measures were calculated as follows: Clinical pregnancy rate = the number of clinical pregnancy cycles/the number of transfer cycles ×100%; Live birth rate = the number of live birth cycles/the number of transfer cycles ×100%; Abortion rate = the number of abortion cycles/the number of clinical pregnancy cycles ×100%; Implantation rate = the number of gestational sacs/the number of transferred embryos ×100%; and Euploidy rate = the number of euploid embryos/the number of embryos with PGT.

Statistical analysis

All analyses were performed using Empower (R) (www.empowerstats.com, X&Y solutions, Inc. Boston MA) and R (http://www.R-project.org). Measurement data are expressed as the mean ± standard deviation (mean ± SD), and a t test (normal distribution) or Kruskal–Wallis rank-sum test (nonnormal distribution) was used for continuous variables. Categorical variables are represented as the number of cases (n) and percentage (%). The rate between groups was compared by chi-square analyses or Fisher's exact test. $P<0.05$ was considered statistically significant.

Results

Comparison of basic clinical data and clinical outcomes among the three groups

A total of 10,176 FET cycles were included in this study, among which 5945, 4094 and 137 FET cycles were included in the D5, D6 and D7 groups, respectively. Compared with the D5 and D6 groups, in the D7 group, there were significant differences in infertility factors and endometrial preparation protocols (P <0.05). Compared with the D5 group, in the D7 group, there were significant differences in female age, male age, infertility duration, the average number of embryos transferred and the number of embryos transferred in the D7 group (P <0.05). The basal follicle-stimulating hormone (FSH) level in the D7 group was significantly different from that in the D6

group (P<0.05). The clinical pregnancy rate and live birth rate were significantly lower in the D7 group than in the D5 and D6 groups, and the differences were significant (P<0.001). The abortion rate was significantly higher in the D7 group than in the D5 and D6 groups (P<0.05) (Table 1).

Basic clinical data and outcomes of FET cycles with D7 blastocysts from different sources

Twelve and 137 D7 blastocysts in the PGT group and non-PGT group, respectively, were transferred. There were significant differences in infertility factors, basal FSH levels and the number of embryos transferred between the two groups (P <0.05). The implantation rate, clinical pregnancy rate and live birth rate in the PGT group were significantly higher than those in the non-PGT group, and the differences were significant (P <0.05). The abortion rate in the PGT group was lower than that in the non-PGT group, and the difference was not significant (P>0.05) (Table 2). The biopsy, report and transfer dates of twelve D7 euploid blastocysts are shown in Table 3. The clinical outcomes are also detailed in Figure 1.

Comparison of the euploidy rates of D5, D6 and D7 blastocysts in the PGT cycles

The detection results of 2261 blastocysts from 581 cycles of PGT-assisted pregnancy in our center from January 2017 to December 2020 were included. The euploidy rate of D7 blastocysts was significantly lower than that of D5 blastocysts (P<0.001). The euploidy rate of D7 blastocysts was also lower than that of D6 blastocysts, but the difference was not significant (P =0.235) (Table 4).

General conditions of live-born neonates and maternal complications during pregnancy after D7 transfer (including PGT cycles)

A total of 14 live births were obtained from D7 blastocysts, including 4 singleton live births in the PGT cycles and 9 singleton live births and 1 twin live birth in the non-PGT cycles. Ten neonates were male, and 4 neonates were female. The delivery mode was cesarean section in 8 cases and natural delivery in 6 cases. IDs 2, 4, and 9 were large for gestational age (LGA), 9 had macrosomia, and

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

	Group D5 (n=5945)	Group D6 (n=4094)	Group D7 (n=137)	*P value*[a]	*P value*[b]
Female age (years)	32.4 ± 4.6	33.2 ± 5.0	33.4 ± 5.2	0.013	0.788
Male age (years)	33.3 ± 5.3	34.3 ± 5.9	34.4 ± 5.0	0.023	0.835
Female body mass index (kg/m^2)	23.8 ± 3.3	23.8 ± 3.9	23.7 ± 3.1	0.878	0.875
Infertility type (%)				0.512	0.431
Primary infertility	2395 (40.3%)	1626 (39.7%)	59 (43.1%)		
Secondary infertility	3550 (59.7%)	2468 (60.3%)	78 (56.9%)		
Main infertility cause (%)				<0.001	<0.001
Female	3469 (58.4%)	2134 (52.1%)	46 (33.6%)		
Male	1039 (17.5%)	944 (23.1%)	67 (48.9%)		
Mixed	1109 (18.7%)	741 (18.1%)	24 (17.5%)		
Other	328 (5.5%)	275 (6.7%)	0 (0.0%)		
Infertility duration (years)	3.2 ± 2.6	3.4 ± 3.0	3.7 ± 3.1	0.023	0.318
Basal FSH (IU/L)	6.2 ± 2.3	5.2 ± 3.1	6.1 ± 2.2	0.520	<0.001
Endometrial preparation method				0.004	<0.001
Artificial cycle	2742 (46.1%)	2671 (65.2%)	45 (32.8%)		
Natural cycle	2190 (36.8%)	944 (23.1%)	68 (49.6%)		
Stimulated cycle	1013 (17.0%)	479 (11.7%)	24 (17.5%)		
Average number of embryos transferred (per patient)	1.193±0.395	1.311±0.463	1.314±0.466	0.003	0.947
The number of embryos transferred (per patient)				<0.001	0.947
1	4797 (80.7%)	2820 (68.9%)	94 (68.6%)		
2	1148 (19.3%)	1274 (31.1%)	43 (31.4%)		
Endometrial thickness (mm)	9.3 ± 1.6	9.4 ± 1.6	9.4 ± 1.9	0.556	0.575
Clinical pregnancy rate	3741 (62.9%)	2104 (51.4%)	19 (13.9%)	<0.001	<0.001
Miscarriage rate	681 (18.2%)	434 (20.6%)	9 (47.4%)	0.001	0.004
Live birth rate	3014 (50.7%)	1658 (40.5%)	10 (7.3%)	<0.001	<0.001

[a]represents Group D7 compared with Group D5, [b]represents Group D7 compared with Group D6; Group D5 represents the D5 FET cycle; Group D6 represents the D6 FET cycle; Group D7 represents the D7 FET cycle.

TABLE 2 Basic clinical data and outcomes of FET cycles with D7 blastocysts from different sources.

	PGT cycles (n=12)	Non-PGT cycles(n=137)	*P value*[c]
Female age (years)	34.2 ± 3.4	33.4 ± 5.2	0.561
Male age (years)	35.2 ± 3.1	34.4 ± 5.0	0.547
Female body mass index (kg/m^2)	24.4 ± 2.2	23.7 ± 3.1	0.477
Infertility type (%)			0.513
Primary infertility	4 (33.3%)	59 (43.1%)	
Secondary infertility	8 (66.7%)	78 (56.9%)	
Main infertility cause (%)			<0.001
Female	3 (25.0%)	80 (58.4%)	
Male	6 (50.0%)	24 (17.5%)	
Mixed	0 (0.0%)	27 (19.7%)	
Other	3 (25.0%)	6 (4.4%)	
Infertility years (years)	4.2 ± 2.7	3.7 ± 3.1	0.548
Basal FSH (IU/L)	4.5 ± 1.6	6.1±2.2	0.013
Endometrial preparation method			0.228
Artificial cycle	6 (50.0%)	46 (33.6%)	
Natural cycle	6 (50.0%)	67 (48.9%)	
Stimulated cycle	0 (0.0%)	24 (17.5%)	
Number of embryos transferred (per patient)			0.021
1	12 (100.0%)	94 (68.6%)	
2	0 (0.0%)	43 (31.4%)	
Endometrial thickness (mm)	9.2 ± 1.4	9.4 ± 1.9	0.743
Implantation rate	5 (41.7%)	20 (11.1%)	0.009
Clinical pregnancy rate	5 (41.7%)	19 (13.9%)	0.012
Miscarriage rate	1 (20.0%)	9 (47.4%)	0.358
Live birth rate	4 (33.3%)	10 (7.3%)	0.003

[c]represents the comparison between PGT cycles and non-PGT cycles.

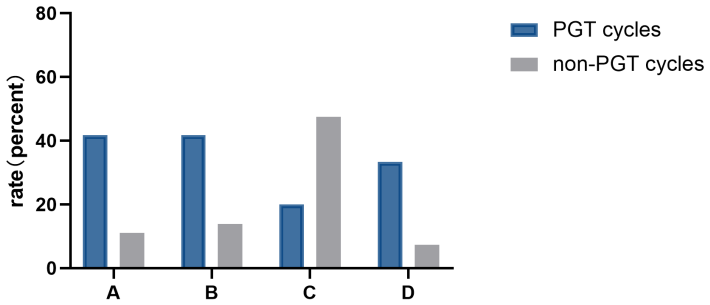
10 was small for gestational age (SGA). In the PGT cycles, 1 premature newborn had neonatal respiratory distress syndrome (NRDS), and the mother had gestational diabetes mellitus (GDM). In the non-PGT cycles, one newborn was premature and had a good general condition; the puerpera had a twin pregnancy and experienced premature rupture of membranes (PROM). The overall prognosis of all neonates was good (Table 5).

Discussion

In this study, a large amount of data and systematic observation and analysis were used to find that although the clinical pregnancy rate, live birth rate and euploidy rate of D7 blastocysts were lower than those of D5/D6 blastocysts and the abortion rate was higher, D7 blastocysts could still progress to

TABLE 3 Biopsy, report and transfer dates of 12 D7 euploid blastocysts.

ID	Biopsy date	Report date	Transfer date
I	November 20, 2018	December 20, 2018	January 18, 2019
II	November 12, 2018	December 10, 2018	April 7, 2019
III	April 02, 2019	May 1, 2019	May 30, 2019
IV	May 29, 2019	June 30, 2019	November 15, 2019
V	August 2, 2019	September 2, 2019	May 4, 2020
VI	October 12, 2019	November 10, 2019	July 31, 2020
VII	June 25, 2019	July 20, 2019	August 14, 2019
VIII	September 04, 2019	October 3, 2019	October 27, 2019
IX	May 19, 2019	June 18, 2019	November 12, 2019
X	September 04, 2019	October 1, 2019	December 7, 2019
XI	October 08, 2019	November 08, 2019	April 6, 2020
XII	May 20, 2020	June 18, 2019	July 20, 2020



Note: A represents the implantation rate; B represents the clinical pregnancy rate, C represents the miscarriage rate, and D represents the live birth rate.

FIGURE 1
Clinical outcomes of D7 FET cycles from different sources.

healthy live births. At the same time, this study was the first to compare the clinical outcomes of D7 blastocysts derived from PGT cycles and non-PGT cycles and showed that the implantation rate, clinical pregnancy rate and live birth rate of PGT cycles were significantly higher than those of non-PGT cycles. This provides a factual basis for whether to use D7 blastocysts and how to make rational decisions in clinical practice.

The results of this study suggested that the parental ages of D7 blastocysts were significantly higher than those of D5 blastocysts, and the authors speculate that the advanced age of couples may be one of the reasons for the slow development of D7 blastocysts. Second, the infertility factors of D7 blastocysts were also significantly different from those of D5/D6 blastocysts. Among these factors, infertility in the D7 group was caused mainly by male factors, and male age may be one of the main factors. Related literature (11) suggests that increased male age is related to changes in epigenetic factors, and changes at the molecular level may affect the development of embryos. Due to the slow development and poor quality of D7 blastocysts, to improve the implantation rate of patients, mostly two blastocysts were transferred in non-PGT cycles. Therefore, the average number of embryos transferred and the number of embryos transferred as D7 blastocysts were significantly higher than those transferred as D5 blastocysts (Table 1).

The results of this study suggest that the transfer of D7 blastocysts can lead to a healthy live birth. When the average number of embryos transferred per cycle is 1.3, the clinical pregnancy rate is 13.9%, the live birth rate is 7.3%, and the abortion rate is 47.4%, which is consistent with the results recently published by Kevin S. Richter et al. (12). Kevin S. Richter et al. (12) studied the FET cycles of 59 D5, 268 D6 and 48 D7 blastocysts and found that the clinical pregnancy rate of D7 blastocysts was only 15%, which was significantly lower than that of D5/D6 blastocysts, and the clinical pregnancy rate was similar to that in this study. The study by Huang et al. (13) included more data, namely, 1961 D5, 4910 D6 and 413 D7 blastocysts. The results showed that the clinical pregnancy rate of D7 blastocysts was 32.9% and that the live birth rate was 26.6%, which were significantly lower than those of D5/D6 blastocysts, and the abortion rate was 19.1% higher than that of D5/D6 blastocysts. At the same time, D7 blastocysts were found to have an increased risk of very large for gestational age (VLGA). Their study mainly reported the obstetric outcomes of D7 blastocysts and did not describe the average number of embryos transferred per cycle in detail. Therefore, the authors speculate that the difference in the average number of embryos transferred may be responsible for the great differences in the clinical pregnancy rate, abortion rate and live birth rate between their study and ours. In addition, due to the small sample size

TABLE 4 Comparison of the euploidy rates of D5, D6 and D7 blastocysts in the PGT cycles.

	D5	D6	D7	*P value*[d]	*P value*[e]
Total	896	1213	152		
euploidy rate				<0.001	0.235
No	459 (51.2%)	787 (64.9%)	106 (69.7%)		
Yes	437 (48.8%)	426 (35.1%)	46 (30.3%)		

[d]represents the comparison between D5 and D7 blastocysts, [e]represents the comparison between D6 and D7 blastocysts.

TABLE 5 General conditions of live-born neonates and maternal complications during pregnancy after D7 transfer (including PGT cycles).

	ID	Number of live births	Gestational age	Newborn sex	Newborn weight (g)	Newborn length (cm)	Neonatal complications	Mode ofdelivery	Pregnancy complications
PGT cycles	1	1	28 w, 4 d	Female	1230	39	NRDS	Natural childbirth	GDM
	2	1	38 w, 2 d	Male	3800	50	Pathological jaundice	Cesarean	NA
	3	1	39 w, 3 d	Female	3300	50	NA	Cesarean	GDM
	4	1	39 w, 6 d	Male	3850	51	NA	Natural childbirth	NA
Non PGT cycles	5	2	35 w, 1 d	Male/ Male	2250/ 2250	48/48	Pathological jaundice/NA	Cesarean	PROM
	6	1	38 w, 1 d	Male	2900	50	NA	Natural childbirth	NA
	7	1	38 w, 5 d	Male	3200	50	NA	Natural childbirth	NA
	8		38 w, 5 d	Male	3460	51	NA	Cesarean	NA
	9	1	39 w, 4 d	Male	4100	52	NA	Cesarean	NA
	10	1	39 w, 5 d	Female	2600	49	NA	Cesarean	NA
	11	1	40 w	Female	3580	50	NA	Cesarean	Anemia during pregnancy
	12	1	40 w, 2 d	Male	3700	52	Pathological jaundice	Cesarean	GDM
	13	1	40 w, 6 d	Male	3400	50	NA	Natural childbirth	NA
	14	1	41 w, 4 d	Male	3360	54	NA	Natural childbirth	NA

NA represents not available.

(only 14 live births after D7 transfer), our study could not conduct specific statistical analysis on the outcomes of singleton pregnancies and listed only the specific situation of each live birth cycle.

Embryo aneuploidy is the main cause of implantation failure, spontaneous abortion, and embryo termination, among other adverse outcomes (14). The implantation rate, clinical pregnancy rate and live birth rate of D7 blastocysts are low, and one of the reasons for the high abortion rate may be the low euploidy rate and high aneuploidy rate of D7 blastocysts. In this study, the euploidy rate of D7 blastocysts was compared with that of D5 and D6 blastocysts during PGT cycles, and it was found that the euploidy rate of D7 blastocysts was only 30.3%. Su et al. (15) examined 151 D7 blastocysts and found that 55 D7 blastocysts were euploid, with a euploidy rate of 36.7%, which was significantly lower than that of D5/D6 blastocysts, consistent with our study results. Samer Alfarawati et al. (16) found that the rate of aneuploidy was higher among blastocysts with slow development, which was also consistent with our results. Another study (17) suggested that the slow development of D7 blastocysts might be caused by their aneuploid character.

Therefore, this study compared the outcomes of D7 blastocyst transfer from PGT cycles and non-PGT cycles and found that the implantation rate, clinical pregnancy rate and live birth rate of D7 blastocysts in PGT cycles were higher than those of D7 blastocysts in non-PGT cycles and that the abortion rate was significantly lower than that in non-PGT cycles. To date, no relevant literature has been published. Although PGT is used mainly by infertile women with chromosome abnormalities, monogenic diseases, repeated implantation failure, recurrent abortion and advanced maternal age (18, 19), embryos derived from PGT cycles have more "abnormal" possibilities than those from non-PGT cycles. In the PGT cycles, the center strictly followed the principle of single blastocyst transfer. However, in the non-PGT cycles, to improve the chances of pregnancy, the center still chose to transfer as many embryos as possible. Consequently, the number of embryos transferred in the non-PGT cycles was significantly higher than that in the PGT cycles. However, after genetic testing, the transfer of embryos diagnosed as "euploid" could still achieve a much higher live birth rate than the transfer of embryos derived from non-PGT cycles. This suggests that biopsy and genetic testing of D7 blastocysts can greatly improve the utilization efficiency of D7 blastocysts and reduce the number of ineffective transfers.

The question of whether preimplantation genetic testing for aneuploidy (PGT-A) should be performed routinely for D7 blastocysts is worth further consideration. PGT-A is used mainly for patients with advanced age, recurrent abortion, chromosomal abnormalities or other conditions. PGT-A is an invasive procedure that is used to improve the effectiveness of assisted reproductive technology (ART) and shorten the time to clinical pregnancy (19). Whitney et al. (20) believed that the ultimate goal of ART was to achieve a healthy live birth and supported routine PGT-A for D7 blastocysts. From this perspective, we need to consider not only the time, effort, and cost of embryo culture, biopsy, and genetic testing (15) but also a patient's time, energy, and emotional and financial capacity. With the gradual introduction of ART into medical insurance in

some areas of China, this perspective may become possible in the future, and more infertile couples will surely benefit from it.

In patients with indications for PGT, As long as the blastocyst is rated as usable, the biopsy operation will be carried out, and the genetic testing will be carried out one week later. After the test result is obtained, the patient will be notified by phone to determine the date of transplantation.

Our study has shown the value of PGT-A in improving the implantation rate, clinical pregnancy rate, and live birth rate after D7 blastocyst transfer. Therefore, D7 blastocyst culture may be considered for patients who must undergo PGT-A to improve their chances of a healthy live birth (15). PGT-A may be a useful tool for screening euploid embryos with slow development. Therefore, embryos that do not form usable blastocysts at D6 should not be generally discarded, and extending embryo culture may improve the clinical outcomes of some patients undergoing ART.

In order to improve the clinical pregnant rate of infertile patients, D7 blastocyst culture would be performanced for patients whose D5/D6 embryos did not form usable blastocysts. At this time, endometrial and embryo development were not synchronized, so we conducted FET for the D7 blastocyst. In this study, we further calculated that the live birth rate of D7 single blastocyst transfer was 7.1%, and the live birth rate of D7 double blastocyst transfer was 10.0%, indicating that the live birth rate of D7 double blastocyst transfer was higher than that of D7 single blastocyst transfer. Therefore, it is recommended to consider D7 double blastocyst transfer for patients without a PGT-A indication and with multiple D7 blastocysts. In contrast, for patients with no PGT-A indication and only single D7 blastocyst formation, the patients and their families should be informed of the low live birth rate, and the next treatment plan should be considered based on the individual situation of the patients.

In summary, although the D7 blastocyst clinical pregnancy rate and live birth rate are low and the abortion rate is high, specialists can consider extending the blastocyst culture time and attempting D7 FET when multiple cycles have failed to yield ideal embryos, D5/D6 embryos have not formed blastocysts (6, 15), the development of D6 embryos is unclear, or embryonic development is slow. PGT for D7 blastocysts may reduce the number of ineffective transfers and improve the outcomes of D7 blastocyst transfer, and this can be implemented according to the conditions of individual patients. D7 blastocyst culture is recommended for patients who must undergo PGT-A (16). It is suggested that double blastocyst transfer be prioritized for patients with D7 blastocysts without PGT-A indications. For patients with only one D7 blastocyst without PGT-A indications, it is recommended to carefully consider the next treatment plan. Additionally, one study (21) suggested the use of D7 blastocysts ≥180 μm in diameter to improve the clinical outcomes of D7 blastocyst transfer. Due to the limited data in our study, whether D7 blastocyst transfer has the risk of epigenetic, innate (22) or acquired developmental abnormalities is not known at present, and this needs to be examined by more rigorous prospective studies with a larger sample size.

Data availability statement

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

Ethics statement

This study was approved by the Institutional Review Board and approved by the Ethics Committee of The Third Affiliated Hospital of Zhengzhou University, which did not require informed consent. The ethics committee approved this study on August 19, 2022, and the approval number is 2022-219-01.

Author contributions

XL, JL and HL contributed to the design of the study. XL, JL, JZ and MD performed the data extraction. YD and SW performed the statistical analyses. XL, JL and HL interpreted the data. XL, JL and HL wrote the manuscript. JL and YG contributed to the critical revision of the article. 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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Prospective multicenter non-interventional real-world study to assess the patterns of use, effectiveness and safety of follitropin delta in routine clinical practice (the PROFILE study)

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Objective: To observe the real-world utilization patterns, effectiveness and safety profile of follitropin delta in women ≥18 years naïve to ovarian stimulation undergoing *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI).

Design: Prospective, multinational, multicenter, observational study. All IVF/ICSI treatment protocols were conducted according to routine clinical practice, including undertaking fresh/frozen transfers. Outcomes included use of dosing algorithm, follitropin delta dosing patterns, ovarian response, pregnancy rates and adverse drug reactions (ADRs).

Results: The first ovarian stimulation cycle using follitropin delta was initiated in 944 women. Mean baseline demographics were: age, 33.5 ± 4.7 years; bodyweight, 67.1 ± 13.6 kg; anti-Müllerian hormone, 20.3 ± 16.1 pmol/L (2.84 ± 2.25 ng/mL). The dosing algorithm was used to calculate the follitropin delta daily starting dose in 893/944 women (94.5%). The mean difference between the calculated and prescribed daily dose was small (0.2 ± 1.40 µg). The mean daily starting follitropin delta dose was 10.4 ± 2.72 µg and the mean total dose administered was 104 µg. Follitropin delta dose adjustments were reported for 57/944 (6.0%) women. The mean number of retrieved oocytes was 10.1 ± 7.03. Ongoing pregnancy at 10–11 weeks was reported for 255 women (27.0% per initiated cycle and 43.1% per fresh transfer [n=592]). Cumulative ongoing pregnancy rate after fresh and/or frozen transfer was 36.4% (344/944). Four

women discontinued follitropin delta due to ADRs. Ovarian hyperstimulation syndrome (OHSS) was the most frequently reported ADR (n=37 [3.9%]); most cases of OHSS were of mild or moderate intensity (n=30 [3.2%]).

Conclusions: This large real-world study of follitropin delta utilization patterns confirms its good pregnancy rates while minimizing OHSS risk during first ovarian stimulation cycle.

KEYWORDS

ovarian stimulation, individualized algorithm-based dosing, follitropin delta, real-world evidence, pregnancy

1 Introduction

Success of *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) techniques used to help couples conceive depends on obtaining enough oocytes to create high-quality embryos for transfer while minimizing the risk of ovarian hyperstimulation syndrome (OHSS). Physicians use different parameters to predict ovarian response, including anti-Müllerian hormone (AMH) levels or antral follicle count (1–3). In clinical practice, treatment individualization is not always based on evidence but rather the physician's experience (4). Follitropin delta (REKOVELLE®, Ferring Pharmaceuticals, Switzerland), is the first recombinant human follicle-stimulating hormone (FSH) to be produced in a human cell line and is also the first FSH that has an approved algorithm based on the woman bodyweight and baseline serum AMH to individualize dosing (5, 6). The dosing algorithm was developed to reduce the risk of extreme hypo- and hyper-ovarian response while maintaining ongoing pregnancy rates compared with conventional FSH dosing strategies (5, 7–14).

Various follitropin preparations are commercially available, including naturally occurring highly purified urofollitropin, recombinant follitropins that are produced using cultured cell lines, and biosimilars (15). Different follitropins share the same amino acid sequence and tertiary protein structure but vary in their post-translational modifications (glycosidic complexity, sialylation and sulfation patterns), which affect *in vivo* bioactivity (16). Follitropin delta's post-translational modifications closely resemble the glycosylation profile of endogenous human FSH, more than recombinant follitropins alfa and beta which are derived from Chinese hamster ovary cell lines (16).

The efficacy and safety of individualized dosing of follitropin delta compared with standard dosing of 150 IU daily recombinant FSH has been established in randomized controlled trials (RCTs). The Phase 3 ESTHER-1 trial demonstrated follitropin delta to be an efficacious and well-tolerated treatment for ovarian stimulation (OS), with a reduced risk of OHSS and a reduced need for gonadotropin-releasing hormone (GnRH) agonist as a preventive intervention for OHSS compared with follitropin alfa (11, 17). Follitropin delta has also demonstrated low immunogenicity potential with second or third repeated OS in the ESTHER-2 trial (18). The Phase 3 STORK trial in Japanese women undergoing IVF/ICSI established non-inferiority between follitropin delta and follitropin beta based on number of oocytes retrieved as well as a favorable benefit-risk with follitropin delta (12). The Phase 3 GRAPE trial established non-inferiority for ongoing pregnancy rates with follitropin delta dosing versus follitropin alfa in Asian women, as well as a significantly higher live birth rate and significantly fewer early OHSS and/or preventive interventions compared to follitropin alfa (13).

Despite this evidence, translating results from RCTs into a clinical setting can be challenging because interventional trials are performed according to stringent treatment protocols, with randomized treatment allocation to reduce risk of bias and strict eligibility criteria to reduce confounding variables. As such, many patients or treatment conditions that are found in daily clinical practice are not always represented in interventional trials. Moreover, some trials investigating drugs for OS may have protocol-driven treatment pathways and pre-determined decision points that affect, or relate to, specific outcomes (19, 20). Protocols for OS are also hugely variable (21). The aim of our single arm, no comparator, observational study, PROFILE, is to report real-world treatment patterns of follitropin delta, including the use of the individualized dosing algorithm, as well as effectiveness and safety profile, in a broad range of women naïve to IVF and ICSI undergoing up to three cycles with follitropin delta.

2 Materials and methods

2.1 Study design

PROFILE was a prospective, multicenter real-world, observational study in women who had not previously undergone IVF/ICSI treatment. The study was performed in compliance with the Declaration of Helsinki, current Guidelines

for Good Pharmacoepidemiology Practice and other national laws applicable in the countries where the study took place, including local institutional review board ethics approval. All women provided written informed consent as part of the enrolment process. Women were enrolled only after the decision to treat with follitropin delta had been made. No aspect of this study interfered with or influenced routine clinical procedures, or the medications prescribed to participating women. All data were collected as part of routine clinical practice at each study site. No study drugs were reimbursed or provided by the study sponsor. Each woman could continue in the study for a maximum of three treatment cycles. The ClinicalTrials.gov identifier is NCT03393780.

2.2 Study participants

In countries where follitropin delta had marketing approval and was available at specialist reproduction medicine clinics, women prescribed follitropin delta for their first IVF/ICSI treatment were consecutively invited to participate in the study. Women who were ≥18 years, IVF/ICSI treatment-naïve and scheduled for OS with follitropin delta for their first cycle of IVF/ICSI using fresh or frozen ejaculated sperm from a male partner or sperm donor were eligible for inclusion. Women who were already participating in an ongoing interventional clinical trial which required any treatment or follow up were excluded from enrolment. Women with any contraindications for treatment with follitropin delta, and women who were planning to become oocyte donors or undergoing OS for fertility preservation were also excluded from enrolment.

2.3 Study drug

As this was a post-authorization, non-interventional observational study, participating physicians could decide all drug doses and regimens for the participating women, including for follitropin delta. All participating physicians were provided with the approved follitropin delta starting dose algorithm (via an on-line tool or App; Ferring Pharmaceuticals, Switzerland), which is based on a woman's body weight and serum AMH. The approved starting daily dose of follitropin delta for women with AMH <15 pmol/L is 12 µg, irrespective of bodyweight. For women with AMH ≥15 pmol/L the daily dose is decreased from 0.19 to 0.10 µg/kg according to increasing AMH concentration until AMH ≥40 pmol/L (6). Follitropin delta was administered subcutaneously using its pen injection device.

A recent (baseline) AMH measurement was acquired for each participating woman using local laboratory facilities. At the time of the study, the approved AMH assay available to use with the follitropin delta dosing algorithm was the Elecsys® AMH Plus (Roche Diagnostics International, Switzerland) with a measuring range from 0.01 to 23 ng/mL (0.07 to 164 pmol/L), and repeatability and intermediate precision of 1.7–2.6% and 2.1–2.9%, respectively (22).

2.4 Outcomes

The primary endpoint was the real-world treatment patterns of follitropin delta, including starting daily dose, number of days of treatment, deviations from the approved dosing schedule as per the summary of product characteristics (per-label), use of dosing algorithm, and use of other treatments during OS, such as GnRH protocol, triggering methods of follicle maturation and luteal phase support.

The key secondary endpoint of ovarian response in Cycle 1 (total number of oocytes retrieved) was documented for all women and for four subgroups based on baseline serum AMH concentration (<7, ≥7 and <15, ≥15 and ≤35 and >35 pmol/L). AMH concentration is a known predictor of ovarian response (1–3). Pregnancy outcomes were recorded, comprising human chorionic gonadotropin [hCG] test, clinical pregnancy defined as at least one gestational sac 5–6 weeks after transfer, vital pregnancy defined as at least one intrauterine gestational sac with fetal heartbeat 5–6 weeks after transfer and ongoing pregnancy (at least one intrauterine viable fetus 10–11 weeks after transfer). Pregnancy data were reported for all women who initiated Cycle 1 by GnRH protocol subgroup (antagonist or agonist), as well as follitropin delta monotherapy and mixed FSH therapy subgroups.

Other secondary endpoints comprised number of women with cycle cancellation for Cycle 1 (including reasons for cancellation) and description of preventive interventions used for potential OHSS for Cycle 1. The number of women with early and late OHSS of severe intensity was recorded for all initiated cycles as well as time from triggering to OHSS occurrence, OHSS duration, duration of hospitalization, medical and surgical interventions, and the OHSS outcomes. Treatment for OHSS and preventive treatment for potential OHSS were conducted according to local practices. All adverse drug reactions (ADRs) and serious ADRs, including OHSS, were reported using the Medical Dictionary for Regulatory Activities (MedDRA) preferred terms (version 23.1). Serious ADRs were defined as an ADR that resulted in death, or was life-threatening, required in-patient hospitalization or prolonged any existing hospitalization, resulted in persistent or significant disability, was a congenital anomaly/birth defect or was an important medical event requiring intervention to prevent any of the previous definitions of a serious ADR.

2.5 Statistical analysis and determining sample size

Dosing patterns and effectiveness were assessed for Cycle 1. Safety was reported for all cycles. Descriptive statistics were used

for all outcomes. Pregnancy outcomes were reported for all women who initiated ovarian stimulation with follitropin delta in Cycle 1. The rate of ongoing pregnancy and cumulative ongoing pregnancy were also reported per embryo/blastocyst transfer. At the time of study termination, if a participant had a vital pregnancy reported as 'Yes' and ongoing pregnancy was missing, the ongoing pregnancy was regarded as 'Pending'. If the last vital pregnancy was 'Yes' and the last ongoing pregnancy result from the same fresh or frozen transfer was missing, the cumulative ongoing pregnancy was regarded as 'Pending.'

A study sample size of between 1000–1200 participants was calculated based on an adverse drug reaction rate range of 2.5–50%, expected drop-out of 20% and a precision (certainty of results) of 0.9–3.1%.

3 Results

3.1 Study participants and enrollment

A total of 1258 women were screened, of whom 1013 (80.5%) met the inclusion and exclusion criteria and were enrolled in the study between March 2018 and October 2020. The study was terminated early by the study sponsor due to the COVID-19 pandemic during which many fertility clinics closed or provided reduced services, but an adequate number of women had enrolled to fulfil the analysis of the primary endpoint. There were 34 study sites across 10 countries. The largest proportion of women was enrolled in Belgium (37.3%), followed by the Netherlands (14.5%), Germany (14.2%) and Italy (7.6%). Patients were also recruited in Australia, Austria, Canada, Poland, Spain and United Kingdom. Of the 245/1258 women who were not enrolled, the most common reason was non-consent to participate, or they did not return to the clinic (n=137 women; Figure 1). A total of 69 women discontinued the study prior to starting their first OS cycle and 944 women started their first OS cycle (Cycle 1), of whom 157 and 29 women also initiated Cycle 2 and Cycle 3, respectively. The most common reason for discontinuation prior to Cycle 2 was pregnancy (n=368). Four spontaneous pregnancies were reported during Cycle 1, and another 20 spontaneous pregnancies were reported prior to women commencing Cycle 2.

The 944 women who initiated Cycle 1 had a mean age of 33.5 ± 4.7 years and mean bodyweight of 67.1 ± 13.6 kg (Table 1). Their mean baseline AMH concentration was 20.3 ± 16.1 pmol/L (2.84 ± 2.25 ng/mL); 17.6% of women had a low AMH concentration of <7 pmol/L (0.98 ng/mL) and 15.3% had a high AMH of >35 pmol/L (4.9 ng/mL). The main type of infertility was primary infertility reported by 671 women (71.1%) and the mean duration of infertility was 2.7 years. The most common reason for infertility was male factor, which was reported by 411 women (43.5%), followed by unexplained infertility, reported by 227 women (24.0%). The mean menstrual cycle duration was 30.3 days. Women who initiated Cycles 2 and 3 had an overall comparable reproductive history as Cycle 1 (data not shown).

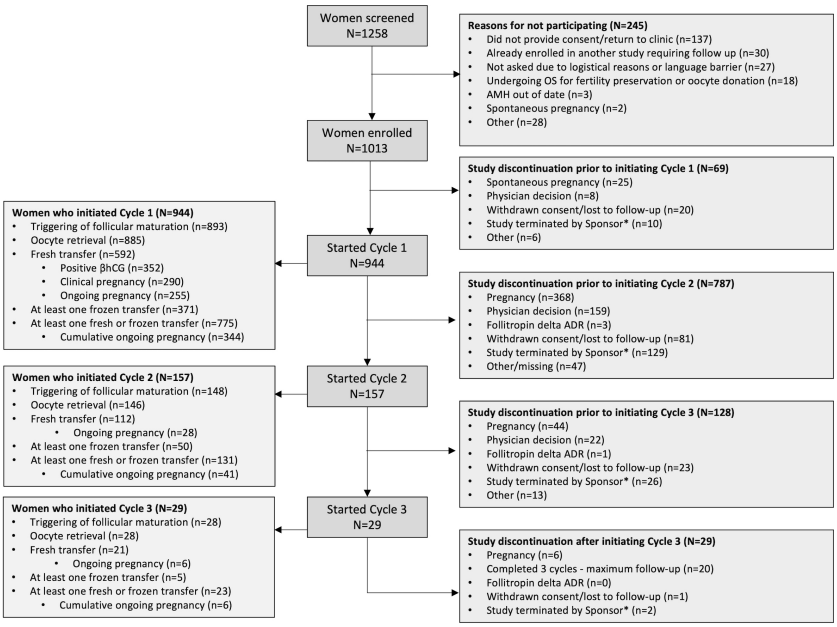

FIGURE 1
Patient study flow *Patient follow-up was truncated at the global cut-off date of 02 October 2020. ADR, adverse drug reaction; OS, ovarian stimulation.

3.2 Follitropin delta dosing patterns

In Cycle 1, 706/944 women (74.8%) received follitropin delta following the label indication, i.e., as monotherapy without a starting dose deviation and/or adjustment during stimulation (Figure 2).

3.2.1 Overview of starting dose, total dose and duration of treatment for Cycle 1

For the 944 women who initiated Cycle 1, the mean starting dose was 10.4 µg, the mean total dose administered was 104 µg and the mean duration of treatment was 10.0 days (Table 2).

TABLE 1 Demographics and baseline characteristics for Cycle 1.

Patient characteristic	All (N=944)
Age, years	33.5 ± 4.7
Body mass index, kg/m^2 (n=937)	24.2 ± 4.6
Bodyweight, kg	67.1 ± 13.6
Bodyweight category	
<50 kg	42 (4.4)
≥50 and < 60 kg	256 (27.1)
≥60 and <70 kg	299 (31.7)
≥70 and <80 kg	165 (17.5)
≥80 and <90 kg	108 (11.4)
≥90 and <100 kg	39 (4.1)
≥100 kg	22 (2.3)
Missing	13 (1.4)
Baseline AMH (pmol/L)	20.3 ± 16.1 16.4 (8.8–27.1)
Baseline AMH category, n (%)	
<7 pmol/L	166 (17.6)
≥7 and <15 pmol/L	259 (27.4)
≥15 and ≤35 pmol/L	375 (39.7)
>35 pmol/L	144 (15.3)
Duration of infertility, years	2.7 ± 2.1
Type of infertility	
Primary infertility	671 (71.1)
Secondary infertility	272 (28.8)
Missing	1 (0.1)
Reason(s) for infertility, n (%)*	
Unexplained infertility	227 (24.0)
Tubal infertility	134 (14.2)
Male factor	411 (43.5)
Anovulatory infertility WHO Group I	25 (2.6)
Anovulatory infertility WHO Group II	64 (6.8)
Endometriosis	104 (11.0)
Other	176 (18.6)
Missing	4 (0.4)

Data are mean ± SD, median (range) or n (%).
N, number of patients; SD, standard deviation.
7 pmol/L = 0.98 ng/mL; 15 pmol/L = 2.1 ng/mL; 35 pmol/L = 4.9 ng/mL.
Percentages calculated using total number of women in study (N).
*Percentages sum to >100% because women could be included in more than one category.

3.2.2 Use of approved dosing algorithm for calculating starting dose for Cycle 1

Nearly all women (893/944 [94.5%]) were prescribed follitropin delta after their physician had calculated the starting dose according to the dosing algorithm, although some physicians then adjusted the prescribed starting dose. The mean difference between the daily starting dose of follitropin delta prescribed by the treating physician and the dose calculated by the per-label algorithm was small (± 0.21 µg, including women with no starting dose deviation). Most women (822/944, 87.1%) were prescribed follitropin delta within 0.33 µg (1 click of pen) of the dose calculated using the approved dosing algorithm.

3.2.3 Deviations from per-label dosing during Cycle 1

Three main types of deviations were observed: **1**) prescribed dose was different from the calculated starting daily dose (i.e., starting dose deviations), reported for 165 women (17.5%), with half of these within 0.66 µg (1 or 2 clicks of pen, n=86 [9.1%]) and one-third within 0.33 µg (1 click of pen, n=59 [6.3%]); **2)** daily dose changes during stimulation (i.e., dose adjustments), reported for 57 (6.0%) women (38 women received dose increases and 22 received dose decreases during their follitropin delta dosing period); and **3)** addition of another gonadotropin during OS (i.e., regimen deviation: change from monotherapy with follitropin delta to a mixed FSH regimen), reported for 33 women (3.5%). Women who received follitropin delta as part of a mixed FSH regimen were more likely to have a starting dose deviation or dose adjustments during stimulation. As so few women received a mixed FSH therapy regimen, we reported ovarian response and pregnancy outcomes for the whole subgroup but not separately according to GnRH protocol.

Reasons for starting dose deviations included physicians prescribing lower follitropin delta doses to avoid OHSS (based on the physician's clinical experience) or higher doses to achieve a better ovarian response for women with very low baseline AMH levels. Similarly, reasons for dose increases during stimulation were normally due to insufficient initial ovarian response (e.g., low antral follicle count at Day 3 of cycle) and dose decreases were made due to potential high ovarian responses (e.g., elevated estradiol levels or high antral follicle count at Day 3 of cycle). A few women were reported to have dose adjustments due to changes to bodyweight (n=2) or had updated AMH levels reported after they had started their cycle (n=3). Reasons for prescribing follitropin delta as part of a mixed FSH were not reported.

3.2.4 Dosing according to GnRH protocol use during Cycle 1

A total of 848 women (89.8%) received a GnRH antagonist protocol and 96 (10.2%) received a GnRH agonist protocol; the

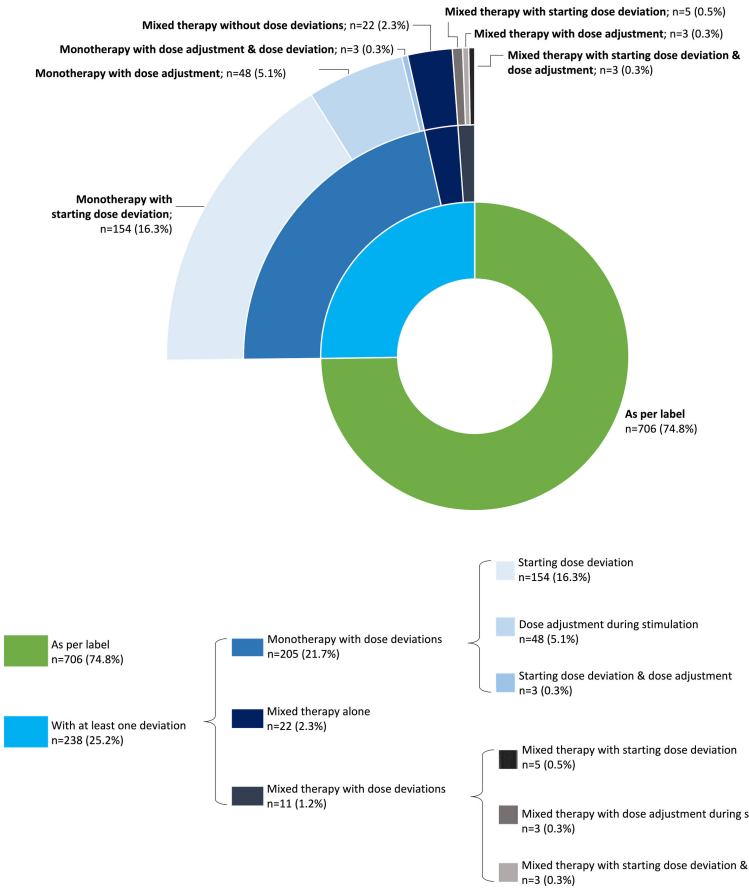

FIGURE 2
Main deviations with follitropin delta during Cycle 1. The figure depicts the hierarchical distribution of deviations in follitropin delta use at Cycle 1. The inner circle differentiates between women who received follitropin delta stimulation therapy as per label (in green) and the women who had at least one deviation in the expected treatment pattern (in blue). The first outer circle presents the group of women with a regimen deviation (i.e., mixed FSH therapy; in dark blue), a dose deviation (including starting dose deviations and dose adjustments during stimulation; in lighter blue) and women who had both regimen and dose deviations (in grey). The second outer circle presents the distribution according to the type of dose deviation (starting dose deviation or dose adjustment during stimulation). FSH, follicle-stimulating hormone.

mean total dose of follitropin delta (for monotherapy and mixed FSH regimens) was 102.5 µg and 116.8 µg for the antagonist and agonist groups, respectively. For women who received follitropin delta as monotherapy in a GnRH antagonist protocol, their mean total dose was 102.2 µg (mean daily starting dose 10.1 µg; n=827). For women who received follitropin delta as monotherapy in a GnRH agonist protocol, their mean total dose was 118.7 µg (mean daily starting dose 10.8 µg; n=84). The median duration of treatment with follitropin delta was 10 days for women using an antagonist protocol and 11 days for women using an agonist protocol. A slightly larger proportion of women who received an agonist protocol had a baseline AMH of <7 pmol/L (<0.98 ng/mL) compared with those who received an antagonist protocol (20.8% and 17.2%, respectively; Figure 3A).

3.2.5 Dosing according to IVF/ICSI, triggering and fresh/frozen transfer procedures for Cycle 1

There was no dosing difference for follitropin delta according to different protocols used for trigger of final follicular maturation, type of transfer procedure or luteal phase support during Cycle 1 (data not shown).

3.3 IVF/ICSI and triggering procedures for Cycle 1

Among women with available data (n=879), the most frequent fertilization technique was ICSI, which was performed for 577 women (61.1%). IVF was used for

TABLE 2 Follitropin delta treatment patterns during Cycle 1.

	Follitropin delta as FSH monotherapy		Follitropin delta as part of combination FSH therapy (N=33)	All(N=944)
	GnRH antagonist (N=827)	GnRH agonist (N=84)		
Overall follitropin delta dosing				
Daily starting dose prescribed (µg)	10.3 ± 2.76	10.8 ± 2.02	11.1 ± 3.12	10.4 ± 2.72
Duration of stimulation (days)	9.9 ± 2.24	10.9 ± 2.06	10.2 ± 2.57	10.0 ± 2.25
Total dose administered (µg)	102.2 ± 34.56	118.7 ± 35.05	110.6 ± 37.71	104.0 ± 35.01
Prescribed daily starting dose based on approved algorithm[a]	779 (94.2)	81 (96.4)	32 (97.0)	892 (94.5)
Dose deviations				
Prescribed daily starting dose not based on the approved algorithm[a]	48 (5.8)	3 (3.6)	1 (3.0)	52 (5.5)
Dose deviations from dose calculated using approved algorithm and prescribed daily starting dose (µg)				
n	814	84	33	931
Dose deviation between calculated and prescribed dose	0.21 ± 1.42	0.1 ± 0.88	0.6 ± 1.90	0.2 ± 1.40
Higher dose (>0.33 µg)[a]	60 (7.3)	2 (2.4)	4 (12.1)	66 (7.0)
Nearly the same dose (± 0.33 µg)[a]	715 (86.5)	79 (94.0)	28 (84.8)	822 (87.1)
Lower dose (> −0.33 µg)[a]	39 (4.7)	3 (3.6)	1 (3.0)	43 (4.6)
Daily dose adjusted during ovarian stimulation[a]	44 (5.3)	7 (8.3)	6 (18.2)	57 (6.0)
If daily dose adjusted, type of adjustment[b,c]				
Increased	31 (3.7)	3 (3.6)	4 (12.1)	38 (4.0)
Decreased	13 (1.6)	6 (7.4)	3 (9.1)	22 (2.3)

Data are mean ± SD or n (%).
[a]Percentages calculated using total number of women in study (N).
[b]Percentages calculated from patients with daily dose adjusted (n).
[c]Percentages sum to >100% because women could be included in more than one category.
GnRH, gonadotrophin-releasing hormone; n, number of patients in specific category; N, number of patients; SD, standard deviation.

216 women (22.9%) and the remaining 86 women (9.1%) received both IVF and ICSI. Triggering of final follicular maturation at Cycle 1 was carried out for 893 women (Table S1).

3.4 Ovarian response for Cycle 1

A total of 885/944 women (93.8%) underwent oocyte pick-up. The mean number of retrieved oocytes per woman in Cycle 1 was 10.1. Women with baseline AMH <7 pmol/L were more likely to have a lower ovarian response (Figure 3B). Follitropin delta dose deviations versus no dose deviations (data not shown) and the use of GnRH antagonist versus GnRH agonist were not associated with differences in ovarian response (Figure 3C). An acceptable ovarian response (4–19 retrieved oocytes) was attained by 702/944 women (74.4%). The mean number of retrieved oocytes decreased by increasing age category, ranging from 11.8 ± 7.64 oocytes retrieved, for women <35 years old to 5.3 ± 4.66 retrieved oocytes for women >40 years old.

3.5 Transfer procedures for Cycle 1

A total of 775/944 women (82.1%) received at least one embryo/blastocyst transfer (fresh and/or frozen; mean number of transfers: 1.6 ± 0.96, range 1–5), without clear differences between GnRH antagonist and agonist protocols. Approximately two-thirds of women (62.7% [n=592]) had a fresh transfer, just over one-third (39.5% [n=371]) had at least one frozen transfer and nearly one-fifth (17.9% n=169) had no transfer. There were 221 women who had all their embryos/blastocysts frozen, with the most frequently reported reasons being either the clinic policy and pre-agreed

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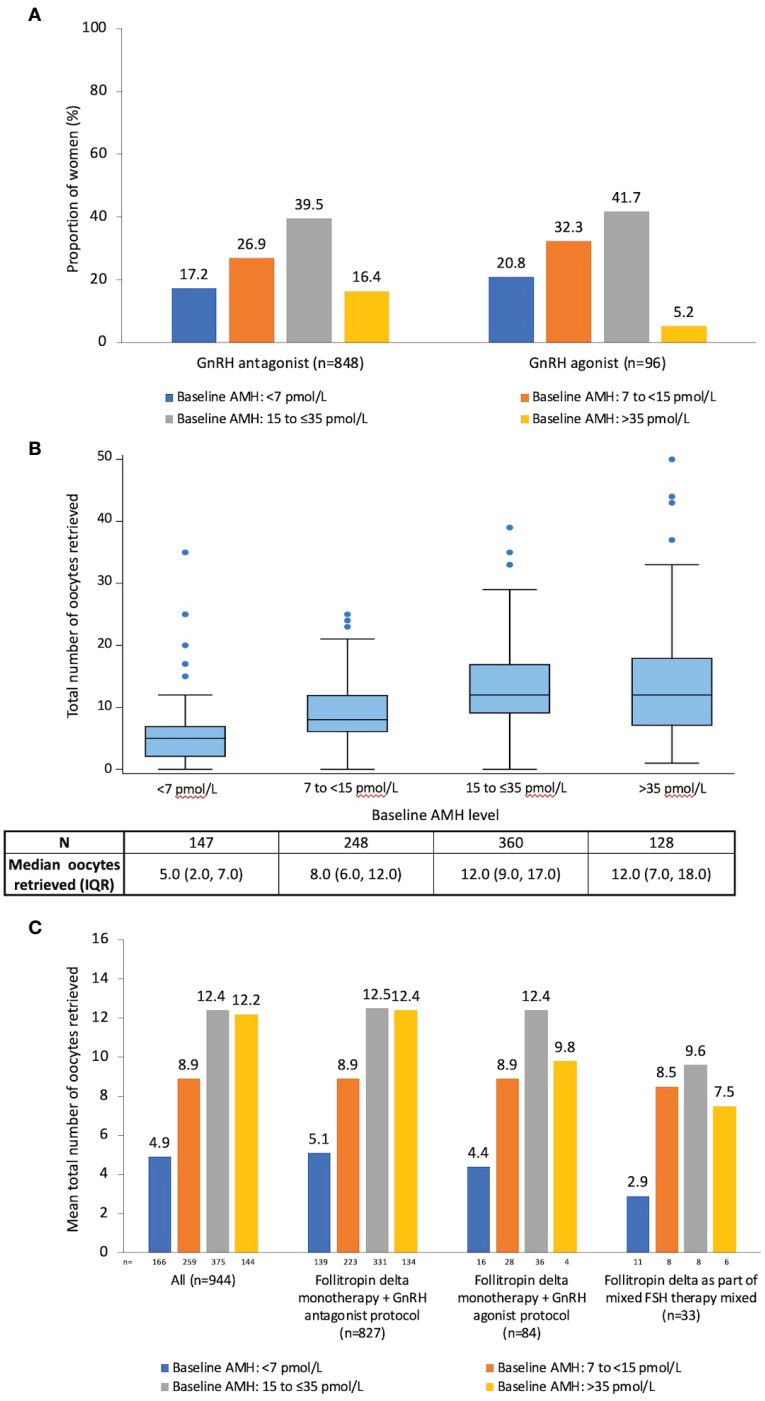

N	147	248	360	128
Median oocytes retrieved (IQR)	5.0 (2.0, 7.0)	8.0 (6.0, 12.0)	12.0 (9.0, 17.0)	12.0 (7.0, 18.0)

FIGURE 3
Baseline AMH subgroups and ovarian response for Cycle 1. **(A)** Women grouped by baseline AMH subgroup and GnRH protocol (n=944); **(B)** Distribution of the total number of retrieved oocytes according to baseline AMH subgroup for all women who underwent oocyte retrieval (n=885). For each category, the box defines the 25th and 75th percentiles of the distribution (i.e., the interquartile range), with the line inside the box representing the median. The whiskers represent the 5th and 95th percentiles of the distribution. Values outside the whisker boundaries are represented as individual dots; **(C)** Mean number of retrieved oocytes by AMH and GnRH protocols for all women who initiated Cycle 1 (n=944). AMH, anti-Müllerian hormone; GnRH, gonadotropin-releasing hormone; IQR, interquartile range.

treatment plan, related to the COVID-19 pandemic or the women's unfavorable progesterone levels. During Cycle 1 (fresh and/or frozen), 666 women received luteal phase support, the majority of whom received progesterone (n=579).

3.6 Pregnancy outcomes for Cycle 1

Pregnancy outcomes by GnRH protocol for Cycle 1 are presented in Table 3. After a fresh transfer, 352 women were reported to have a positive βhCG test (37.3% [352/944]). Clinical and vital pregnancies at 5–6 weeks were reported for 290 (30.7%) and 279 (29.6%) women, respectively. There was a trend for rates of positive βhCG, clinical pregnancy and vital pregnancy to be slightly lower for the women with baseline serum AMH <7 pmol/L regardless of which GnRH protocol was used (data not shown). The ongoing pregnancy rate was 27.0% (n=255) for those who initiated Cycle 1, and 43.1% per fresh transfer. Cumulative ongoing pregnancy rate was 36.4% (n=344) per initiated cycle, and 44.4% per transfer. Fifteen women reported pregnancy loss (spontaneous abortion, n=13; elective abortion, n=2).

3.7 Cycle cancellation for Cycle 1

Most women (n=889 [81.3%]) successfully completed Cycle 1 with follitropin delta (Table S2). A total of 5.8% of women (n=55) had their cycle cancelled prior to oocyte collection and 12.9% women (n=122) had their cycle cancelled after oocyte collection. The most common reason for cancellation prior to oocyte collection was poor ovarian response (n=32; 3.5%), followed by excessive ovarian response (n=3; 0.3%). The most common reason for cancellation after oocyte collection was 'Other' reason (n=41; 4.3%), for whom 17 women had the reason listed as to prevent or avoid OHSS and the rest were mostly related to clinical complications, such additional embryonic screening for genetic mutation(s), hydrosalpinx, post-pick-up bleeding, spontaneous pregnancy, no semen available, or endometrial polyp. Eleven women (1.2%) had cycles cancelled after pickup due to OHSS.

3.8 Interventions used to prevent potential early OHSS during Cycle 1

All treatment decisions for preventing potential early OHSS were according to local practices. Overall, 156 women (16.5%) received preventive interventions for potential early OHSS (before Day 9 after triggering). Preventive interventions were administered to 142/827 women (17.2%) who had received a monotherapy antagonist protocol; 7/84 (8.3%) who had received a monotherapy agonist protocol; 4/21 (19.0%) who had received a mixed antagonist protocol and 3/12 (25.0%) who had received a mixed agonist protocol. Overall, preventive interventions for potential early OHSS used during Cycle 1 were coasting (n=8), triggering of final follicular maturation with GnRH agonist with fresh transfer (n=12), triggering of final follicular maturation with GnRH agonist with a freeze-all strategy (n=107), dopamine agonist (n=10), colloid infusion (n=4), plasma expander (n=1) and the remaining 27 women mostly received a combination of the previous interventions. Women who required a dopamine agonist, colloid infusion or plasma expander were likely to have had early OHSS which needed treatment, whereas the decision to use a freeze-all strategy may have been due to a combination of clinical factors, not just to prevent or avoid OHSS (see reasons for cycle cancellation above). The most frequently used preventive intervention for women who had received a follitropin delta monotherapy plus antagonist protocol was triggering of final follicular maturation with GnRH agonist with a freeze-all strategy (n=105/827 [12.7%]). Of the seven women who had received a follitropin delta monotherapy plus GnRH agonist protocol and who had received preventive intervention for potential early OHSS, six were confirmed not to have received any agonist trigger (one woman had missing data). There were 10 cycle cancellations among the 156 women who received preventive interventions for potential early OHSS.

3.9 Safety for all cycles

For the safety analysis, 944 women underwent a total of 1130 OS cycles. ADRs are summarized in Table 4. Overall, 49 women (5.2%) reported 58 ADRs, with four women experiencing six ADRs leading to treatment and study discontinuation (OHSS, n=2 events; vomiting, n=1 event; headache, n=1 event; rash, n=1 event and premature ovulation, n=1 event). Most ADRs (51/58 [87.9%]) were mild or moderate in intensity. During the whole study period the occurrence of ADRs was low, with most types of ADR occurring in one or two women (≤0.2%), except for OHSS. No deaths were reported during the study.

3.9.1 OHSS occurrence during all cycles

The most frequently reported ADR was OHSS (n=37 [3.9%]), which lasted a median of 9 days; most cases of OHSS were of mild or moderate intensity (n=30 [3.2%]). After triggering of follicular maturation, OHSS presentation was a median of 6 days for 32 women who had timing of OHSS reported, with 20 OHSS events (54.1%) occurring at or before 9 days after triggering, and 12 events (32.4%) occurring after 9 days. Overall, seven women (0.7%) had OHSS of severe intensity, which lasted a median duration of 9 days, three of which occurred ≤9 days after triggering of follicular maturation and three occurred after 9 days (timing was missing for the seventh woman with severe OHSS). Nine women were hospitalized with OHSS, for a median duration of 6.0 days. In

TABLE 3 Pregnancy outcomes by GnRH protocol for Cycle 1.

	Follitropin delta as FSH monotherapy		Follitropin delta as part of combination FSH therapy (N=33)	Overall (N=944)
	GnRH antagonist (N=827)	GnRH agonist (N=84)		
Patients with fresh transfer, n (%)[a]	515 (62.3)	60 (71.4)	17 (51.5)	592 (62.7)
Positive βhCG test, n (%)[a]	317 (38.3)	25 (29.8)	10 (30.3)	352 (37.3)
Clinical pregnancy, n (%)[a,b]	258 (31.2)	22 (26.2)	10 (30.3)	290 (30.7)
Vital pregnancy[c]	250 (30.2)	*21 (25.0)*	8 (24.2)	279 (29.6)
Ongoing pregnancy, n (%)[a,d]	229 (27.7)	19 (22.6)	7 (21.2)	255 (27.0)
Pending ongoing pregancy result	5 (0.6)	1 (1.2)	1 (3.0)	7 (0.7)
Pregnancy rate per fresh transfer[f]	44.5%	31.7%	41.2%	43.1%
Implantation rate per started cycle, %[e]	34%	30%	30%	33%
Patients with at least one transfer (fresh and/or frozen transfer), n (%)[a]	683 (82.6)	67 (79.8)	25 (75.6)	775 (82.1)
Cumulative ongoing pregnancy, n (%)[a,g]	310 (37.5)	26 (31.0)	8 (24.2)	344 (36.4)
Pending ongoing pregnancy result	9 (1.1)	1 (1.2)	1 (3.0)	11 (1.2)
Pregnancy rate per transfer[f]	45.4%	38.8%	32.0%	44.4%

[a]Percentages calculated using total number of women in study (N).
[b]Clinical pregnancy, defined as at least 1 gestational sac 5–6 weeks after transfer.
[c]Vital pregnancy, defined as at least 1 intrauterine gestational sac with fetal heartbeat 5–6 weeks after transfer.
[d]Ongoing pregnancy, defined as at least 1 intrauterine viable fetus 10–11 weeks after transfer.
[e]Implantation rate, defined as the proportion of transferred embryos/blastocysts that resulted in intrauterine viable fetuses at 10-11 weeks after transfer. This only includes women with fresh transfer. Some women had multiple embryos/blastocysts transferred.
[f]Pregnancy rates per transfer were calculated using the number of ongoing pregnancies per number of embryo/blastocyst transfers.
[g]Cumulative ongoing pregnancy includes the number of women with at least one ongoing pregnancy following a fresh transfer and/or any frozen transfer using embryos from Cycle 1.
βhCG, beta human chorionic gonadotropin; FSH, follicle stimulating hormone; GnRH, gonadotrophin-releasing hormone.

total, 16 of the 37 women who developed OHSS had received preventive treatment. All 37 women recovered from their OHSS episode without sequalae.

4 Discussion

The PROFILE study is the first real-world multinational observational study to explore utilization patterns, effectiveness and safety of follitropin delta in daily clinical practice. Follitropin delta is the first and only FSH used for OS that uses an individualized fixed daily dose based on the woman's bodyweight and AMH levels. Most of the participants received follitropin delta as monotherapy without starting dose deviations or dose adjustments (i.e., according to the approved label). Physicians used the follitropin delta dosing algorithm for 95% of participants, although some made minor adjustments to the prescribed starting dose or adjusted the dose during the OS cycle based on clinical factors. In PROFILE, the mean total dose of follitropin delta (104 µg) was slightly higher than observed in randomized clinical trials, in which the mean total dose was 90.0 µg, 83.5 µg and 77.5 µg for the ESTHER-1, STORK and GRAPE trials, respectively (11–13). The higher total dose observed in PROFILE can be explained by the differences in duration of stimulation (~9 days in ESTHER-1, GRAPE and STORK compared with 10 days in PROFILE) as well as differences in participating women's bodyweight (mean bodyweight was ~10 kg lower for women participating in GRAPE and STORK, and 2.4 kg lower for women participating in ESTHER-1 compared with PROFILE) and baseline AMH levels (median AMH concentrations were higher for women in the GRAPE [23.4 pmol/kg] and STORK [18.2 pmol/kg] trials compared with PROFILE [16.4 pmol/kg]). Moreover, the ESTHER-1, GRAPE and STORK trials only included women with a BMI between 17.5 and 32 kg/m^2 whereas PROFILE had no BMI limit, allowing obese women to enroll. Regardless of the differences in follitropin delta utilization among these studies, PROFILE demonstrates that the follitropin delta dosing algorithm based on bodyweight and AMH levels allows for acceptable ovarian responses and ongoing pregnancy rates that were similar to, or higher than, the rates observed in RCTs with follitropin delta (11, 13, 18, 23). PROFILE is in line with the findings of a recent retrospective study of 360 women undergoing ovarian stimulation with follitropin delta for IVF/ICSI at eight German fertility clinics (24).

Nearly all women who received either starting dose deviations or dose modifications during Cycle 1 were prescribed a daily dose of follitropin delta within 0.33 µg or 0.66 µg of the algorithm-calculated dose (1 or 2 clicks of the injection pen) and importantly, women with these small deviations in follitropin delta dosing showed no discernable

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TABLE 4 Adverse drug reactions.

	Participating women (N=944)	
	N (%)[a]	Number of events
Women with at least one ADR	49 (5.2)	58
Women with at least one ADR leading to treatment withdrawal	4 (0.4)	6
Women with at least one ADR with severe intensity[b]	7 (0.7)	7
Women with at least one serious ADR[b]	12 (1.3)	12
Women with at least one serious ADR leading to treatment withdrawal	0	0
Women with at least one serious ADR with severe intensity[b]	5 (0.5)	5
Women with at least one serious ADR leading to death	0	0
All ADRs (any severity)		
Ovarian hyperstimulation syndrome	37 (3.9)	37
Headache	2 (0.2)	2
Mood altered	2 (0.2)	2
Fatigue	2 (0.2)	2
Vomiting	2 (0.2)	2
Affect lability	1 (0.1)	1
Anxiety	1 (0.1)	1
Diarrhea	1 (0.1)	1
Dry skin	1 (0.1)	1
Endometriosis	1 (0.1)	1
High response to ovarian stimulation	1 (0.1)	1
Mood swings	1 (0.1)	1
Nausea	1 (0.1)	1
Premature ovulation	1 (0.1)	1
Pruritus	1 (0.1)	1
Rash	2 (0.2)	2
Swelling	1 (0.1)	1

[a]Percentages calculated using total number of women in study (N).
[b]All reported ADRs with severe intensity and all reported serious ADRs were cases of OHSS, and nine of these women with OHSS were admitted to hospital. See text for more details.
ADR, adverse drug reaction; OHSS, ovarian hyperstimulation syndrome.

difference in ovarian response compared with those who received follitropin delta as per the approved label regimen. The ovarian response, analyzed by AMH subgroup, confirms that the dosing algorithm for follitropin delta is effective at producing a predictable ovarian response in real-world clinical settings, regardless of the type of GnRH protocol used. Moreover, most physicians did not make follitropin delta dose modifications during stimulation.

As expected, women with AMH <7 pmol/L had fewer oocytes retrieved indicating that ovarian reserve is an important factor for predicting the number of retrieved oocytes. Previous studies show that higher doses of FSH for women with low ovarian reserve do not result higher live birth rates (25, 26).

A total of 43% women who underwent a fresh transfer had an ongoing pregnancy at Cycle 1 (27% of women who initiated Cycle 1), and the cumulative ongoing pregnancy rate was 36.4% for initiated cycle. This demonstrates that OS with an individualized follitropin delta dosing regimen results in a high rate of pregnancies in real-world clinical practice, supporting pregnancy rates observed in RCTs (11–14). There were no identified signals for reported pregnancy loss.

As PROFILE was a non-interventional study, investigators treated women at risk of OHSS as they would normally do according to local clinical practice. Although national guidelines are generally similar, there are subtle differences in OHSS severity classification and treatment guidance, including when hospitalization is necessary (27–29). As such, country-specific guidelines should be taken into consideration if comparisons are made between PROFILE and other trials with regards to the treatment of women with OHSS. When considering all cases of OHSS, a similar proportion of women experienced any OHSS in PROFILE compared with RCTs for follitropin delta, ESTHER-1 (3.9% in PROFILE and 3.5% in the first cycle of ESTHER-1); however, there was greater use of preventive interventions for potential early OHSS among women enrolled in the PROFILE study compared with the ESTHER-1 trial (16.5% in PROFILE and 2.3% in ESTHER-1) (11). This was possibly due the stricter use of *per protocol* criteria for preventive interventions for OHSS in the

ESTHER-1 trial and reflects a more cautious approach among physicians when it comes to prescribing preventive interventions for potential early OHSS in clinical practice. In addition, the PROFILE study's inclusion/exclusion criteria allowed enrolment of women with a broad range of comorbid health conditions, including polycystic ovary syndrome and metabolic disorders, representing a wide range of women requiring fertility treatment with confounding factors for OHSS risk that were not considered for this study. In both ESTHER-1 and 2, follitropin delta resulted in a lower incidence of any OHSS than the comparator, follitropin alfa (3.5% vs 4.8%) (17).

In PROFILE, most patients (~90%) received a GnRH suppression protocol with an GnRH antagonist, in line with the evidence from RCTs for follitropin delta (11, 12, 17). Although a relatively small proportion of women (~10% of the study population) received a GnRH agonist protocol, our results from the PROFILE study are one of the first reports of real-world use of combining follitropin delta with an agonist protocol. Among women who received an agonist protocol, a slightly higher proportion had a baseline AMH of <7 pmol/L (<0.98 ng/mL) compared with those who received an antagonist protocol. We observed a similar mean number of oocytes collected for women who underwent an agonist protocol as for those who had an antagonist protocol, which may be due to the lower ovarian reserve among women who received an agonist protocol. Normally, women who receive agonist protocols potentially have more oocytes to be retrieved compared with antagonist protocols (30). Long GnRH agonist protocols are generally associated with one more oocyte retrieved (vs antagonist protocols), one extra day of stimulation and a higher total dose of exogenous FSH (that is equivalent to the extra day of dosing) (31, 32). Our study aligns with the dosing regimens of follitropin delta when used with an agonist protocol, but not the one extra oocyte retrieved. In addition, fewer women who received an agonist protocol received a preventive intervention for potential early OHSS than those who received an antagonist protocol, which again could have been because these women were more likely to have a low ovarian response and therefore at lower risk of OHSS. Generally, long GnRH agonist protocols are associated with a higher risk of OHSS than antagonist protocols (33). A recent RCT of follitropin delta used with a long GnRH agonist protocol showed that the women had a mean of 12.5 oocytes retrieved and an ongoing pregnancy rate of 43% (n=104) per started cycle, supporting our results that follitropin delta is effective when used with a long GnRH agonist protocol (23). Another RCT is currently ongoing to compare the use of follitropin delta in GnRH antagonist versus agonist protocols (BEYOND, NCT03809429).

4.1 Study strengths and limitations

The main strengths of this study are its large size, heterogenous population representing the broad range of patients seen in reproductive health clinics and its prospective design. PROFILE comprised 944 women undergoing OS for IVF/ICSI fertility treatment with a broad range of characteristics who followed routine clinical practice in multiple countries and clinics; however, enrolment was not balanced across countries meaning that there may be a bias towards treatment protocols used in countries with the highest enrolment. For women with OHSS, severity grade was not recorded due to differences in local practice guidelines for treating and grading OHSS. As such, we do not know how many women would have been graded as having severe OHSS.

We also did not anticipate physicians prescribing a mixed FSH regimen during the study. Although only a small proportion of participants received a mixed FSH regimen, the reasons for prescribing follitropin delta as part of a mixed FSH regimen were not recorded, nor were the doses of the other administered FSH preparations used for OS.

Another potential limitation of our study is that we did not record live birth rates or neonatal outcomes. Although these outcomes are now part of the core outcomes for infertility research (34), enrollment for the PROFILE study started before these outcomes had been published. Nonetheless, live birth rates after ovarian stimulation with follitropin delta have previously been established in Phase 3 studies and were almost the same as ongoing pregnancy rates in these trials (11–13). Pregnancy and neonatal outcomes after OS with follitropin delta have also previously been reported (14). For this observational study, the ongoing pregnancy rate was deemed sufficient to confirm the effectiveness of follitropin delta when compared to previously published RCTs.

Although we had originally planned to observe up to three consecutive OS cycles with follitropin delta, the COVID-19 pandemic led to the temporary closure of fertility clinics and we made the decision to terminate the study early once we had enough participants for the primary outcome analysis; however, this meant that many participants could not start subsequent OS cycles. As such, too few participants started Cycles 2 and 3 to allow meaningful analysis of these data.

Real-world observational studies, such as PROFILE, complement data from RCTs, and provide reassurance about effectiveness and safety in a broader range of women compared to the strict inclusion/exclusion criteria often necessary for RCTs to prevent confounding variables. Although clinical decisions are normally based on data from gold standard RCTs, evidence of real-world effectiveness is particularly important for policy makers and payers when deciding access to treatments. Nonetheless, there are a few publications that discuss the pharmacoeconomic impact of follitropins and most compare follitropin alfa to its biosimilars (35–37). As such, further research is ongoing to assess the overall cost effectiveness of follitropin delta when used for OS as part of IVF/ICSI therapy, including any potential cost savings in reduced rates of OHSS in the first OS cycle, compared with other follitropins.

4.2 Overall conclusions

This first large real-world study among a broad range of women naïve to OS supports the efficacy and safety profile of follitropin delta previously demonstrated in randomized controlled trials (11–13, 17). In PROFILE, nearly all patients (95%) had their starting dose calculated using the approved algorithm and most women (87%) received follitropin delta within 0.33 µg (one pen click) of the algorithm-recommended dose. Most women received a GnRH antagonist protocol. Real-world use of individualized dosing of follitropin delta is effective with 74.4% of women attaining 4–19 retrieved oocytes in their first OS cycle. During the first cycle, the ongoing pregnancy rate per fresh transfer was 43%, confirming the efficacy of follitropin delta demonstrated in pivotal clinical trials (11–13). Discontinuation of follitropin delta was rare, with only four women stopping the drug before the end of their dosing regimen. No new safety signals for follitropin delta were reported, and OHSS incidence was within the expected range for the broad range of women undergoing their first OS cycle.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Melbourne IVF Human Research Ethics Committee, Australia; Bellberry Human Research Ethics Committee, Australia; IVFAustralia Ethics Committee, Australia; Ethikkommission der Medizinischen Universität Innsbruck, Austria; Commissie Medische Ethiek, Vrije Universiteit Brussels, Belgium; Cliques universitaires de Bruxelles, Hopital Erasme, Belgium; Universitat zu Lubeck ethics committee, Germany; Veritas Review Board, Quebec, Canada; Single Regional Ethics Committee, Scientific Institute for Research, Hospitalization and Healthcare, *Via* Franco Gallini, Italy; Comitato Etico Lazio 2, Rome, Italy; Comitato Etico Regionale per la Sperimentazione Clinica della Regione Toscana, Italy; Comitato Etico di Area Vasta Sud Est, Toscana, Italy; Il Comitato Etico dell'Ospedale San Raffaele - Milano Istituto di Ricovero e Cura a Carattere Scientifico, Milan, Italy; Medische Ethische Toetsings Commissie, Erasmus MC Universitair Medisch Centrum Rotterdam, Netherlands; Diakonessenhuis Utrecht - Zeist - Doorn, Netherlands; Komisja Bioetyczna, Kliniki iLaboratoria Medyczne Invicta, Rajska, Gdansk, Poland; Fundacio Puigvert ethics committee, Barcelona, Spain; IVIRMA, Barcelona, Spain; and NHS Health Research Authority London- Surry Research Ethics committee, United Kingdom. The patients/participants provided their written informed consent to participate in this study.

Author contributions

CB: study conception, study design, data acquisition, data analysis, drafting and critically reviewing multiple versions of the manuscript, and approval of the final version. GG: study conception, study design, data acquisition, data analysis, drafting and critically reviewing multiple versions of the manuscript, and approval of the final version. RR: data acquisition, critically reviewing multiple versions of the manuscript, and approval of final version. PL: study design, data analysis, statistical analysis plan, drafting and critically reviewing multiple versions of the manuscript, and approval of final version. YLYS: data analysis, drafting and reviewing multiple versions of the manuscript, and approval of final version. SR: study design, data analysis, statistical analysis plan, draft and critically reviewing multiple versions of the manuscript, and approval of final version. JSEL: data acquisition, drafting and critically reviewing multiple versions of the manuscript, and approval of the final version. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.992677/full#supplementary-material

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Global trends in polycystic ovary syndrome research: A 10-year bibliometric analysis

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Background: Polycystic ovary syndrome (PCOS) is one of the most common reproductive metabolic disorders in women, significantly affecting the biological functionalities of ovaries. This disease has garnered enormous interest from researchers. However, there is a lack of a comprehensive research concerning assessing the current status and future trends in PCOS field. This study uses bibliometric tools to comprehensively analyze the PCOS-related research progress based on the literature in the past decade.

Methods: The reported PCOS literature in the past decade is downloaded from the Web of Science database. The bibliometric software is applied to analyze the co-authorship, co-citation, and co-occurrence status.

Results: A total of 9936 publications imported into bibliometric tools for analysis show a sharp increase in the annual citations. The USA is dominant in terms of contribution in the field of PCOS, while China is making a significant contribution to the advancement of this field. Monash University is the most prolific institution with the highest H-index value. The contribution of University of Adelaide must be acknowledged. Legro RS and Teede HJ are the most active and influential authors in recent times, while Azziz R is the most contributed pioneer in this field. The Journal of *Clinical Endocrinology & Metabolism* is the most active journal with the highest number of publications and citations. The pathogenesis of PCOS had been a long-term forefront of research. In recent years, the health management in PCOS prevention and long-term complications was attracting more and more attention. The keywords like "gut microbiota", "microRNAs", "apoptosis",

"Myo-inositol", "TNF-alpha", "androgen receptor", and "Vitamin D-deficient" are considered the latest research topics.

Conclusion: The study comprehensively analyzes the current status and global trends in the PCOS field, providing a significant reference for researchers to explore this field effectively.

KEYWORDS

polycystic ovary syndrome, current status, global research trends, research hotspots, bibliometrics

Highlights

The study comprehensively analyzes the current status and global trends in the PCOS field, providing a significant reference for researchers to explore this field effectively.

Introduction

Polycystic Ovary Syndrome (PCOS) is one of the most common heterogeneous endocrine disorders in women of reproductive age, involving endocrine, reproductive, and metabolic systems. The underlying etiology of this disease is highly complicated with the key clinical manifestations of hyperandrogenemia (clinical and/or biochemical), oligo/anovulation, and polycystic ovaries on ultrasound (1). Based on the NIH 1990 guide and Rotterdam 2003 criteria, the global prevalence rate of PCOS is in the range of 4-21% (2), in which 5.6% of the PCOS cases account for the Chinese women aged 19-45 years (3). This significant variance could be due to differences in races and diagnostic criteria. Notably, the global incidence rate of PCOS has shown a rapidly increasing trend annually along with the severe long-term complications with age, including infertility, obstetrical problems, type 2 diabetes mellitus, cardiovascular diseases, endometrial cancer, psychological problems, resulting in a deprived life quality (4–8). Considering these aspects, PCOS has garnered enormous interest from researchers in various fields at home and abroad.

In virtue of the magnitude of complications and the complexity, as well as the uncertainty of PCOS pathogenesis, enormous literature of original research and review articles have been reported concerning epidemiology, pathophysiology, diagnosis, and treatment of PCOS. Nevertheless, it is challenging to summarize and analyze the advancements in the field on a large scale by the traditional systematic reviews. To a considerable extent, bibliometric analysis has emerged as an essential research tool for quantitatively evaluating the scholarly literature and efficiently predicting the forefront hotspots in global scientific research based on mathematical models and statistical techniques (9). Although the bibliometric analysis has been widely applied in different fields of medicine, there has been only a single article on bibliometric analysis of PCOS presented data from 3 years ago (10). In an attempt to evaluate contributions to this field, a bibliometric analysis was applied to qualitatively and quantitatively analyze the latest PCOS literature published in the past decade (2012–2021) and track the trend and hotspots in the future.

Materials and methods

Data source and search strategy

The bibliometric study was conducted based on a literature search using the Web of Science Core Collection (1985-present). The original data were downloaded and extracted from the Science Citation Index Expanded database (SCIE, 1999-present, last accessed October 28, 2022). The detailed search was performed using items as follows: TS=(Polycystic Ovary Syndrome OR Ovary Syndrome, Polycystic OR Syndrome, Polycystic Ovary OR Stein-Leventhal Syndrome OR Stein Leventhal Syndrome OR Syndrome, Stein-Leventhal OR Sclerocystic Ovarian Degeneration OR Ovarian Degeneration, Sclerocystic OR Sclerocystic Ovary Syndrome OR Polycystic Ovarian Syndrome OR Ovarian Syndrome, Polycystic OR Sclerocystic Ovaries OR Ovary, Sclerocystic OR Sclerocystic Ovary), with the publication language restricted to English. The timespan for retrieval was set as 2012 to 2021, and the document type was limited to Article or Review. Notably, ethical approval was not required for this analysis as all the data used in this study were downloaded from the public database, and no human participants were involved. A flowchart illustrating the literature search and selection process is displayed in Figure 1.

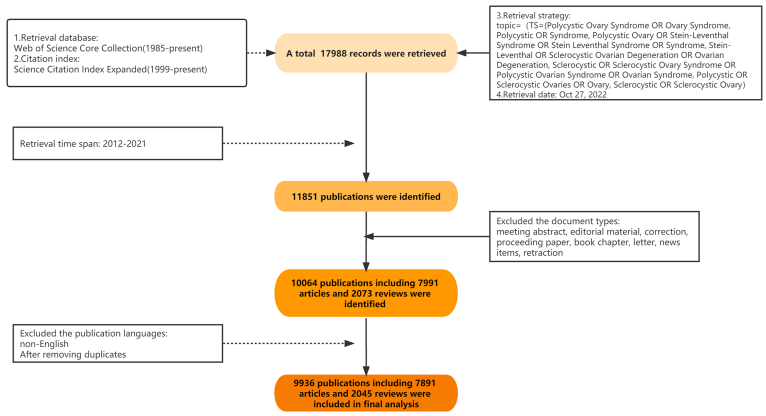

FIGURE 1
Flowchart of literature search and selection process.

Data extraction and collection

According to the aforementioned search criteria, all records of qualified literature were initially downloaded as a plain text format. Because CiteSpace could only identify the files named with "download_*.txt", all files needed to be renamed and placed in a folder named "input". Then, the files were imported into CiteSpace 5.8.R3 (Chaomei Chen, Drexel University, USA) for removing duplicates. Further, the duplicate reports were identified and removed, and all data were manually inspected thrice to ensure the accuracy of the results. Using the "citation report" function in Web of Science, the total number of citations, average citations of each item (ACI), and Hirsch index (H-index) were obtained. ACI and H-index are the two important indicators to measure the impact of individuals, publications, or institutions. The impact factor (IF) and Journal Citation Reports (JCR) category quartile rankings (Q1–Q4) of journals were extracted from the online journals retrieval platform (https://www.medsci.cn/sci/index.do).

Bibliometric analysis

In this study, the CiteSpace (version 5.8.R3) (11) and VOSviewer (version 1.6.18) (12) and a free online tool of literature analysis (https://bibliometric.com/) were applied in the bibliometric data analysis and network visualization due to their complementary advantages. Throughout the process of data visualization, some data needed to be cleaned or summarized. For example, the data from Taiwan or Hong Kong was pooled into China, and the data from England, Northern Ireland, Scotland, and Wales was merged into the United Kingdom. In the keyword analysis, some similar keywords were merged into one.

CiteSpace, a Java-based software, was developed by Professor Chaomei Chen for information visualization. This software was used to establish the cooperation relationships of institutions, the co-occurring network for subject categories, co-citation analysis and dual-map overlay analysis for journals, and bursts analysis for references and keywords. In the parameters settings of CiteSpace, the time slice was set as one-year, and the note type was selected as author, institution, country, keyword, and reference, respectively. In addition, the Top N per slice was set as 50. The color-coded nodes and edges were created in the network, with a different color assigned to each year. The high degree of betweenness centrality (BC≥0.1) was represented by a purple ring in a node, indicating the number of links of one node relative to another (13). A dual-map overlay analysis in CiteSpace could provide the citing trajectories displaying the dynamics of previous cross-discipline study activities (14).

The VOSviewer, a software tool for constructing and visualizing bibliometric networks, was developed by the Centre for Science and Technology Studies at the Leiden University in Leiden, the Netherlands. By setting the two parameters of "Create a map based on bibliographic data" and "Read data from bibliographic database files", this software tool was used for the following visual analyses of co-authorship of countries/regions, institutions, and authors, co-citation of authors, and co-occurrence of keywords. The visualizing networks were generated in VOSviewer. The total link strength (TLS) is a valuable parameter applied in the VOSviewer to measure the strength of associations among countries, authors, journals, and keywords. It should be noted that the greater the TLS, the higher the cooperation or co-occurrence of the two notes (15).

In this study, the online tool of literature analysis (https://bibliometric.com/) was used to analyze the cooperation among countries/regions. The downloaded data from Web of Science database was used as the data source.

In addition, an online mapping platform (http://www.bioinformatics.com.cn/) was used to produce the world map

displaying the contribution of each country, and Microsoft Excel v2016 were applied to for data collection and plotting graphs.

Results

Analysis of global trends of publications and citations

The number of publications and citations are the essential indicators of the development trends in this bibliometric analysis. Overall, 9936 publications met the filtering criteria to be imported into bibliometric tools for subsequent analysis, including 7891 original articles and 2045 reviews. Similarly, annual citations also showed a sharp increasing trend from 548 in 2012 to 47038 by 2021 (Supplementary Figure S1).

Analysis of the contributions of counties/regions

Notably, all the reported publications related to PCOS research were distributed among 140 countries/regions (Figure 2). The top 20 high-yield countries with H-index are listed in Supplementary Table S1. Among various countries, the highest number of publications was reported from China with 2154 articles, followed by the USA with 2101, while the rest have published less than 700 articles. From the perspective of the H-index, the countries with the highest indices were the USA (114), the UK (72), Australia (69), Italy (66) and China (63).

Supplementary Figures S2-S3 show the visualization of cooperative relationships among countries/regions. Similar to H-index, the top five active countries with the greatest TLS included the USA (1351 times), the UK (940 times), Italy (636 times), Australia (618 times), and China (571 times). Among these top five active countries list, the USA, the UK and China were most closely related. In addition, the USA, Canada, Australia, and India demonstrated active collaborations, while the UK also showed close cooperation with Qatar and the Netherlands. From the co-authorship overlay visualization map from VOSviewer, the green and yellow nodes presented that the average appearing years were later than others, with the representative countries including China, Iran, India, Australia, and Poland.

Analysis of the productiveness and co-authorship of institutions

The top 15 institutions with the highest number of publications are listed in Supplementary Figure S4. Among them, Monash University was the most productive institution with 241 articles, followed by Shanghai Jiao Tong University (231 articles), Tehran University of Medical Sciences (152 articles), and Shandong University (149 articles). Moreover, in terms of the H-index, as presented in Figure 3A, Monash University (47), University of Adelaide (44), and Karolinska Institute (16) were stood in the top three ranks in the list. As the second most published institution, Shanghai Jiao Tong University showed an H-index of only 32. While the publication of University of Adelaide was not many (148 articles), its H-index was in the top ranks. The top 15 institutions in terms of ACI are shown in Figure 3B. University of Adelaide significantly outranked other institutions working in this field with the highest ACI (56.35), followed by University of Michigan (37.02), Monash University (35.98) and Karolinska Institute (35.38).

The co-authorship relationship among institutions was performed using CiteSpace software (Figure 4) and VOSviewer (Figure 5). It was observed that University of Adelaide was the only

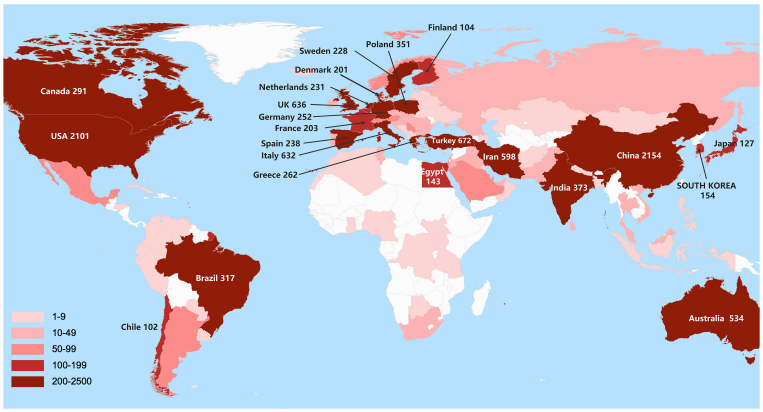

FIGURE 2
A world map displaying the contribution of each country to PCOS research based on publication counts: the darker the color, the more publications, as shown at the bottom left.

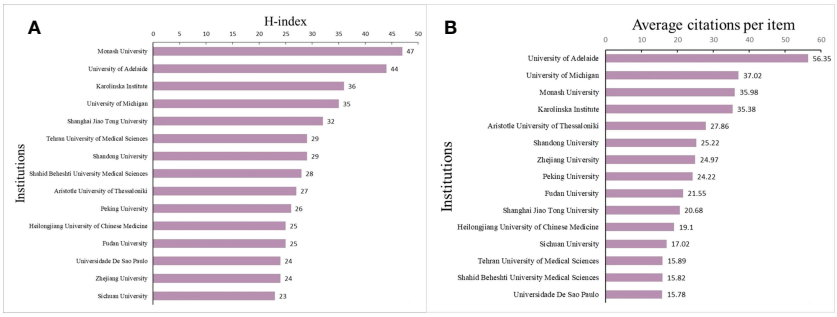

FIGURE 3
(A) The top 15 institutions according to H-index. **(B)** The top 15 institutions according to ACI.

institution with a higher BC value than 0.1 in the purple ring and identified as a critical network hub connecting many institutions, suggesting no substantial influence on other institutions. In this aspect, three significant clusters were clearly identified to dominate the research domain in the co-authorship network map, predominantly concentrated in institutions from Australia, USA, and China, respectively. In addition, many institutions from Iran formed a tight cluster, which was indistinctly placed from the significant clusters. The top 3 institutions with the greatest TLS included Monash University (715), University of Adelaide (498), and University of Pennsylvania (480).

Analysis of funding agencies

The top 15 worldwide funding agencies for the support of PCOS research are summarized in Supplementary Figure S5. Six funding agencies (46% of all studies) were from the USA. National Institutes of Health ranked first, supporting 1020 studies, and the National Natural Science Foundation of China was indicated second, sponsoring 939 studies.

Analysis of productiveness and co-authorship of authors

Figure 6 lists the top 10 most active authors of the PCOS-related publications. Among the list, Prof. Legro RS from the USA was ranked first with 103 articles, followed by Teede HJ from Australia with 99 articles, and Chen ZJ from China with 96 articles. In addition to the number of publications, various indicators, such as H-index and ACI, could help identify the total impact of the author in the form of citations. In terms of H-index, Legro RS (38), Teede HJ (16), Asemi Z (17), and Chen ZJ (18) were listed as the top four highest cited researchers in the field. Legro RS possessed the highest ACI of 74.17 times from ACI, and Teede HJ was the second with the ACI value of 52.19 times, followed by Moran LJ from Australia (40.85 times).

Further, a co-authorship overlay visualization map of authors with a minimum of 20 articles (Figure 7) and a collaboration analysis of core authors in several research clusters (Supplementary Figure S6) were presented using VOSviewer. It was observed from these maps that Legro RS, Stener-Victorin E, Chen ZJ, and Teede HJ were positioned in the

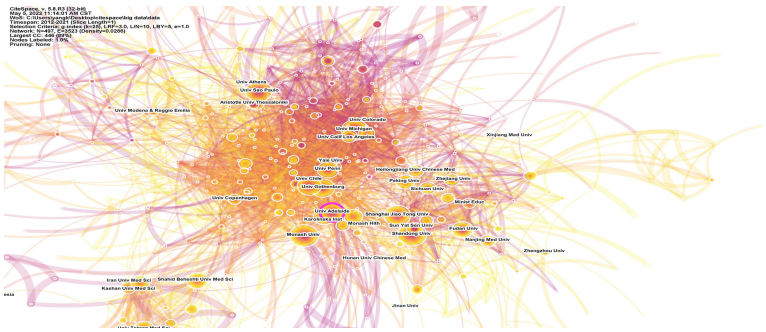

FIGURE 4
The network map of institutions by Citespace. The node size represents the number of publications from the institution. Node with purple ring in the network reflects it with high betweenness centrality (BC ≥ 0.1).

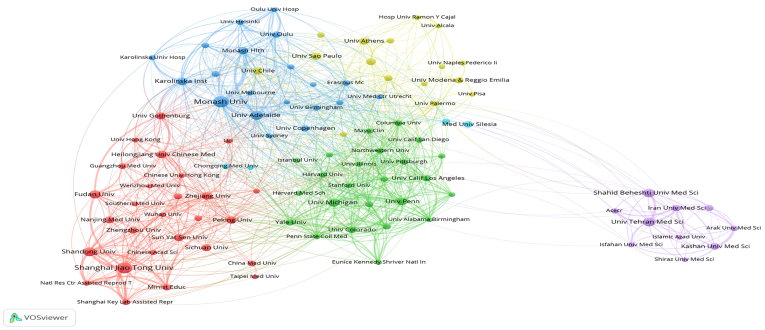

FIGURE 5
Institution co-authorship network visualization map by VOSviewer. The distance between two nodes indicates the relatedness of institutions in terms of co-authorship links. The smaller distance, the stronger relatedness, defined as one cluster with the same color.

central location of the co-authorship clusters, but with a few strong links between them, implicating a need to intensify the collaboration and communication among authors in the domain. In addition, the AAY for each author was labeled with different colors, as shown by the gradient at the bottom right. Among these core authors, the contribution of the relatively young researchers should be acknowledged, such as Morin-Papunen L, Moran LJ, Teede HJ, Hu M, and Sun Y, among others.

The co-citation relationship network map of authors with at least 100 citations was obtained by the VOSviewer (Figure 8), displaying the authors with a significant influence in this field.

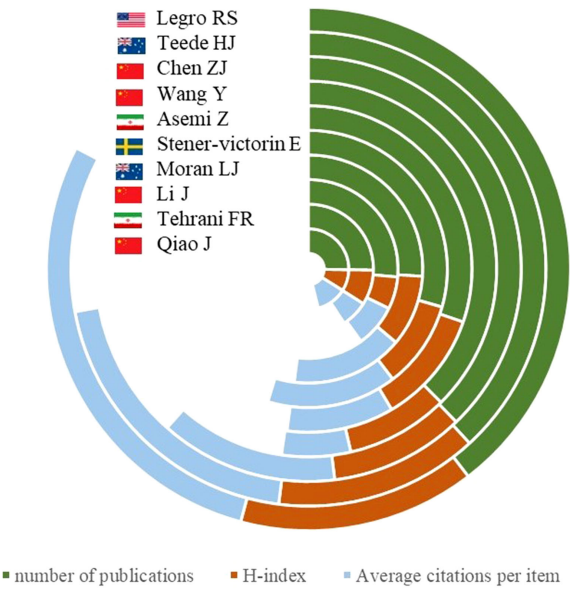

FIGURE 6
The top 10 authors in terms of total number of publications, ACI, and H-index.

The top three authors with the highest TLS included Azziz R, Legro RS, and Diamanti-Kandarakis E. The co-citation relationship of Azziz R with other authors is showed in Supplementary Figure S7. Among these authors with more than 100 citations, Azziz R collaborated with 443 authors worldwide.

Analysis of highly influential journals

The basic information on the top 15 influential journals is summarized in Table 1, including citing articles, the journal impact factor (JIF), and H-index. Around 50% of the articles were published in the top 10 most influential journals. Among them, the top three journals with the highest number of citing articles included the *Journal of Clinical Endocrinology & Metabolism*, *Fertility and Sterility*, and *Human Reproduction*. As the JIF and H-index are the essential parameters to measure the quality and value of a journal, *Lancet* possessed the highest JIF of 44.862 and *New England Journal of Medicine* has achieved the second-highest JIF of 19.075.

The co-citation frequency is another valuable parameter to reflect the impact of a journal. The co-citation visualization network map was displayed by the VOSviewer (Figure 9), with a minimum of 100 citations of a selected journal. It was observed from the results that the network resulted in 690 nodes and 6 clusters. The top 5 most cited journals included *Journal of Clinical Endocrinology & Metabolism*, *Fertility and Sterility*, *Human Reproduction*, *Endocrinology*, and *Human Reproduction Update*.

Analysis of the highest relevant subject categories

A co-occurring analysis of subject categories based on journals was generated using CiteSpace (Figure 10). The top

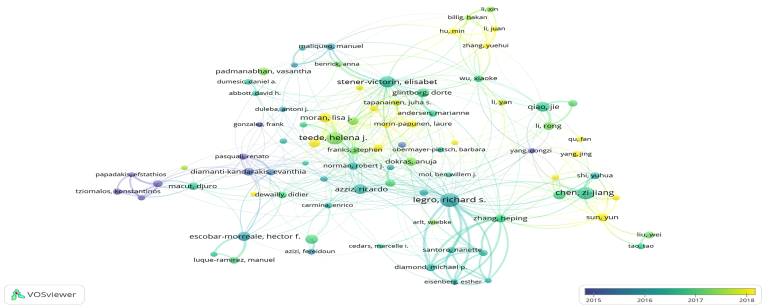

FIGURE 7
A co-authorship overlay visualization map of authors by VOSviewer.

15 relevant subject categories with PCOS are displayed in Supplementary Figure S8. As can be seen, the three most relevant subject categories correlated with PCOS included Endocrinology & Metabolism, Obstetrics & Gynecology, and Reproductive Biology. Among the relevant subject categories with PCOS, four predominant subject categories with a higher BC value than 0.1, included Biochemistry & Molecular Biology, Pharmacology & Pharmacy, Cell Biology, and Research & Experimental Medicine (from high to low). Otherwise, a dual-map overlay of journals was generated using CiteSpace (Figure 11), standing for the citing trajectories for interdisciplinary collaboration. Two primary citation lines tinted in orange were identified. These results indicated that the studies published in Molecular/Biology/Immunology journals mainly cited studies published in the Molecular/Biology/Genetics and Health/Nursing/Medicine journals. In contrast, two main green citation lines showed that a large proportion of articles in Medicine/medical/clinical journals most likely cited articles from the Molecular/Biology/Genetics and Health/Nursing/Medicine journals.

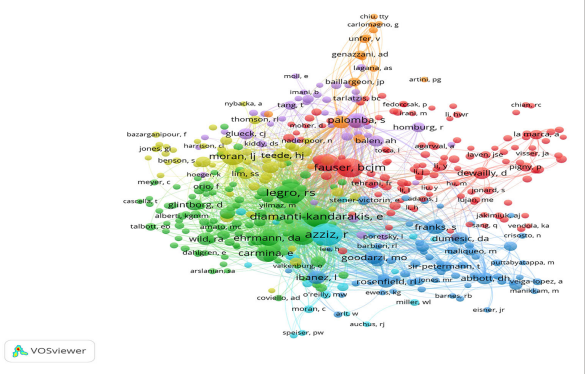

FIGURE 8
The co-citation network map of authors obtained by the VOSviewer. The node size represents the number of citations. The distance between two nodes indicates the relatedness of authors in terms of co-citation link. The smaller distance, the stronger relatedness, defined as one cluster with the same color.

Analysis of highly cited papers

In this context, the top 20 highest cited papers on PCOS were listed in Supplementary Table S2. Among these, the top 5 articles were cited over 900 times. Specifically, a review entitled "Lack of Exercise Is a Major Cause of Chronic Diseases", published in *Comprehensive Physiology* journal, has been cited 1141 times. The second highest cited paper entitled "Cellular and molecular mechanisms of metformin: an overview", published in the *Clinical Science*, has been cited 1114 times.

Co-citation analysis of the references based on citation bursts

Notably, burst detection can significantly classify the cited references that have affected concern among peer researchers. Moreover, the references with citation bursts can assess the developmental frontiers of the domain. The top 25 references with the most robust citation bursts are listed in Table 2. The blue line represented the timeline, and the red segment in the blue timeline indicated the duration that a reference showed a burst. Among these top references, the paper with the most substantial citation burst was authored by Azziz R et al. in 2009. The notable second more vigorous burst written by March WA et al.

Analysis of co–occurring keywords

The analysis of co-occurring keywords is one of the prevalent ways to identify the main topics of a specific subject area. In this study, a total of 20709 keywords were extracted from 9936 publications. After excluding keywords with no

TABLE 1 Top 15 journals with most publications in PCOS research.

Ranking	Journals title	Citing Articles	JIF (2022)	H-index	Publication country
1	*Journal of Clinical Endocrinology & Metabolism*	8706	5.243	61	United Kingdom
2	*Fertility and Sterility*	7364	3.706	49	Netherlands
3	*Human Reproduction*	7240	5.426	52	United Kingdom
4	*Human Reproduction Update*	6620	12.527	42	United Kingdom
5	*Gynecological Endocrinology*	4480	2.027	33	United Kingdom
6	*Clinical Endocrinology*	4289	2.838	32	United Kingdom
7	*PLoS One*	4064	3.041	36	United States
8	*Endocrine Reviews*	3335	17.27	19	United States
9	*Reproductive Biology and Endocrinology*	3150	4.791	28	United Kingdom
10	*European Journal of Endocrinology*	2692	5.702	30	United Kingdom
11	*Endocrinology*	2276	3.988	34	United Kingdom
12	*New England Journal of Medicine*	1303	19.075	7	United States
13	*Diabetes Care*	937	11.378	11	United States
14	*Lancet*	200	44.862	3	United Kingdom
15	*Diabetes*	682	6.072	9	United States

significance and merging keywords with similar meaning, a network visualization map with 518 keywords that occurred at least 30 times was performed using the VOSviewer (Supplementary Figure S9). The top 25 most frequently occurred keywords are displayed in Figure 12. Apart from "polycystic ovary syndrome" and "women", the other keywords with frequent occurrences were mainly selected, focusing on pathogenesis (oxidative stress, AMH, gene expression, and inflammation), characteristics (insulin resistance, obesity, and hyperandrogenism), and complication risks (metabolic syndrome, impaired glucose-tolerance, infertility, and cardiovascular diseases).

In addition, the co-occurrence of keywords was colored according to the AAY using VOSviewer (Figure 13). In this context, several predominantly used keywords, such as "gut microbiota", "microRNAs", "cumulus cells", "apoptosis", "Myo-inositol", "TNF-alpha", "androgen receptor", and "Vitamin D-deficient", showed relatively latest AAY, indicating the potential research topics in the near future.

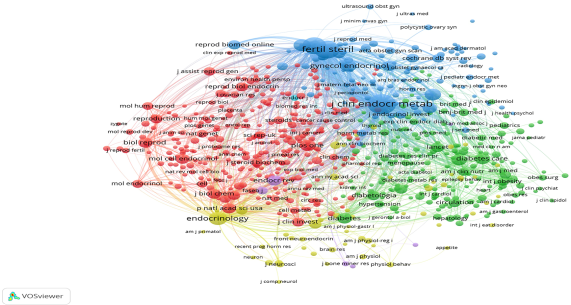

FIGURE 9
The co-citation network visualization map of journals by VOSviewer.

Discussion

Based on the analysis of global trends of publications and citations in the field of PCOS, in the past decade (2012-2021), there had been a gradually increasing trend in the annual publications and a rapidly growing trend of citations. The increasing trends indicated that significant attention had been paid to PCOS by researchers towards improved insights in terms of the pathogenesis of PCOS.

Among the top 20 high-yield countries, the USA was the leading country in the academic impact and quality of publications. Although the number of publications from China was highest, the quality was suboptimal, predominantly due to the lack of sufficient citations as one new entrant. Thus, much effort is still needed by China to enhance the quality of the publications and strengthen further collaborations with other countries.

As the most productive institution and with the highest H-index, Monash University consistently brought international leadership in this field. One of his most important achievements was that he spearheaded development and implementation of International evidence-based guideline for

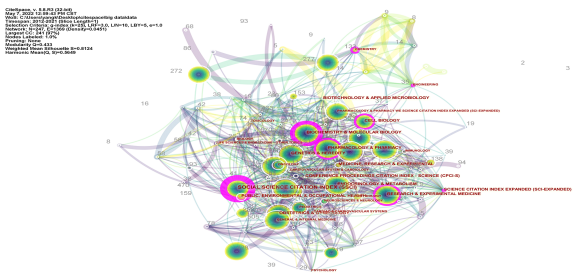

FIGURE 10
A co-occurring network map of subject categories generated by Citespace.

the assessment and management of polycystic ovary syndrome 2018. University of Adelaide, although not high in publication counts, ranked first in ACI and second in H-index. Robinson Research Institute at University of Adelaide had a collective of internationally renowned researchers in reproductive health, obstetrics, and gynecology. The likely predominant reason could be that several consensus guidelines from this institution have gained considerable attention. For instance, an article reported the third PCOS consensus workshop on women's health in 2010, followed by the two previous ESHRE/ASRM-sponsored PCOS consensus. It clarified some knowledge gaps in understanding the women's health of PCOS and addressed diverse care aspects during the reproductive and post-reproductive years (6). In addition, a systematic review and meta-analysis substantially explored the association between PCOS and obesity, presenting the prevention and management of overweight and obesity in the clinical management of PCOS (17). More often, one of those was considered an international evidence-based guideline regarding some recommendations on assessing and managing PCOS (18).

The co-authorship network analysis mainly provided three significant cooperation network, predominantly concentrated in institutions from Australia, USA, and China, respectively. Of these, Monash University had strongest collaborative ability, followed by the University of Adelaide and the University of Pennsylvania. However, the USA, Australia and China as the leaders in this domain, needed to strengthened further collaboration and communication in different research institutions, specifically with institutions from different countries, to eliminate the academic barriers. One additional tight cluster, formed by many institutions from Iran, was indistinctly placed from the above significant clusters. For instance, a study related to a large-sample epidemiological investigation on PCOS prevalence study based on community from Shahid Beheshti University Medical Sciences has been cited 154 times (19). Notably, another research team from Kashan University of Medical Sciences reported the effect of calcium plus vitamin D supplementation on glucose metabolism and lipid profiles in PCOS for the first time (20), which has been cited 83 times.

Financial support is considered an essential pillar for the developing PCOS research. As can be seen, 80% of the identified articles were supported by these funding agencies in the USA and China. Greater investments was associated with more rapid progress of the field.

Regarding the most prolific authors, Legro RS and Teede HJ were the two most contributed pioneers in this field. In 1999, Legro RS and colleagues published a study about the prevalence and risk of impaired glucose intolerance and type 2 diabetes mellitus in PCOS women. The authors reported that the prevalence of impaired glucose intolerance and type 2 diabetes mellitus were significantly augmented in the PCOS women (21), which has been cited 1285 citations so far. Additionally, Legro RS developed a clinical practice guideline for the diagnosis and treatment of PCOS with additional expert panelists from the

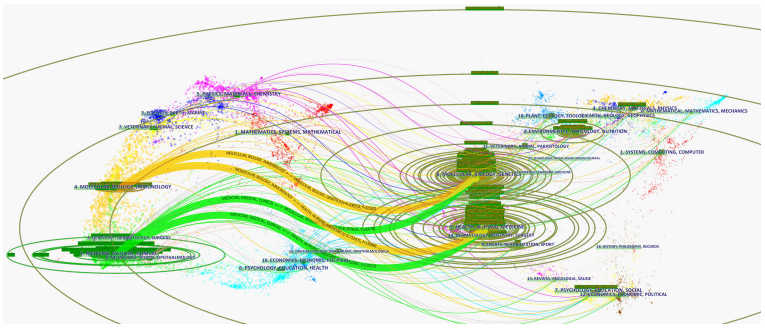

FIGURE 11
The dual-map overlay of journals stood for the topic distribution of academic journals involving PCOS research (generated by Citespace). The colored connecting lines represent citation relationship. The cited journals were on the right, and the citing journals were on the left.

TABLE 2 The top 30 references co-citation with the strongest citation bursts.

References	Year	Strength	Begin	End	2012 - 2021
Azziz R, 2009, FERTIL STERIL, V91, P456, DOI 10.1016/j.fertnstert.2008.06.035	2009	72.2	2012	2014	
March WA, 2010, HUM REPROD, V25, P544, DOI 10.1093/humrep/dep399	2010	53.9	2012	2015	
Wild RA, 2010, J CLIN ENDOCR METAB, V95, P2038, DOI 10.1210/jc.2009-2724	2010	53.61	2012	2015	
Goodarzi MO, 2011, NAT REV ENDOCRINOL, V7, P219, DOI 10.1038/nrendo.2010.217	2011	41.32	2012	2016	
Moran LJ, 2010, HUM REPROD UPDATE, V16, P347, DOI 10.1093/humupd/dmq001	2010	36.07	2012	2015	
Chen ZJ, 2011, NAT GENET, V43, P55, DOI 10.1038/ng.732	2011	24.74	2012	2016	
Teede H, 2010, BMC MED, V8, P0, DOI 10.1186/1741-7015-8-41	2010	23.64	2012	2015	
Palomba S, 2009, ENDOCR REV, V30, P1, DOI 10.1210/er.2008-0030	2009	22.42	2012	2014	
Piouka A, 2009, AM J PHYSIOL-ENDOC M, V296, P0, DOI 10.1152/ajpendo.90684.2008	2009	21.32	2012	2014	
Fauser BCJM, 2012, FERTIL STERIL, V97, P28, DOI 10.1016/j.fertnstert.2011.09.024	2012	47.38	2013	2017	
Diamanti-Kandarakis E, 2012, ENDOCR REV, V33, P981, DOI 10.1210/er.2011-1034	2012	48.25	2014	2017	
Shi YY, 2012, NAT GENET, V44, P1020, DOI 10.1038/ng.2384	2012	21.17	2014	2017	
Yildiz BO, 2012, HUM REPROD, V27, P3067, DOI 10.1093/humrep/des232	2012	21.03	2014	2017	
Stepto NK, 2013, HUM REPROD, V28, P777, DOI 10.1093/humrep/des463	2013	19.17	2014	2018	
Lim SS, 2012, HUM REPROD UPDATE, V18, P618, DOI 10.1093/humupd/dms030	2012	19.17	2014	2017	
Legro RS, 2013, J CLIN ENDOCR METAB, V98, P4565, DOI 10.1210/jc.2013-2350	2013	59.23	2015	2018	
Li R, 2013, HUM REPROD, V28, P2562, DOI 10.1093/humrep/det262	2013	20.53	2015	2018	
Conway G, 2014, EUR J ENDOCRINOL, V171, P0, DOI 10.1530/EJE-14-0253	2014	25.5	2016	2019	
Sirmans SM, 2014, CLIN EPIDEMIOL, V6, P1, DOI 10.2147/CLEP.S37559	2014	20.64	2016	2019	
Dumesic DA, 2015, ENDOCR REV, V36, P487, DOI 10.1210/er.2015-1018	2015	29.67	2017	2021	
Goodman NF, 2015, ENDOCR PRACT, V21, P1415, DOI 10.4158/EP15748.DSCPT2	2015	19.19	2017	2021	
Bozdag G, 2016, HUM REPROD, V31, P2841, DOI 10.1093/humrep/dew218	2016	41.34	2018	2021	
Azziz R, 2016, NAT REV DIS PRIMERS, V2, P0, DOI 10.1038/nrdp.2016.57	2016	39.54	2018	2021	
Rosenfield RL, 2016, ENDOCR REV, V37, P467, DOI 10.1210/er.2015-1104	2016	40.79	2019	2021	
Teede HJ, 2018, FERTIL STERIL, V110, P364, DOI 10.1016/j.fertnstert.2018.05.004	2018	37.11	2019	2021	
Teede HJ, 2018, HUM REPROD, V33, P1602, DOI 10.1093/humrep/dey256	2018	28.37	2019	2021	
Teede HJ, 2018, CLIN ENDOCRINOL, V89, P251, DOI 10.1111/cen.13795	2018	34.01	2019	2021	
Lizneva D, 2016, FERTIL STERIL, V106, P6, DOI 10.1016/j.fertnstert.2016.05.003	2016	35.11	2019	2021	
Tata B, 2018, NAT MED, V24, P834, DOI 10.1038/s41591-018-0035-5	2018	21.66	2019	2021	
Day F, 2018, PLOS GENET, V14, P0, DOI 10.1371/journal.pgen.1007813	2018	20.13	2019	2021	

Endocrine Society in 2013 (22), which has been cited 878 times. Meanwhile, Teede HJ and group found a high prevalence of IR in PCOS, related to BMI but not visceral fat (23). Alternatively, Teede HJ et al. summarized the guideline recommendations for assessing and managing PCOS (18), providing broader implications in terms of clear advice for clinicians.

The author co-citation relationship refers to the appearance of two authors in the bibliography of a third citing article, revealing the rigidity of the research directions and the impact of the authors (24). As the top-cited author, Prof. Azziz R is an internationally renowned expert in the fields of reproduction and endocrinology, working primarily at the University of Alabama at Birmingham. He was one of the makers of the Rotterdam criteria of PCOS in 2003 and the AES criteria in 2006. Prof. Legro RS, the director of the Department of Obstetrics and Gynecology at Penn State Health, contributions have been mentioned above. Diamanti-Kandarakis E is full professor of the Department of Endocrinology and Diabetes at Hygeia

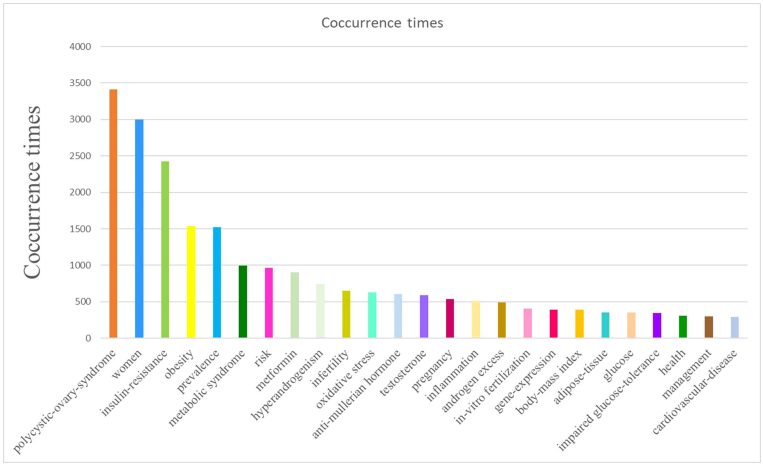

FIGURE 12
The top 25 keywords with the most frequent occurrences.

Hospital, Athens, Greece. In 2009, Diamanti-Kandarakis E and Azziz R et al. collaborated to develop the Androgen Excess-PCOS Society (AE-PCOS) criteria, in which hyperandrogenism was the principal aspect in diagnosis in combination with ovarian dysfunction. Further, more emphasis should be given for the contribution of the relatively young researchers.

A comprehensive analysis of journal indicators substantially provides a conducive reference for researchers to quickly find articles or submit manuscripts (25). The top three citing journals in the field of PCOS were the *Journal of Clinical Endocrinology & Metabolism*, *Fertility and Sterility*, and *Human Reproduction*. *Lancet* and *New England Journal of Medicine* were the two journals with the highest JIF. The two journals were the world's leading medical journals involving all aspects of human health with global coverage in focus. Notably, most publishers were from the UK and the USA except one from the Netherlands. The plausible reason for having no journals from Asian countries might be non-English native language. Nevertheless, China must create some international journals to strengthen its academic impact in the field. Applaudingly, there was immense contribution to the investment in research and development funds from China. Among the top 5 most cited journals, *Fertility and Sterility*, *Human Reproduction*, and *Human Reproduction Update* mainly concerned the field of reproductive medicine. At the same time, *Journal of Clinical Endocrinology & Metabolism* and *Endocrinology* highlighted current topics in endocrinology and metabolism. Notably, the vast amounts of high-quality studies published in these journals have garnered significant attention from researchers working in the area of PCOS.

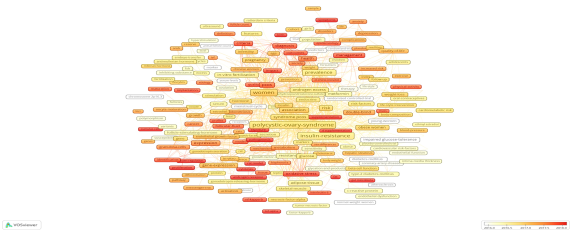

FIGURE 13
Overlay visualization map of keywords with co-occurrence. The color of each note is based on the average year (AAY) of keyword, as shown by the color gradient at the bottom right.

The number of citations of a paper could directly reflect its influence in the field. The highest cited review, entitled "Lack of Exercise Is a Major Cause of Chronic Diseases", published in *Comprehensive Physiology* journal, discussed that lack of exercise was the primary cause of most chronic diseases like PCOS, indicating that physical activity could substantially prevent and treat PCOS. The second highest cited paper entitled "Cellular and molecular mechanisms of metformin: an overview", published in the *Clinical Science*, discussed that metformin could restore ovarian function in PCOS by improving the insulin sensitivity of the ovarian cells and further exploring its underlying molecular mechanisms. The third article, entitled "Diagnosis and Treatment of Polycystic Ovary Syndrome: An Endocrine Society Clinical Practice Guideline", was illustrated in the discussion in "the most prolific authors" section. The review, entitled "Exercise as medicine - evidence for prescribing exercise as therapy in 26 different chronic diseases", stated for the importance of exercise as medicine in the treatment of PCOS. In another article, titled "Insulin Resistance and the Polycystic Ovary Syndrome Revisited: An Update on Mechanisms and Implications", it described the main probable mechanism of insulin resistance in the pathogenesis of PCOS, independent of

obesity, and the contribution of androgens to insulin resistance in PCOS.

As the reference with the most robust citation bursts, Azziz R et al. in 2009 (26) indicated an expert consensus about the clinical diagnostic criteria of PCOS, which should be defined by the presence of hyperandrogenism (clinical and/or biochemical), together with ovarian dysfunction (oligo-anovulation and/or polycystic ovaries), and the exclusion of related disorders. As the second more vigorous burst, March WA et al. was a large retrospective cohort study concerning the prevalence of PCOS under conflicting diagnostic criteria (27). In this study, the authors found that the prevalence of PCOS using the NIH criteria (8.7 ± 2.0%) was down to twice that obtained with the Rotterdam (17.8 ± 2.8%) and AES criteria (12.0 ± 2.4%). These references with citation bursts could reflect the development of PCOS research during the period from 2012 to 2021.

The hotspots of PCOS research before 2016 were predominantly concentrated on the continuous improvement of diagnostic criteria, and the exploration of etiology and pathogenesis. Then, it was shifted to the impact caused on health across the lifespan as a complex disease with multiple complications (28). Consequently, the significant attention of researchers was turned to the prevention of PCOS and the monitoring and management of long-term complications.

Meanwhile, some in-depth findings provided new insights into the pathogenesis of PCOS, in the cases of some new risk loci for PCOS were recognized (29). Notably, several references with continuous bursts in recent years indicated that these topics were of paramount concerns in the PCOS research. Among them, the pathogenesis of the disease was still one of the focused areas. For instance, Dumesic DA et al. (1) analyzed the pathophysiology of PCOS in terms of molecular genetics and proposed the significant contribution of epigenetic studies to the development of PCOS. In another instance, Rosenfield RL et al. (30) demonstrated that typical functional ovarian hyperandrogenism (FOH) typically showed a higher prevalence of PCOS than atypical FOH. The most common provocative factors of typical FOH included obesity and insulin resistance. Tata B and colleagues demonstrated the critical role of prenatal exposure to AMH excess and subsequent aberrant signaling from the GnRH receptor in the neuroendocrine abnormalities of PCOS (31). The large-scale genome-related studies after 2018 significantly contributed to further investigating pathological processes in PCOS research (32). In addition, the assessment and management of PCOS have garnered increasing attention, resulting in the introduction of the corresponding guidelines, including the evaluation for the risk of its complications, lifestyle management, emotional well-being, and weight loss (18, 33).

It can be seen from the analysis of co-occurring keywords that the glycolipid metabolism disorder played a crucial role in the pathogenesis and progression of PCOS. Hence, metformin showed promising effects in the its application for PCOS treatment. Considering these attributes, the prevalence and the health management of PCOS have become the research hotspots with global attention.

In addition, keywords with relatively latest AAY showed indicating the potential research topics in the near future. For instance, microRNAs are often referred to as endogenous, small non-coding RNAs, which are differentially expressed in serum, whole blood, adipose tissues, granulosa cells, follicular fluid, and other tissues of PCOS patients (34). Previous reports indicated that the differentially expressed microRNAs played significant roles in the pathogenesis of various diseases, including insulin signaling processes, inflammation-related pathways, cell proliferation, and apoptosis, among others (16, 35, 36). Consequently, we stringently believe that microRNAs are of great significance in investigating the molecular mechanism of PCOS pathogenesis as novel potential biomarkers.

Limitations

Despite the success in exploring the bibliometric analysis of PCOS research, this study suffers from some limitations. First, the data sources were only downloaded from the WoSCC database, but not other relevant databases, which would undoubtedly miss some related studies. Second, SCIE-based reports were only considered, meaning that the excellent findings written in other languages from the non-English-speaking countries were underestimated, for instance, China, the most productive country. Third, some of the high-quality papers published in recent times might have been missed due to low citation frequency. Therefore, continuous efforts are required to focus on the latest studies from other databases, including the non-English language publications.

Conclusion

In conclusion, this study has summarized the bibliometric analysis focusing on the current status and global trends in the PCOS research over the past decade (2012-2021). To date, the USA is leading the position in the field of PCOS in terms of the total number of publications and total citation frequency, which could be due to excellent funding sources and adequate facilities. Nevertheless, China will undoubtedly make its contribution to the advancement of this field with adequate funding support. Among various institutions, Monash University was the most prolific institution with the highest H-index value. Notably, the contribution of University of Adelaide should be highly acknowledged. Among the world-renowned researchers, Legro RS and Teede HJ were the most active and influential authors in recent years, while Azziz R was the most contributing pioneer in this field. In these journals analyzed, the *Journal of Clinical*

Endocrinology & Metabolism was the most active journal with the highest number of publications and citations in the PCOS research. According to the current analysis, PCOS pathogenesis has become a long-term forefront of research. In recent years, the health management in PCOS prevention and long-term complications have attracted significant attention from researchers. Besides that, the latest research hotspots include "gut microbiota", "microRNAs", "apoptosis", "Myo-inositol", "TNF-alpha", "androgen receptor", and "Vitamin D-deficient", warranting further focus of this research.

Data availability statement

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

Author contributions

NS designed the study, analyzed the data, and wrote the manuscript. H-BM supervised the study 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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1027945/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Global trends in the number of annual publications and citations on PCOS research from 2012 to 2021.

SUPPLEMENTARY FIGURE 2
The visualization of cooperative relationships among countries/regions. Thicker lines represent stronger cooperation.

SUPPLEMENTARY FIGURE 3
Country co-authorship overlay visualization map generated by VOSviewer. The size of each note represents the number of publications. Collaboration between the two countries becomes more close as the line thickens, which is measured by TLS. The color of each note is based on the average appearing year (AAY) of the country, as shown by the gradient at the bottom right.

SUPPLEMENTARY FIGURE 4
The top 15 most prolific institutions.

SUPPLEMENTARY FIGURE 5
The top 15 funding agencies for the support of PCOS research.

SUPPLEMENTARY FIGURE 6
A collaboration analysis of core authors in the several research clusters.

SUPPLEMENTARY FIGURE 7
The co-citation relationship of Azziz R with other authors.

SUPPLEMENTARY FIGURE 8
The top 15 most prolific subject categories with BC value.

SUPPLEMENTARY FIGURE 9
A network visualization map of keywords co-occurrence analysis. The size of the nodes corresponds to the number of occurrences. The distance between two nodes is representative of the relatedness of co-occurrence links.

SUPPLEMENTARY TABLE 1
Top 20 high-yield countries/regions related to PCOS research. *Rank, based on the number of total publications.*

SUPPLEMENTARY TABLE 2
The top 20 highest cited papers on PCOS.

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Vanishing twin syndrome is associated with first-trimester intrauterine hematoma in twin pregnancies after *in vitro* fertilization

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Research question: Is there an association between intrauterine hematoma (IUH), vanishing twin syndrome (VTS), and subsequent complications in twin pregnancies after *in vitro* fertilization (IVF)? What are the risk factors for these complications?

Design: Women who presented with two live gestational sacs following double embryo transfer were included. Patients with systematic diseases, artificial fetal reduction, and incomplete data were excluded. Further stratification of IUH pregnancies was performed according to IUH-related characteristics (i.e., volume, changing pattern, and relationship with fetal cardiac activities). The primary outcome was the incidence of VTS, while adverse outcomes in the surviving singleton and the gestational age of VTS were secondary outcomes.

Results: The incidence of IUH was 13.8%. A total of 1,078 twin pregnancies including 539 IUH pregnancies and 539 non-IUH pregnancies were included. IUH pregnancy was associated with higher risks of VTS (26.9% *vs.* 18.7%, p = 0.001) as well as a higher incidence of preterm birth (p = 0.001, crude OR = 1.98, 95% CI 1.28–3.09, adjusted OR = 1.19, 95% CI 1.09–1.24), threatened abortion (p < 0.001, crude OR = 9.12, 95% CI 2.90–28.69, adjusted OR = 6.63, 95% CI 1.69–14.67), and postpartum hemorrhage (p = 0.024, crude OR = 3.13, 95% CI 1.09–8.99, adjusted OR = 1.16, 95% CI 1.08–1.32) in the surviving singleton. There was no significant difference in risks of other complications. The absence of fetal cardiac activities at the diagnosis of IUH predicted VTS (p < 0.001, crude OR 4.67, 95% CI 3.67–5.78, adjusted OR 3.33, 95% CI 1.56–5.14) and fetal loss at smaller gestational age (7.81 ± 2.10 *vs.* 11.39 ± 5.60 weeks, p < 0.001), while an IUH with an increasing volume did not increase the risk of VTS

but might induce threatened abortion in the surviving fetus ($p < 0.001$, crude OR 1.84, 95% CI 1.32–2.55, adjusted OR 1.72, 95% CI 1.13–2.13).

Conclusions: IUH was a risk factor for VTS in twin pregnancies following double embryo transfer and elevated the risks of threatened abortion, preterm birth, and postpartum hemorrhage in the surviving singleton. The absence of fetal cardiac activities at the diagnosis of IUH elevated the risks of VTS, while an IUH with an increasing volume was associated with threatened abortion without elevating the risks of VTS. An IUH diagnosed before the presence of fetal cardiac activities also resulted in an earlier miscarriage. The study suggests that attention be paid to twin pregnancies with first-trimester IUH to prevent VTS and subsequent adverse perinatal outcomes.

Highlights: First-trimester intrauterine hematoma (IUH) following double embryo transfer is associated with a higher incidence of vanishing twin syndrome (VTS) and elevated subsequent risk of threatened abortion, preterm birth, and postpartum hemorrhage in the surviving singleton. Other perinatal outcomes were not associated with the diagnosis of first-trimester IUH. The absence of fetal cardiac activities at the diagnosis of IUH was of predictive value toward VTS, while an IUH with an increasing size was associated with threatened abortion without elevating the risk of VTS. Incomplete fetal cardiac activities and earlier detection of an IUH might also predict miscarriage at smaller gestational age.

KEYWORDS

intrauterine hematoma, twin pregnancy, vanishing twin syndrome, fetal cardiac activities, threatened abortion

Introduction

Intrauterine hematoma (IUH) is a common gestational complication characterized by a hypoechoic or anechoic crescent-shaped area found through ultrasonic examinations. The incidence rate of IUH varies from 0.46% to 39.5% (1–3), as different definitions of IUH, inclusion criteria, and ultrasound equipment were applied in diverse study populations (4). Symptoms associated with intrauterine hematoma included vaginal bleeding and pelvic pain, while some cases could be independent of the patient's subjective symptoms (2, 5). In previous studies, a positive correlation between the presence of an IUH and an uplifting incidence of singleton miscarriages and other adverse perinatal outcomes or insignificant results were reported. Tuuli et al. suggested that women who experienced first-trimester IUH were at a twofold increased risk of both early and late pregnancy loss (6–8). Other perinatal outcomes including placental abruption, preterm premature rupture of the membrane (PPROM), pre-eclampsia, fetal restriction, and preterm delivery were also shown to be of higher incidence (6, 9, 10). However, discrepant findings were reported in some studies where IUH was not associated with adverse pregnancy outcomes (1, 2, 11). Additional controversies lie in the effect of IUH-related characteristics (i.e., volume, location, gestational age at diagnosis, duration, and the presence of vaginal bleeding), as some of these characteristics were shown to impact pregnancy outcomes, whereas other cohorts found no direct correlations (4, 12). Notably, existing literature was mainly about singleton pregnancies following spontaneous conception, and very little research focused on vanishing twin syndrome (VTS) during twin pregnancies following assisted reproduction, which was the theme of our study.

With the growing prevalence of infertility (13) and extensive application of assisted reproductive technology (ART), the rate of twin pregnancy has been increasing accompanying the use of double embryo transfer, which was being performed as a routine ART method in China (14). In prior studies, several studies reported that the frequency of IUH was 12.1%–22.4% in the *in vitro* fertilization (IVF) group (15), which was significantly higher compared with the frequency of 11%–12.4% in the non-IVF group (7, 16), indicating that assisted conception was an independent risk factor for high IUH rates (16, 17).

Vanishing twin syndrome, defined as the spontaneous miscarriage of one fetus in the uterine, was estimated to occur in 36% of twin pregnancies (18). Specifically, the occurrence of VTS might also elevate the risk of several perinatal complications, putting both the mother and the fetus at risk (19).

As both IVF and twin pregnancy were found to be associated with either higher rates of IUH or the occurrence of VTS, few reports featured the potential effect of IUH on twin pregnancies after *in vitro* fertilization–embryo transfer (IVF-ET) and the role of IUH-related characteristics in the development of VTS and subsequent complications. Therefore, the purpose of this study was to investigate whether IUH played an important role in altering the risks of VTS and follow-up adverse pregnancy outcomes in pregnant women after IVF-ET and to examine the effect of different IUH-related characteristics (i.e., volume, gestational age at diagnosis, and presence of cardiac activity) on pregnancy complications.

Materials and methods

Participants

This was a retrospective observational cohort study conducted in two reproductive centers, the Peking University Third Hospital and Xiamen University Women and Children's Hospital. The study was approved by the Ethics Committee of Peking University Third Hospital (reference number: IRB00006761-M2020572) and the Ethics Committee of Xiamen University Women and Children's Hospital (reference number: KY-2022-015-K01).

We reviewed the clinical records and laboratory records of all patients diagnosed with twin pregnancies at the first ultrasound examination after ART between January 2016 and December 2018. In the two centers, all patients underwent routine examination of serum human chorionic gonadotropin (HCG) at 14 and 21 days after embryo transfer and received trans-vaginal sonogram (TVS) at 5 0/7 to 10 6/7 weeks of gestation to identify early pregnancy, subsequent TVS was routinely performed at 2-week intervals until 10–11 weeks of gestation if the pregnancy continued to progress. Further ultrasound examinations and laboratory tests were performed according to standard obstetrical guidelines after 11–12 weeks of gestation (20). Notably, no patients in our study had extra TVS due to the diagnosis of an IUH or the occurrence of uncomfortable symptoms such as vaginal bleeding.

In our study, an IUH was defined by the crescent-shaped collection of fluid between the chorionic membrane and the myometrium, with the largest diameter being determined as the largest of the three orthogonal measurements obtained from the fluid collection area. Non-IUH was defined as the absence of an IUH during the whole gestation period. Gestational age was calculated based on the time of embryo implantation, while the presence of vanishing twin syndrome was defined as vanished or viable fetal intrauterine demise in one of the twins. Gestational age at miscarriage, however, was determined by ultrasound estimation according to the volume of the demise fetal bud. The diagnosis of intrauterine hematoma, measurement of IUH-related characteristics, and the determination of first-trimester pregnancy complications were completed by experienced ultrasound practitioners.

Our inclusion criteria were the presence of two gestational sacs determined by trans-vaginal ultrasound during 5 0/7 to 10 6/7 weeks of gestation. Exclusion criteria included 1) patients with existing maternal high blood pressure, endocrine, and coagulation disorders as well as those with fetal structural malformations and chromosomal abnormalities; 2) patients with uterine factor infertility; 3) fetal reduction; 4) monoamniotic twin pregnancy; 5) lost to follow-up. Therefore, 539 IUH pregnancies were recruited in this study, and 539 non-IUH pregnancies were included after matching with the IUH group in terms of maternal age (± 5 years), cycle type, and stage of the embryo during implantation (Figure 1).

The IUH group was further classified into subgroups according to the occurring time of an IUH (before or after the presence of fetal cardiac activities) or the changing pattern of the volume of an IUH. The presence of fetal cardiac activities was evaluated *via* an electric database, as all pregnancies were categorized based on whether cardiac activities of the two fetuses were observed at the diagnosis of an IUH. The changing pattern of an IUH was defined by the consecutive ultrasound record, which kept track of the three orthogonal measurements of an IUH and was categorized into those with an increasing volume and those with a decreasing volume. To be exact, we calculated the volume by multiplying three orthogonal measurements of an IUH (measure 1 × measure 2 × measure 3) and multiplying these values by the constant 0.52, as suggested by Campbel (21). Patients who did not obtain at least two ultrasound records were not included in these subgroup analyses, as further classification of IUH pregnancies was performed only in cases with complete data.

Data collection

Demographic features and clinical information including patients' medical history, laboratory findings, and ultrasound records were obtained through electrical medical records. Maternal age, body mass index (BMI), cause of infertility, and gestational history were collected as demographic features, while current assisted reproduction records including cycle type (fresh embryo transfer and frozen embryo transfer), stage of the embryo, method of fertilization (*in vitro* fertilization and intracytoplasmic sperm injection), controlled ovarian stimulation (COS) protocols, and pregnancy results were reviewed. Ultrasound records featuring the condition of fetuses

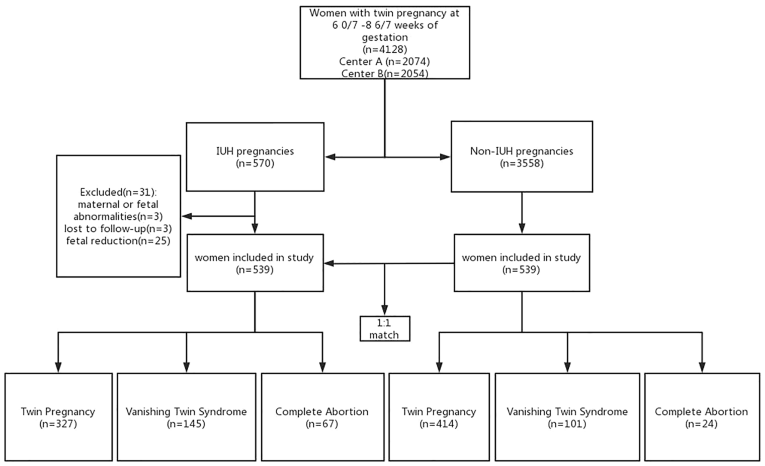

FIGURE 1
Flowchart showing the study selection of women with or without an IUH pregnancy and the incidence of pregnancy outcomes categorized by the number of live births. Center A, Peking University Third Hospital; Center B, Xiamen University Women and Children's Hospital; IUH, intrauterine hematoma.

and IUH-related characteristics were also documented through the electrical database of the hospital.

Outcomes measured

Several outcome measures were investigated in this study: the primary outcomes included the rate of VTS, while the secondary outcomes featured the application of cesarean section, the incidence of pre-eclampsia, fetal growth restriction, placental abruption, PROM, postpartum hemorrhage, and threatened abortion, accompanied with neonatal outcomes including low birth weight (<2,500 g), macrosomia (>4,000 g), and preterm birth (<37 weeks). Data related to all outcome measures mentioned above were acquired through either the hospital's electric database (if the patient delivered in Peking University Third Hospital or Xiamen University Women and Children's Hospital) or telephone interview (if the patient did not deliver in Peking University Third Hospital or Xiamen University Women and Children's Hospital) at 24, 28, and 37 weeks of gestation and 42 days after childbirth.

Statistical analysis

Statistical analysis was performed by using SPSS version 26.0 and R 4.0.4. The continuous variables in accordance with normal distribution were presented with mean ± standard deviations, and an independent t-test was used to compare the results of different studying groups. Those that did not fit the normal distribution were shown as median (25% quartile, 75% quartile) and were compared using the Kruskal–Wallis test. The categorical variables, however, were described as numbers (%) and were compared using the chi-square test. Risk analysis was demonstrated through crude odds ratios (ORs) and 95% confidence intervals (CIs), while adjusted odds ratios were calculated after applying a logistic regression model to adjust for potential cofounders. Correlations between different variables were analyzed using a restricted cubic spline (smooth curve). *p*-Values were considered significant at less than 0.05.

Results

Demographic characteristics, elevated risks of VTS, threatened abortion, and postpartum hemorrhage in IUH compared with non-IUH pregnancies

A total of 4,128 pregnant women were diagnosed as twin pregnancy at 6 0/7 to 8 6/7 weeks of gestation after ART cycles were carried out in Peking University Third Hospital Reproductive Center (2,074 women) and Xiamen University Women and Children's Hospital Reproductive Center (2,054 women) between January 2016 and December 2018. A total of 570 pregnancies were discovered with IUH in the first trimester, and the total incidence rate of IUH was 13.8% (570/4128) in two centers, with 12.1% (250/2074) in Peking University Third Hospital Reproductive Center and 15.6% (320/2055) in Xiamen University Women and Children's Hospital.

After a retrospective review of all records of 570 twin pregnancies with IUH, 539 women were involved based on the inclusion and exclusion criteria (Center A, n = 238; Center B, n =

301). A total of 539 non-IUH twin pregnancies were included after matching with maternal age, cycle type, and stage of the embryo during implantation of IUH twin women at the proportion of 1:1 (Center A, n = 272; Center B, n = 267) (Figure 1).

The demographic characteristics and baseline medical information are shown in Table 1, and no differences were observed in patients from different centers except for maternal BMI (Supplementary Table 1). In addition, IUH and non-IUH patients received their first ultrasound scan at similar gestational weeks without statistical significance (6.93 ± 0.68 *vs.* 6.88 ± 0.58, $p = 0.194$) and had undergone a similar number of ultrasound scans during the first trimester of pregnancy (2.18 ± 0.56 *vs.* 2.15 ± 0.89, $p = 0.508$). Compared with the non-IUH group, patients who were diagnosed with an IUH had higher rates of VTS. In the IUH group, 12.4% (67/539) patients suffered from complete spontaneous abortion, and 26.9% (145/539) patients had experienced vanishing twin syndrome, while the rate in the non-IUH group was 4.5% (24/539) and 18.7%(101/539) respectively. Other demographic features and pregnancy characteristics were statistically insignificant (Table 1).

Comparing other subsequent pregnancy outcomes after VTS between IUH and non-IUH pregnancies

Vanishing twin syndrome was considered to be the most important adverse pregnancy outcome in IUH pregnancies, as the ratio of fetal loss was higher in the IUH group compared with the non-IUH group. To further analyze whether the occurrence of IUH was associated with other adverse pregnancy outcomes in the second or third trimester, we investigated the subsequent perinatal outcomes in patients experiencing first-trimester VTS. Aside from elevated risks of threatened abortion, preterm birth, and postpartum hemorrhage in the surviving singleton, other complications including fetal growth restriction, low birth weight, and placental abruption were not statistically significant between IUH and non-IUH pregnancies (Table 2). In general, our data suggested that the occurrence of IUH is associated with the occurrence of threatened abortion, preterm birth, and postpartum hemorrhage followed by the incidence of VTS, while other adverse outcomes were not elevated by first-trimester IUH.

Correlation between IUH-related characteristics and perinatal outcomes in IUH pregnancies

In order to further investigate how IUH-related characteristics affect VTS and other perinatal outcomes in twin pregnancies, subgroup analyses were performed. First, we tried to analyze whether the occurring time of IUH influenced pregnancy outcomes. All twin pregnancies with an IUH were classified into two groups based on whether IUH was diagnosed before or after the cardiac activity of the fetuses. Among 145 IUH pregnancies with VTS, 142 pregnancies were included while 3 were excluded due to a lack of ultrasound information regarding the discovery of fetal cardiac activities. It was suggested that the presence of an IUH before the discovery of fetal cardiac activities was more likely to result in vanishing twin syndrome (89.5% *vs.* 20.1%, $p < 0.001$, crude OR 4.67, 95% CI 3.67–5.78, adjusted OR 3.33, 95% CI 1.56–5.14) compared with an IUH after fetal cardiac activities. In IUH pregnancies experiencing VTS, a similar analysis was performed to determine the correlation between perinatal outcomes and an IUH diagnosed before or after the establishment of fetal cardiac activities. It turned out that no significant differences were found in other adverse pregnancy outcomes subsequent to VTS in the surviving fetus (Supplementary Table 2).

Moreover, in order to analyze how the changing pattern of an IUH affects pregnancy outcomes, we classified IUH pregnancies into two subgroups according to whether the volume of hematoma was increasing or decreasing. A total of 128 pregnancies including 64 pregnancies with an increasing IUH and 64 with a decreasing IUH were included, while 17 were excluded with only one valid documentation of accurate IUH size. We found that the changing pattern of an IUH was not associated with a significant difference in VTS rates, while IUH pregnancies with an increasing volume of hematoma were prone to experience higher possibilities of several adverse pregnancy outcomes than those with a decreasing volume of hematoma. In the surviving singleton, the incidence of threatened abortion was found to be higher ($p < 0.001$, RR 1.84, 95% CI 1.32–2.55) in the former group where the volume of IUH increased during pregnancy. The related data are shown in Table 3.

The association between gestational age at VTS and IUH-related characteristics

Noticing the higher risks of miscarriage in IUH pregnancies, we applied a non-linear regression model to study the correlation between the gestational age at VTS and IUH-related characteristics including the volume of an IUH, gestational age at IUH diagnosis, presence of fetal cardiac activities at IUH diagnosis, and a changing volume of an IUH. As a result, we found that an IUH diagnosis after the presence of fetal cardiac activities ($p < 0.001$, $R^2 = 0.222$) and at later gestational age ($p < 0.001$, $R^2 = 0.262$) might be associated with later miscarriage, whereas no additional correlations were found between the gestational age at a fetal loss and other IUH characteristics (Figure 2).

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TABLE 1 Demographic characteristics and clinical information in patients with and without intrauterine hematoma.

Characteristics	IUH (n = 539)	Non-IUH (n = 539)	*p*-Value
Maternal age (years)	30.86 ± 4.09	30.56 ± 4.02	0.670
Maternal BMI (kg/m²)	22.92 ± 3.25	22.82 ± 3.78	0.778
Duration of infertility (years)	3.72 ± 2.95	3.87 ± 2.86	0.592
Previous gestations			
0	179 (33.2%)	143 (26.5%)	0.053
1	151 (28.0%)	158 (29.3%)	
≥2	209 (38.8%)	238 (44.2%)	
Previous pregnancies			
0	457 (84.8%)	451 (83.7%)	0.877
1	67 (12.5%)	73 (13.6%)	
≥2	15 (2.7%)	15 (2.7%)	
Previous ART cycles			
0	332 (61.6%)	328 (60.9%)	0.803
≥1	207 (38.4%)	211 (39.1%)	
Etiology of infertility			
Female	176 (32.6%)	176 (32.6%)	0.640
Male	190 (35.3%)	191 (35.4%)	
Both	109 (20.2%)	100 (18.6%)	
Idiopathic	64 (11.9%)	72 (13.4%)	
PCOS			
Yes	70 (13.0%)	56 (10.3%)	0.224
No	469 (87.0%)	473 (89.7%)	
Hydrosalpinx			
Yes	149 (27.6%)	130 (24.1%)	0.186
No	390 (72.4%)	409 (75.9%)	
Endometriosis (confirmed by pathology)			
Yes	14 (2.6%)	11 (2.1%)	0.544
No	525 (97.4%)	528 (97.9%)	
Fertilization method			
ICSI	196 (36.4%)	213 (39.5%)	0.286
IVF	343 (63.6%)	326 (60.5%)	
Cycle type			
Fresh	406 (75.3%)	418 (77.6%)	0.389
Frozen	133 (24.7%)	121 (22.4%)	
Stage of embryo			

(Continued)

TABLE 1 Continued

Characteristics	IUH (n = 539)	Non-IUH (n = 539)	*p*-Value
Cleavage stage	513 (95.2%)	514 (95.3%)	0.886
Blastocyst	26 (4.8%)	25 (4.7%)	
COS protocol			
Antagonist protocol	261 (48.4%)	276 (51.2%)	0.361
Agonist protocol	278 (51.6%)	263 (48.8%)	
Pregnancy outcome			
Complete spontaneous abortion	67 (12.4%)	24 (4.5%)	<0.001***
Vanishing twin syndrome	145 (26.9%)	101 (18.7%)	<0.001***
Surviving twin pregnancy	327 (60.7%)	414 (76.8%)	<0.001***

BMI, body mass index; IUH, intrauterine hematoma; ART, assisted reproductive technology; ICSI, intracytoplasmic sperm injection; IVF, in vitro fertilization; COS, controlled ovarian stimulation; PCOS, polycystic ovary syndrome.
***p < 0.001.

Discussion

This retrospective cohort study indicated that twin pregnancies with IUH after *in vitro* fertilization were primarily associated with higher risks of vanishing twin syndrome. The presence of an IUH was also an essential risk factor for preterm birth, postpartum hemorrhage, and threatened abortion in the surviving singleton during the second or third trimester of pregnancy without affecting the incidence of other adverse perinatal outcomes. Moreover, an IUH diagnosis before the presence of fetal cardiac activities was capable of predicting a higher incidence of VTS and smaller gestational age at miscarriage. An IUH with an increasing size was not associated with a higher incidence of VTS but was at higher risk of threatened abortion.

In this retrospective cohort study, the rate of an IUH in twin pregnancy following ART was approximately 13.8%, which exceeded that of the report by Naqvi et al., where the incidence of IUH in twin pregnancy following both ART and spontaneous conception was 8.9% (22). However, it was lower than the 21.0% incidence rate of IUH in twin pregnancies reported by Ji et al. (21) In the cohort study, we found that IUH in the first trimester was associated with higher risks of vanishing twin syndrome, as well as

TABLE 2 Incidence and risk of pregnancy complications in VTS patients with and without intrauterine hematoma.

	IUH (n = 145)	Non-IUH (n = 101)	*p*-Value	Crude OR	Adjusted OR‡
Preterm birth	57 (39.3%)	20 (19.8%)	0.001**	1.98 (1.28, 3.09)	1.19 (1.09, 1.24)
Cesarean section	91 (62.8%)	58 (57.4%)	0.428	1.09 (0.89, 1.35)	NS
Low birth weight	21 (14.5%)	14 (13.9%)	1.000	1.05 (0.56, 1.96)	NS
Macrosomia	5 (3.4%)	7 (6.9%)	0.240	0.50 (0.16, 1.52)	NS
Pre-eclampsia	5 (3.4%)	3 (3.0%)	1.000	1.16 (0.28, 4.75)	NS
Fetal distress	4 (2.8%)	3 (3.0%)	1.000	0.93 (0.21, 4.06)	NS
Fetal growth restriction	4 (2.8%)	0 (0.0%)	0.146	–	–
Placental abruption	3 (2.1%)	0 (0.0%)	0.271	–	–
PROM	23 (15.9%)	9 (8.9%)	0.126	1.78 (0.86, 3.69)	NS
Postpartum hemorrhage	18 (12.4%)	4 (4.0%)	0.024*	3.13 (1.09, 8.99)	1.16 (1.08, 1.32)
Threatened abortion	39 (26.9%)	3 (3.0%)	<0.001***	9.12 (2.90, 28.69)	6.63 (1.69, 14.67)

IUH, intrauterine hematoma; OR, odds ratio; CI, confidence Intervals; PROM, preterm premature rupture of membranes; NS, not significant.
*p < 0.05; **p < 0.01; ***p < 0.001.
‡Adjusted ORs were obtained after matching for age, fertilization method, cycle type, stage of transferred embryos, and previous medical history.

TABLE 3 Incidence and risks of pregnancy complications in IUH pregnancies with an increasing or decreasing volume of hematoma.

	IUH with an increasing volume (n = 64)	IUH with a decreasing volume (n = 64)	*p*-Value	Crude OR	Adjusted OR‡
Preterm birth[F]	5 (7.8%)	4 (6.3%)	0.743	1.24 (0.36–4.51)	NS
Cesarean section	37 (57.8%)	43 (67.2%)	0.355	0.86 (0.67–1.14)	NS
Low birth weight	10 (15.6%)	8 (12.5%)	0.800	1.25 (0.53–2.96)	NS
Macrosomia	1 (1.6%)	3 (4.7%)	0.619	0.33 (0.04–3.12)	NS
Pre-eclampsia	2 (3.1%)	1 (1.6%)	1	2.00 (0.19–21.51)	NS
Fetal distress	3 (4.7%)	0 (0.0%)	0.244	–	–
Fetal growth restriction	3 (4.7%)	1 (1.6%)	0.619	3.00 (0.32-28.08)	NS
Placental abruption	3 (4.7%)	0 (0.0%)	0.244	–	–
PROM	12 (18.8%)	9 (14.1%)	0.634	1.33 (0.60–2.94)	NS
Postpartum hemorrhage	6 (9.4%)	7 (10.9%)	1	0.86 (0.31–2.41)	NS
Threatened abortion	47 (74.6%)	26 (40.6%)	<0.001***	1.84 (1.32–2.55)	1.72 (1.13–2.13)
Gestational anemia	13 (20.3%)	11 (17.2%)	0.821	1.18 (0.57–2.44)	

IUH, intrauterine hematoma; OR, odds ratio; CI, confidence interval; PROM, premature rupture of membranes; NS, not significant.
[F]Analysis performed only in cases with complete data.
‡Adjusted ORs were obtained after matching for age, fertilization method, cycle type, and stage of transferred embryos.
***p < 0.001.

preterm birth, threatened abortion, and postpartum hemorrhage in the surviving singleton during the second or third trimester. In singleton pregnancy, most of the research suggested that the presence of IUH in the first and second trimesters was associated with miscarriage in singleton pregnancies (6–8). Some existing literature showed that increased risks of fetal loss, pre-eclampsia, placental abruption, and preterm premature rupture of membranes were observed in IUH singleton pregnancies (3, 10, 23).

However, in twin pregnancies after IVF/intracytoplasmic sperm injection (IVF/ICSI), very few studies featured the relevance of IUH in early pregnancy and adverse pregnancy outcomes. Ji suggested that the presence of IUH was associated with the loss of one or both of the fetuses before 20 weeks of gestation, whereas the conception method, IUH size, and previous miscarriage were not independently associated with such fetal loss (21). Different from our study where all twin pregnancies were achieved through IVF/ICSI, Ji's study population was half conceived naturally and half by an assisted reproductive technique including IVF/ICSI, intrauterine insemination (IUI), and ovulation induction treatment. Another study reported that the finding of a first-trimester subchorionic hematoma (SCH) and the size of the SCH were not associated with adverse pregnancy outcomes in women with twin pregnancies after 24 weeks of gestation. Nonetheless, if the SCH is associated with vaginal bleeding, there is an increased risk of preterm birth. Compared with our study, Mariam's research did not address the pregnancy outcomes before 24 weeks of gestation, and patients with pregnancy loss prior to 24 weeks of gestation were excluded from the study (22).

In twin pregnancies following the ART method, which comprised approximately 30% of newborns in China, the incidence of fetal loss was reported to be higher than that of singleton pregnancies (24). In previous reports, Zhu et al. suggested a rate of 9.5% vanishing twin in IVF/ICSI twin pregnancy (19) in our center and that VTS was an independent factor for a higher risk of low birth weight (LBW), preterm birth (PTB), small for gestational age (SGA), and perinatal mortality in the surviving singleton. Similarly, more evidence indicated that VTS resulted in poor pregnancy outcomes for the surviving singleton when compared with initial singleton pregnancy in both ART and spontaneous conception (18, 25–27). Additionally, Marton et al. revealed that vanishing twin pregnancies had a lower prevalence and a worse perinatal outcome after IVF-ICSI as compared with those of their spontaneously conceived counterparts (27), and Nigel et al. indicated that the pregnancy outcomes of the surviving singletons that experienced VTS was similar whether by cleavage-stage or blastocyst-stage embryo transfers during fresh IVF cycles (25).

Prior studies did not differentiate the diagnosis of an IUH in the presence or absence of fetal cardiac activities, while in our study, evidence had shown that the absence of fetal cardiac activities yielded predictive value for higher risks of VTS and miscarriage at smaller gestational age since the presence cardiac activities was

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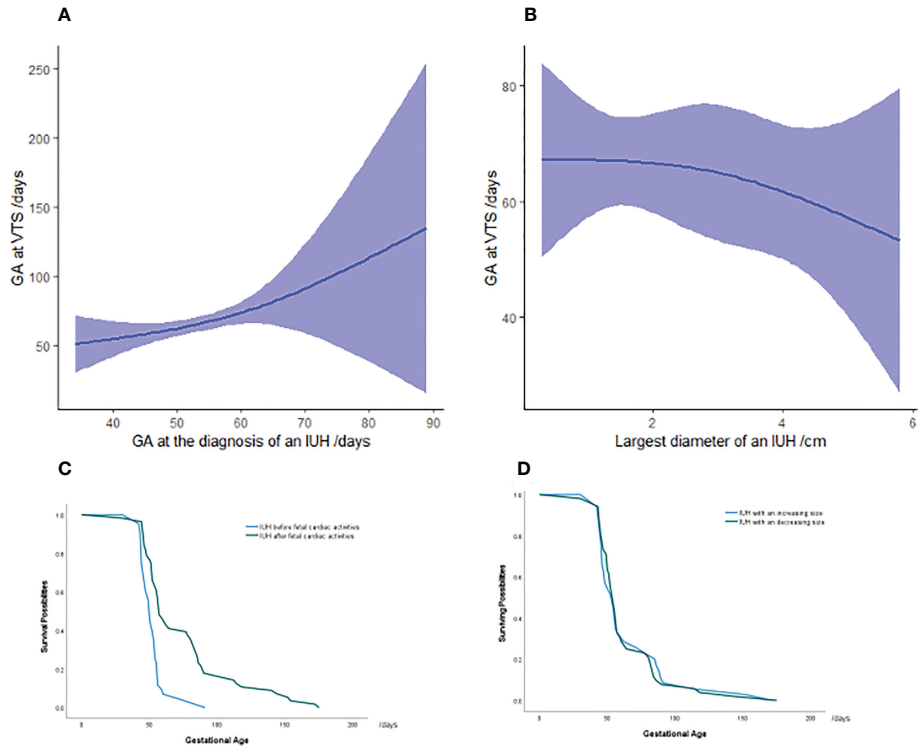

FIGURE 2
Association between gestational age at miscarriage and IUH characteristics in vanishing twin syndrome patients **(A, B)** Survival analysis in pregnancies with an IUH diagnosis at the presence or absence of fetal cardiac activities **(C)** and an increasing or decreasing volume of hematoma **(D)** in VTS patients. The green and blue lines represent the surviving possibilities of fetuses in vanishing twin syndrome as gestational age progressed. The majority of VTS occurred between 50 and 100 days of gestation, and the y-value represents the percentage of remaining vanishing twins who will be miscarried later. Each color symbolizes IUH patients with the hematoma diagnosed before or after fetal cardiac activity and the either increasing or decreasing volume of an IUH. GA, gestational age; IUH, intrauterine hematoma.

found to be a symbol of the viability of a fetus in previous reports (28). However, the trend for increased risks of threatened abortion and earlier fetal loss was observed in patients with an increasing volume of IUH. This positive correlation had not been reported in prior studies, as most literature mainly focused on the single detection of an IUH through ultrasound.

The underlying mechanisms for the elevated risks of VTS in IUH pregnancies included secondary mechanical trauma caused by the presence of an IUH, shallow trophoblast invasion, impaired angiogenesis, and resultant friable vessels (29). An IUH might also affect the receptivity of endometrium, which therefore interfered with proper blastocyst–endometrial communication (30) and eventually caused either threatened abortion or VTS. Another explanation might be the fact that the hematoma was caused by the tearing of marginal veins in the placenta (29), which subsequently led to premature perfusion of the intervillous space, causing insufficient development and adaptation of the placenta to cope with oxidative stress and imbalance of free radicals. Such oxidative disorders were followed by the degeneration of syncytiotrophoblast and eventually resulted in VTS, which was the primary yet most essential outcome measure of our study (31). Preterm birth threatened abortion and postpartum hemorrhage could possibly be elevated subsequently, as Zhu et al. reported a trend of more frequent yet severe complications in the surviving singleton following the occurrence of vanishing twin syndrome (19). The presence of threatened abortion in early pregnancy might also bring higher risks of other adverse outcomes such as fetal growth restrictions, pre-eclampsia, PROM, and placental abruption due to the common pathway of underlying placental dysfunction (32).

With regard to the role of fetal cardiac activities, we hypothesized that embryos with cardiac developing activities in a delayed fashion or early formation of an IUH were prone to fail, as multiple organ functions that helped to safeguard the survival of the fetus were less complete in the early stage of embryonic development and were more vulnerable to oxidative stress and the function of mother–fetus interface (33, 34). The enlarging volume of an IUH, however, might associate with higher insufficiency in angiogenesis, a larger scale of endometrium receptivity defects, and heavier mechanical stress of the hematoma (12). The continuing rupture of blood vessels, ongoing detachment of chorionic membrane from the decidua, and the expansion of damage to the definitive villous tissue (30), which

promoted the dilation of an IUH, accounted for more severe placental defect and eventually led to VTS and threatened abortion.

In terms of the gestational age at the diagnosis of an IUH and the gestational age at miscarriage, no consensus had been reached in previous studies. Xiang et al. suggested in their 2014 review that most studies featuring the clinical significance of IUH failed to reveal the impact of its concrete characteristics such as size and gestational age at diagnosis; others' conclusions were limited to the broader application due to the sample size or uncontrolled design (3). In the existing literature, some studies illustrated that an IUH identified in early pregnancy was more likely to result in spontaneous abortion (35), whereas others claimed only those occurring after a certain timeline at gestation yielded deleterious results (36). However, no clear mechanism explaining the timing threshold of the genesis of an IUH and its impact on early miscarriage had yet been reported. We suggested that this correlation might be explained by the idea that earlier development of an IUH probably deteriorated the early development of the placenta or the formation of endometrium receptivity, which further led to the miscarriage of fetuses. Additionally, fetal viability was not formed yet in smaller gestational age and was more vulnerable to the insufficiency of the placenta and the endometrium, resulting in early miscarriage (15, 29, 37).

On the basis of our results, this study provided new knowledge regarding both antenatal and neonatal outcomes of IUH pregnancies following double embryo transfer in assisted conception. Compared with prior studies, our study had the following advantages: a) a large sample volume was applied. b) We analyzed the adverse perinatal outcomes in surviving singleton pregnancy following VTS, establishing the timeline between the occurrence of VTS and pregnancy complications in the surviving fetus during the second or third trimester of pregnancy. c) The cycle types, stage of the embryo, and maternal age were controlled within the electric database, as the role of different cycle types in the onset of IUH had been debated in prior studies. It was reported in some studies that frozen–thawed embryo transfer and blastocyst transfer were risk factors for IUH (24), while Zhou reported that fresh embryo transfer may contribute to IUH onset in IVF/ICSI patients (13). d) Further classifications by the presence or absence of fetal cardiac activities and the changing pattern in the volume of an IUH were performed and analyzed separately in terms of pregnancy outcomes. e) We visualized the correlation between gestational age at miscarriage and different IUH characteristics using a non-linear regression model (smooth curve), as well as the survival possibilities in different groups categorized by those IUH characteristics.

The limitation of this study was the retrospective design. Ultrasound scans during the second or third trimester were not routinely undertaken by ART practitioners, so IUH characteristics subsequent to the first trimester were lacking, making it unable to obtain detailed changing patterns of the volume of an IUH and specific IUH characteristics at the time of delivery (38). Therefore, the major limitation of our study was that IUH characteristics of the second and third trimesters have not been analyzed. Meanwhile, information regarding pharmaceuticals being used during the whole gestational period was not addressed, and the effect of the drug on IUH or adverse pregnancy outcomes might be neglected, as it had been reported that the use of low-dose aspirin might be associated with an increased occurrence and persistence of IUH during the first trimester, regardless of fertility diagnosis or method of fertility treatment (39). In addition, a large number of IUH patients who had undergone artificial fetal reduction were not included in this study. Further prospective studies consisting of more accurate and complete data will be necessary.

To sum up, we speculated that IUH was associated with vanishing twin syndrome and adverse pregnancy complications in the surviving singleton, while the identification of fetal cardiac activities and the measurement of the volume of an IUH presented early warning signs for adverse pregnancy outcomes in both the mother and the fetuses. These findings demonstrated that special attention should be given to twin pregnancies with an IUH in the first trimester to prevent or attend to adverse pregnancy outcomes including VTS, postpartum hemorrhage, and threatened abortion.

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 study was reviewed and approved by the Ethics Committee of Peking University Third Hospital (Reference number: IRB00006761-M2020572) and the Ethics Committee of Xiamen University Women and Children's Hospital (Reference number: KY-2022-015-K01).

Author contributions

Study design and concept: YG, CM, and JZ. Data collection and analysis: YG, SL, JS, and XL. Interpretation of data and critical revision of the manuscript: YG, CM, and JZ. 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.

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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.2022.1062303/full#supplementary-material

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Comparison of two different starting dose of rhFSH in GnRH antagonist protocol for patients with normal ovarian reserve

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Objective: To evaluate different starting doses of recombinant human follicle-stimulating hormone (rhFSH) on pregnancy outcomes for patients with normal ovarian reserve during gonadotropin- releasing hormone antagonist (GnRH-ant) protocol-controlled ovarian stimulation of *in vitro* fertilization (IVF) cycles.

Methods: In this retrospective study, a total of 1138 patients undergoing IVF cycles following the GnRH-ant protocol were enrolled. Patients were divided into two groups according to the starting dose of rhFSH. 617 patients received a starting dose of rhFSH of 150 IU, and 521 patients received a starting dose of rhFSH of 225 IU. We compared demographic characteristics, ovarian stimulation and embryological characteristics, and pregnancy and birth outcomes between the two groups. Multivariate logistic regression analysis was performed to examine the possible effects of the known potential confounding factors on pregnancy outcomes.

Results: The number of oocytes retrieved in the 150 IU rhFSH group was significantly lower than those in the 225 IU rhFSH group. There was no significant difference between the two groups referring to embryological characteristics. The proportion of fresh embryo transfer in the 150 IU rhFSH group was significantly higher than that in the 225 IU rhFSH group (48.30% vs. 40.90%), and there was no difference in the risk of ovarian hyperstimulation syndrome and pregnancy outcomes between the two groups.

Conclusions: In conclusion, the starting dose of rhFSH of 150 IU for ovarian stimulation has a similar pregnancy outcome as starting dose of rhFSH of 225 IU in GnRH-ant protocol for patients with normal ovarian reserve. Considering the potential cost-effectiveness and shorter time to live birth, the starting dose of rhFSH of 150 IU may be more suitable than 225 IU.

KEYWORDS

GnRH antagonist protocol, starting dose of rhFSH, live birth rate, normal ovarian reserve, *in vitro* fertilization

1 Introduction

Gonadotropin-releasing hormone agonist (GnRH-a) protocol and gonadotropin-releasing hormone antagonist (GnRH-ant) protocol have been widely applied in controlled ovarian stimulation (COS) for *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI) cycles; they are comparable in clinical outcomes, obstetric and perinatal outcomes (1). GnRH-ant protocol, which was discovered in the 1990s, is increasingly favored in clinical practice because of its physiological advantages (2). Compared with the GnRH-a protocol, the GnRH-ant protocol competitively binds to the receptor of the pituitary gland and causes rapid suppression of gonadotropin release without "flare-up" effect. Meanwhile, the GnRH-ant protocol can effectively reduce the consumption of gonadotropin and greatly shorten the treatment time, and reduce the risk of ovarian hyperstimulation syndrome (OHSS) (3). Recombinant human follicle-stimulating hormone (rhFSH) is the key hormone that stimulates the development of multiple follicles during COS to obtain an adequate number of oocytes and embryos (4). Individualization of the starting dose of rhFSH is considered standard clinical practice. The optimum dose of rhFSH required to exceed the "FSH threshold" during the therapeutic "FSH window", which is an inconclusive medication management, ranges from 100 IU to 300 IU (5). Starting dose of rhFSH selection mainly depends on the patient's characteristics and ovarian reserve, which is affected by various biomarkers, including basal follicle-stimulating hormone (FSH), antral follicle count (AFC), and anti-Müllerian hormone (AMH) (6, 7). Therefore, a starting dose of rhFSH selection is often dependent on the clinical experience of specialists.

Classification of patients according to their ovarian reserve is the basis for selecting an appropriate starting dose of rhFSH. In 2022, the Chinese Medical Doctor Association (CMDA) promulgated the "Expert Consensus on Standardized Application of gonadotropin-releasing hormone antagonist in Assisted Reproductive Technology" (8), which divides the population into normal ovarian reserve (NOR), high ovarian reserve (HOR), diminished ovarian reserve (DOR), according to ovarian reserve. Patients meeting the following criteria were defined as NOR in the consensus, including age < 35 years; basal FSH level < 10 IU/L; AMH level 1.1-4.0 ng/L; and AFC 7-15. For NOR patients, the main treatment goals are to reduce the time to ovulation induction, shorten the time to live birth and increase the pregnancy rate of fresh embryo transfer. The recommended starting dose of rhFSH in the consensus is 150-225 IU for the NOR patients. Generally, the number of oocytes retrieved depends on the dose of rhFSH (9). However, individual women's responses vary (10). GnRH-ant protocol was only used in China in 2013, and clinical experience is relatively lacking. The starting dose of rhFSH of 150 or 225 IU is a broad dose range, and it is unclear whether there is a difference in pregnancy outcomes between the two starting doses of rhFSH. This study aimed to investigate whether IVF and pregnancy outcomes would change in the NOR patients between the two starting doses of rhFSH.

2 Material and methods

2.1 Patients

Individuals who completed an autologous IVF/ICSI cycle and received a GnRH-ant protocol treated at the authors' reproductive clinic from January 2014 to June 2021 were included in this single-center retrospective cohort study. The study was authorized by the local institutional review board (Reproductive Ethics Committee of The Affiliated Hospital of Shandong University of Traditional Chinese Medicine, approval no. SDTCM/E2204-02, dated April 2, 2022) and was undertaken at a public tertiary referral university hospital.

The inclusion criteria were as follows: (a) age < 35 years; (b) FSH level < 10 IU/L; (c) AMH level 1.1-4.0 ng/L; (d) basal AFC 7-15.

The exclusion criteria were as follows: (a) patients with polycystic ovary syndrome; (b) patients with ovarian insufficiency; (c) body mass index (BMI) > 28; (d) patients with an abnormal uterine cavity that affected embryo implantation; (e) patients with recurrent implantation failure; (f) patients who required a genetic diagnosis before embryo implantation.

A total of 1138 patients were included in this study. Patients were divided into two groups according to the starting dose of rhFSH. 617 patients received a starting dose of 150 IU rhFSH, and 521 patients received a starting dose of 225 IU rhFSH (Figure 1).

2.2 Controlled ovarian stimulation protocols

All participants underwent IVF/ICSI treatment using a GnRH-ant protocol. Recombinant human follicle stimulating hormone (Gonal-F, Merck Serono, Switzerland) is administered on the second or third day of the menstrual cycle at a dose of 100-300 IU per day, depending on the woman's age, FSH, AFC, and AMH.

During COS, participants were monitored for follicular recruitment and growth and endometrial thickness by serial transvaginal ultrasound and blood hormone tests, including estradiol (E2), progesterone (P4) and luteinizing hormone (LH)

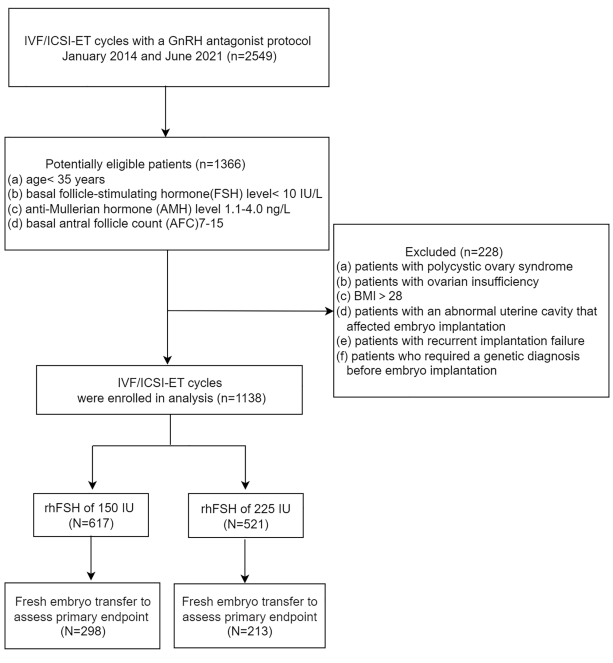

FIGURE 1
Flowchart of this study.

plasma levels. The dose of rhFSH may be increased or decreased according to the follicular development of the patient during COS, within the range of 50 IU. Cetrorelix (Merck Serono, Switzerland) of 0.25 mg/day was initiated until the trigger day, when the dominant follicle diameter was ≥14 mm, E2 ≥400 pg/ml. Human chorionic gonadotropin (hCG, Lizhu, Zhuhai, China) or GnRH agonist (triptorelin acetate; France) combined hCG (dual trigger) was administered to trigger the maturation of oocytes when there were three follicles measuring 18 mm or more in diameter.

Oocyte pick-up (OPU) was performed by transvaginal ultrasound-guided needle aspiration 35-36 hours following triggering, followed by standard IVF/ICSI as previously reported (11). Fresh embryo transfer was carried out 3 days (cleavage embryo) or 5 days (blastocyst) after OPU. Whole embryos were frozen if patients with a high risk of OHSS, high progesterone level (progesterone ≥1.5-2 ng/ml), severe hydrosalpinx, or endometrial polyp. Embryo grading was done following the proceedings of the Istanbul consensus (12). High-quality embryos were characterized as those that reached at least the six-cell stage with<20% fragmentation. Oral progesterone combined vaginal progesterone or intramuscular progesterone was used for luteal support since the day of OPU for fresh transfer cycles.

2.3 Outcome measures

We compared IVF and pregnancy outcomes at a different starting dose of rhFSH. Our primary outcome measure was live birth, which we defined as the delivery of at least one infant with breathing and heartbeat, regardless of gestational age. The secondary outcomes included positive pregnancy rate, biochemical pregnancy rate, clinical pregnancy rate, ectopic pregnancy rate, ongoing pregnancy rate, miscarriage rate, live birth rate and IVF outcomes. Positive pregnancy is defined as a serum-hCG level of at least 10 mIU/mL. Biochemical pregnancy loss is described as undetected pregnancy losses that are recorded only *via* a positive pregnancy test (serum hCG level 10 mIU/mL). After 10 gestational weeks, clinical pregnancy is defined as an intrauterine gestational sac with fetal heartbeat identified through transvaginal ultrasonography. Ectopic pregnancy is described as a pregnancy that occurs outside the uterine cavity. We thereby define ongoing pregnancy as a viable intrauterine pregnancy of at least 12 weeks, confirmed on an ultrasound scan (13). Miscarriage refers to the termination of pregnancy before 28 weeks of gestation and a fetus weighing less than 1000 g (14). OHSS was diagnosed according to the latest classification criteria (15).

2.4 Statistical analysis

All statistical analyses were performed with the SPSS 25.0 statistical software (IBM, Chicago, IL, USA). The K-S test was used for the normality test. Continuous variables are expressed as mean ± SD or median (IQR), and Categorical variables are expressed as number (n) and percentage (%). Mann-Whitney U test or Student's t-tests were used for continuous variables, and the Chi-square test was used for categorical variables. Various factors affecting clinical outcomes were identified by univariate logistic regression analysis. Multivariate logistic regression analysis was performed to examine the possible effects of the following known potential confounding factors on pregnancy outcomes, including age, BMI, AMH, infertility type (primary or secondary), starting dose of rhFSH, ovulation trigger protocol, the total dose of Cetrorelix. A P-value < 0.05 was considered statistically significant.

3 Result

3.1 Baseline characteristics of the study population

A total of 1138 ovarian stimulation cycles with GnRH-ant protocol were included in this study, including 617 cycles a starting dose of rhFSH of 150 IU and 521 cycles with 225 IU. Patients' baseline characteristics are detailed in Table 1. There were no statistically significant differences in the Mean age, duration of infertility, basal FSH, LH, E2, P4, AMH level, gravidity, parity, miscarriage, BMI, AFC, etiology of infertility, types of infertility between the two groups (all P > 0.05). The method of fertilization was similar between the two groups (P > 0.05).

3.2 Ovarian stimulation outcomes

The characteristics of ovarian stimulation are presented in Table 2. The total dose of rhFSH in the 150 IU group was significantly lower than that in the 225 IU group (1517.63 ± 283.65 vs. 2218.62 ± 402.61, p<0.01) (Figure 2), and there was no difference in the stimulation duration of rhFSH administration. There was no difference in the total dose and duration of Cetrorelix between the two groups. The lag time from ovulation trigger to oocyte aspiration and ovulation trigger protocol, including hCG or GnRH agonist combined hCG, was not significantly different between the two groups. On trigger day, the 225 IU group had significantly higher levels of estradiol (2790.51 ± 1329.37 vs. 3139.98 ± 1403.4, p<0.01) and progesterone (1.11 ± 0.57 vs. 1.2 ± 0.6, p<0.01) than the 150 IU group (Figure 2). There was no severe OHSS in both groups, and mild to moderate OHSS was not statistically significant (3.70% vs 5.40%, p=0.181).

3.3 Embryological outcomes

The characteristics of the embryological are presented in Table 3. The number of oocytes retrieved (10.19 ± 3.6 vs. 11.16 ± 3.67, p<0.01), maturation oocytes (8.63 ± 3.91 vs. 9.6 ± 3.97, p<0.01), and two-pronuclear (2PN) fertilization (6.45 ± 3.52 vs. 7.16 ± 3.7, p<0.01) in the 150 IU group were significantly lower than those in the 225 IU group (Figure 3). There were no significant differences in the number of available embryos, number of high-quality embryos, proportion of high-quality embryos, number of blastocysts, and proportion of

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TABLE 1 Comparison of general data between the two groups.

Groups	150 IU (n = 617)	225 IU (n = 521)	P-value
Age (years)	30.66 ± 3.07	30.75 ± 3.13	0.63
Infertility duration(years)	3.27 ± 2.1	3.23 ± 2.13	0.75
Basal FSH level (IU/L)	6.85 ± 1.48	6.69 ± 1.45	0.06
Basal LH level (IU/L)	5.34 ± 2.69	5.25 ± 2.57	0.59
Basal E2 level (pg/ml)	35.28 ± 9.44	35.01 ± 9.18	0.63
Basal P4 level (ng/ml)	0.91 ± 1.14	0.95 ± 1.31	0.61
AMH (ng/ml)	2.58 ± 0.85	2.61 ± 0.79	0.59
Gravidity (n)	0 (0,1)	0 (0,1)	0.89
Parity (n)	0 (0,0)	0 (0,0)	0.76
Miscarriage (n)	0 (0,0)	0 (0,0)	0.89
BMI (kg/m^2)	23.32 ± 2.63	23.6 ± 2.54	0.07
Antral follicle count	11.18 ± 2.56	11.09 ± 2.51	0.53
Etiology of infertility			0.552
Tubal factor (%)	456/617 (73.90%)	370/521 (71.00%)	
Male factor (%)	121/617 (19.60%)	114/521 (21.90%)	
Others (%)	40/617 (6.50%)	37/521 (7.10%)	
Types of infertility			0.503
primary infertility	311/617 (50.40%)	273/521 (52.40%)	
secondary infertility	306/617 (49.60%)	248/521 (47.60%)	
Method of fertilization			0.346
ICSI	121/617 (19.60%)	114/521 (21.90%)	
IVF	496/617 (80.40%)	407/521 (78.10%)	

FSH, follicle stimulating hormone; LH, luteinizing hormone; E2, estradiol; P4, progesterone; AMH, anti-Müllerian hormone; BMI, body mass index; ICSI, intracytoplasmic single sperm injection; IVF, in vitro fertilization; Data are presented as mean ± SD, median (IQR) and n (%).

TABLE 2 Ovarian stimulation outcomes between the two groups.

Groups	150 IU (n = 617)	225 IU (n = 521)	P-value
Stimulation duration of rhFSH (day)	9.71 ± 1.63	9.85 ± 1.75	0.19
Total rhFSH (IU)	1517.63 ± 283.65	2218.62 ± 402.61	**p<0.01**
Duration of Cetrorelix (day)	5.81 ± 1.52	5.93 ± 1.62	0.18
Total Cetrorelix (mg)	1.45 ± 0.38	1.48 ± 0.41	0.18
LH level on trigger day (IU/L)	2.72 ± 1.96	2.88 ± 2.25	0.2
E2 level on trigger day (pg/ml)	2790.51 ± 1329.37	3139.98 ± 1403.4	**p<0.01**
P4 level on trigger day (ng/ml)	1.11 ± 0.57	1.2 ± 0.6	**p<0.01**
Ovulation trigger protocol			0.744
dual trigger	140/617 (22.70%)	114/521 (21.90%)	
Triggered with hCG	477/617 (77.30%)	407/521 (78.10%)	
Lag time from ovulation trigger to oocyte aspiration	36.11 ± 0.53	36.11 ± 0.55	0.899
OHSS	23/617 (3.70%)	28/521 (5.40%)	0.181

rhFSH, recombinant human follicle-stimulating hormone; LH, luteinizing hormone; E2, estradiol; P4, progesterone; OHSS, ovarian hyperstimulation syndrome. Data are presented as mean ± SD, median (IQR) and n (%).
The bold font indicates that the p value is less than 0.05, which means it has statistical significance.

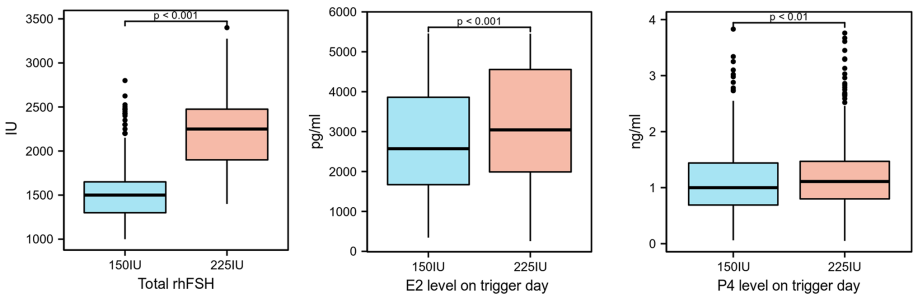

FIGURE 2
Statistically significant indicators of ovarian stimulation outcomes.

blastocysts between the two groups. For ovarian stimulation cycle outcomes, the proportion of fresh embryo transfer in the 150 IU group was significantly higher than that in the 225 IU group (48.30% vs. 40.90%, p=0.035) (Figure 3).

3.4 Pregnancy and birth outcomes

As demonstrated in Table 4, between-group comparisons in both 150 IU and 225 IU groups revealed insignificant differences in positive pregnancy per embryo transfer, biochemical pregnancy loss per positive pregnancy, clinical pregnancy per embryo transfer, ectopic pregnancy per positive pregnancy, ongoing pregnancy per embryo transfer, miscarriage per clinical pregnancy, live birth per embryo transfer, number of live births (all P > 0.05).

A binary logistic regression model was also used to assess the association between starting dose of rhFSH and pregnancy and birth outcomes while adjusting for potential confounders (Table 5). Furthermore, in the crude and adjusted models, the 150 IU group was comparable to the 225 IU group in terms of positive pregnancy, clinical pregnancy, ongoing pregnancy, and live birth (all P > 0.05).

TABLE 3 Embryological outcomes between the two groups.

Groups	150 IU (n = 617)	225 IU (n = 521)	P-value
Number of oocytes retrieved	10.19 ± 3.6	11.16 ± 3.67	**p<0.01**
Maturation oocytes	8.63 ± 3.91	9.6 ± 3.97	**p<0.01**
2PN Fertilization	6.45 ± 3.52	7.16 ± 3.7	**p<0.01**
Number of available embryos	3.95 ± 2.61	4.23 ± 2.72	0.08
Number of high-quality embryos	1.34 ± 1.81	1.35 ± 1.68	0.96
Proportion of high-quality embryos	829/2440 (34.00%)	703/2204 (35.60%)	0.13
Number of blastocysts	1.32 ± 2.41	1.51 ± 2.83	0.23
Proportion of blastocysts	813/2440 (33.30%)	785/2204 (31.90%)	0.10
Ovarian stimulation cycle outcomes			**0.035**
Freeze-all strategy	283/617 (45.90%)	278/521 (53.40%)	
No embryos to transfer	36/617 (5.80%)	30/521 (5.80%)	
Fresh embryo transfer	298/617 (48.30%)	213/521(40.90%)	
Endometrial thickness prior to embryo transfer.	10.84 ± 2.02	10.68 ± 2.02	0.19
Number of embryos transferred			0.218
Single	54/298 (18.10%)	48/213 (22.50%)	
Double	244/298 (81.90%)	165/213 (77.50%)	
Quality of transferred embryos			0.421
Available embryos	131/298 (44.00%)	96/213 (45.10%)	
A high-quality embryo	141/298 (47.30%)	105/213 (49.30%)	
Two high quality embryos	26/298 (8.70%)	12/213 (5.60%)	
Blastocyst transfer	22/298 (7.40%)	24/213 (11.30%)	0.13

2PN, two-pronuclear. Data are presented as mean ± SD, median (IQR) and n (%).
The bold font indicates that the p value is less than 0.05, which means it has statistical significance.

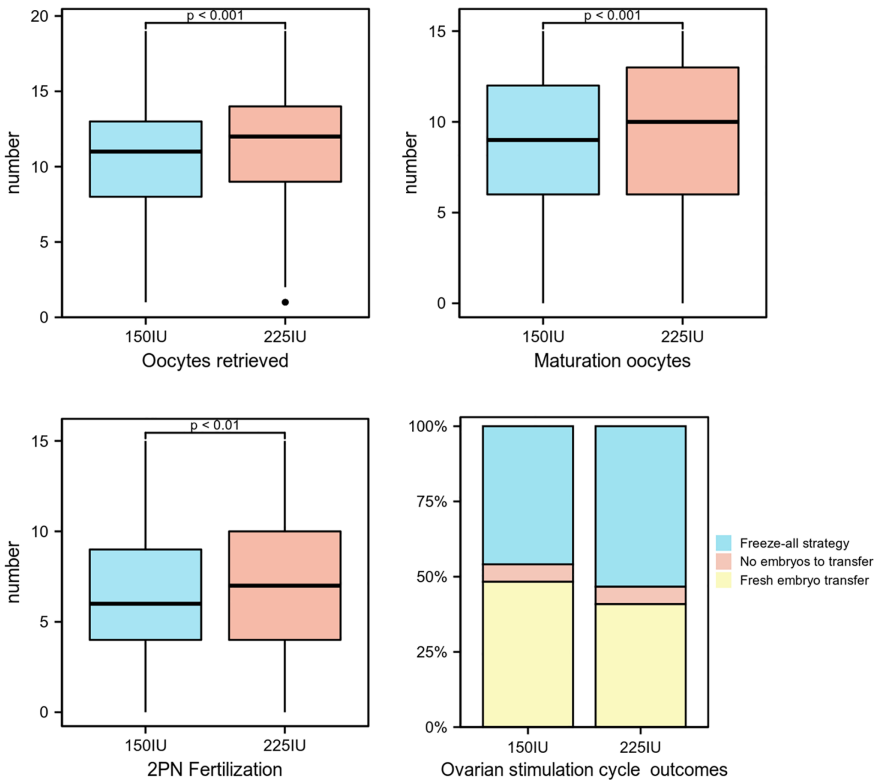

FIGURE 3
Statistically significant indicators of embryological outcomes.

4 Discussion

So far, few studies have evaluated different starting dose of rhFSH on pregnancy outcomes for NOR patients in GnRH antagonist protocol. In this study, we qualified the NOR population in terms of age, FSH, AMH, and AFC following the expert consensus issued by CMDA and compared the IVF and pregnancy outcomes of the recommended starting dose of rhFSH in GnRH antagonist protocol in the consensus.

Compared to previous research, our practice can provide evidence-based guidance to select a starting dose of either 150 IU or 225 IU rhFSH for IVF based on *post-hoc* randomization and a large sample for the NOR population in GnRH antagonist protocol. Data from this study suggest that in NOR patients, a starting dose of rhFSH of 225 IU has no advantage over 150 IU in terms of pregnancy and live birth outcomes.

The dose-dependent increase between the number of retrieved oocytes and rhFSH has been confirmed. For the number of oocytes retrieved, the 225 IU group was significantly more than the 150 IU group (11.16 ± 3.67 vs. 10.19 ± 3.6, p<0.01), which is consistent with the results of a published prospective randomized controlled trial. In a randomized, double-blind, multicenter clinical trial comparing

TABLE 4 Pregnancy and birth outcomes between the two groups.

Groups	150 IU (n = 298)	225 IU (n = 213)	P-value
Positive pregnancy per embryo transfer	153/298 (51.30%)	106/213 (49.80%)	0.725
Biochemical pregnancy loss per positive pregnancy	10/153 (6.50%)	9/106 (8.50%)	0.553
Clinical pregnancy per embryo transfer	143/298 (48.00%)	97/213 (45.50%)	0.585
Ectopic pregnancy per positive pregnancy	11/153 (7.20%)	6/106 (5.70%)	0.625
Ongoing pregnancy per embryo transfer	132/298 (44.30%)	91/213 (42.70%)	0.724
Miscarriage per clinical pregnancy	21/143 (14.70%)	13/97 (13.40%)	0.780
Live birth per embryo transfer	122/298 (40.90%)	84/213 (39.40%)	0.733
Number of live births			0.794
singletons	91/122 (74.60%)	64/84 (76.20%)	
twins	31/122 (25.40%)	20/84 (23.80%)	

TABLE 5 Binary logistics regression analysis with pregnancy and birth outcomes as the influencing factor.

	starting dose of rhFSH	Crude model		Adjusted model	
		OR (95% CI)	P-value	OR (95% CI)	P-value
Positive pregnancy	150 IU	Reference		Reference	
	225 IU	1.065 (0.749-1.514)	0.725	1.02 (0.711-1.464)	0.915
Clinical pregnancy	150 IU	Reference		Reference	
	225 IU	1.103 (0.775-1.57)	0.585	1.074 (0.748-1.542)	0.699
Ongoing pregnancy	150 IU	Reference		Reference	
	225 IU	1.066 (0.748-1.52)	0.724	1.036 (0.719-1.491)	0.85
Live birth	150 IU	Reference		Reference	
	225 IU	1.065 (0.743-1.524)	0.733	1.043 (0.721-1.51)	0.822

CI, confidence interval.
Analyses were adjusted for age, BMI, AMH, infertility type (primary or secondary), starting dose of rhFSH, ovulation trigger protocol, total dose of Cetrorelix.

starting dose of 150 and 200 IU of rhFSH, the results showed that the 200 IU group had an average of 0.6 more oocytes than the 150 IU group (16). In another prospective randomized study comparing daily doses of 150 and 225 IU, the results showed that the 225 IU group had an average of 1.9 more oocytes than the 150 IU group (17). However, the patient in these studies included advanced maternal age, and there is a consensus that women over 35 are defined as advanced maternal age. Also, the sample sizes of the two articles were small, and pregnancy and live birth outcomes were not followed up.

In studies using higher rhFSH over 225 IU, the results indicated no significant differences in the number of oocytes retrieved and pregnancy outcomes (18). The mild increase in the number of oocytes retrieved in the high-dose group did not even increase the number of available embryos, either in our study or in previous studies.

In addition, no improvement in pregnancy outcomes was found in any of these studies. Therefore, the value of increasing the total number of retrieved oocytes should not be overemphasized for NOR patients.

GnRH analogues inhibit endogenous LH surges and prevent early follicular ovulation and follicular luteinization. Despite the use of GnRH analogs during COS, Premature progesterone elevation still occurred due to high doses of rhFSH, high E2 levels, and the simultaneous development of multiple follicles (19, 20). Meanwhile, the simultaneous development of multiple follicles leads to an increase in serum estradiol levels during COS (21). In our study, progesterone levels were significantly higher in the 225 IU group than in the 150 IU group on the trigger day (1.11 ± 0.57 vs. 1.2 ± 0.6, p=0.01).

Two large retrospective cohort studies showed a negative impact on pregnancy outcomes in women undergoing IVF when progesterone > 1.5-2 ng/mL (22, 23).

The putative negative effect of premature progesterone elevation is embryo-endometrial asynchrony which is critical for successful implantation (24).

Elevated progesterone levels on the trigger day were negatively correlated with live births in the fresh embryos transfer cycle but not in the subsequent frozen embryo transfer cycles (25, 26). Therefore, the freeze-all strategy was performed to reduce the effect of elevated progesterone on live birth rates when progesterone exceeds 1.5-2ng/ml (27). In this study, the freeze-all strategy was significantly higher in the 225 IU group than in the 150 IU group, which was associated with more patients in the 225 IU group having elevated progesterone. A systematic review showed that the freeze-all strategy was not superior to fresh embryo transfer in terms of live birth rate (28, 29). Conversely, the risk of maternal hypertensive disorders of pregnancy, of having a large-for-gestational-age baby and a higher birth weight of the children born may be increased following the freeze-all strategy (30). By design, the time to pregnancy is shorter in the conventional strategy than in the freeze-all strategy when the cumulative live birth rate is comparable, as embryo transfer is delayed in a 'freeze-all' strategy (31). At the same time, the freeze-all strategy increases the financial burden on patients (32). Therefore, the freeze-all strategy is unsuitable for all patients (33). The fresh embryo transfer should be adopted as much as possible without affecting live births, considering clinical safety and convenience (34).

OHSS is a potentially life-threatening iatrogenic complication during COS (35). A study analyzing 256,381 *in vitro* fertilization cycles showed that retrieval of >15 oocytes significantly increase OHSS risk during COS (36). In our study, we excluded patients with a high prevalence of OHSS, such as those with polycystic ovary syndrome, and limited the number of AFC in the patients. Therefore, there was no severe OHSS occurring between the two groups, and mild to moderate OHSS was not statistically significant.

Live birth is the principal clinical outcome following IVF. Previous studies have shown that when the total dose of rhFSH is more than 2500 IU, it negatively affects the live birth rate in fresh embryo transfer (37). This suggests that the CMDA consensus recommendation of a starting rhFSH dose of 150 or 225 IU is equally safe and reliable for NOR patients. In our study, the total dose of rhFSH dose did not exceed this upper limit in either group. Also, there was no difference in the live birth rate between the two groups in fresh embryo transfer. This suggests that a starting dose of rhFSH of 150 or 225 IU is equally safe and reliable for NOR patients.

A potential limitation of the study is that adjustments in rhFSH dose were permitted during COS (38), with adjustments limited to 50

IU. The dose adjustment of rhFSH is part of daily clinical practice during COS, and a systematic review covering 10 years showed that the proportion was about 45% (39). In addition, the dose adjustment of rhFSH did not affect the live birth (40). In spite of all the efforts to control bias, this study is inherently limited by the review of a retrospectively collected data set. Despite these limitations, our study still provides clinicians with a reasonable option for starting dose of rhFSH in the NOR population.

5 Conclusion

In conclusion, for the NOR patients following the GnRH-ant protocol, the starting dose of rhFSH of 225 IU slightly increased the number of oocytes retrieved compared to the starting dose of rhFSH of 150 IU, at the cost of an extra approximate 700 IU of rhFSH during COS. However, there was no significant difference in the number of available embryos and live birth rate in fresh embryo transfer. Notably, the fresh embryo transfer rate was higher in the 150 IU group than in the 225 IU group. Considering the potential cost-effectiveness and shorter time to live birth, the starting dose of rhFSH of 150 IU may be more suitable than 225 IU.

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 study was authorized by the local institutional review board (Reproductive Ethics Committee of The Affiliated Hospital of Shandong University of Traditional Chinese Medicine, approval no. SDTCM/E2204-02, dated April 2, 2022) and was undertaken at a public tertiary referral university hospital.

Author contributions

Z-CJ collected data and developed the manuscript. Y-QL and RL guided the design and reviewed the manuscript. SH, Q-CX, and KY contributed to data collection. P-XW and S-ML assisted in data analysis. Z-GS and YG guided the design and implementation of the study. 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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The serum follicle stimulating hormone-to-luteinizing hormone ratios can predict assisted reproductive technology outcomes in women undergoing gonadotropin releasing hormone antagonist protocol

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Background: The basal follicle stimulating hormone (FSH)/luteinizing hormone (LH) ratio is a useful predictor of ovarian response. In this study, we investigated whether the FSH/LH ratios during the entire controlled ovarian stimulation (COS) can be used as effective predictors of outcomes in women undergoing *in vitro* fertilization (IVF) treatment using the gonadotropin releasing hormone antagonist (GnRH-ant) protocol.

Methods: A total of 1,681 women undergoing their first GnRH-ant protocol were enrolled in this retrospective cohort study. A Poisson regression model was used to analyze the association between the FSH/LH ratios during COS and embryological outcomes. Receiver operating characteristic analysis was performed to determine the optimal cutoff values for poor responders (≤ 5 oocytes) or poor reproductive potential (≤ 3 available embryos). A nomogram model was constructed to provide a tool for predicting the cycle outcomes of individual IVF treatments.

Results: The FSH/LH ratios (at the basal day, stimulation day 6 (SD6) and trigger day) were significantly correlated with the embryological outcomes. The basal FSH/LH ratio was the most reliable predictor of poor responders with a cutoff value of 1.875 (area under the curve (AUC) = 72.3%, $P < 0.05$), or of poor reproductive potential with a cutoff value of 2.515 (AUC = 66.3%, $P < 0.05$). The SD6 FSH/LH ratio predicted poor reproductive potential with a cutoff value of 4.14 (AUC = 63.8%, $P < 0.05$). The trigger day FSH/LH ratio predicted poor responders with a cutoff value of 9.665 (AUC = 63.1%, $P < 0.05$). The basal FSH/LH ratio, combined with the SD6 and trigger day FSH/LH ratios, slightly increased these AUC values and improved the prediction sensitivity. The nomogram provides a reliable model with which to assess the risk of poor response or poor reproductive potential directly based on the combined indicators.

Conclusions: FSH/LH ratios are useful predictors of poor ovarian response or reproductive potential throughout the entire COS with the GnRH antagonist protocol. Our findings also provide insights into the potential for LH supplementation and regimen adjustment during COS to achieve improved outcomes.

KEYWORDS

embryological outcomes, GnRH antagonist (GnRH-ant) protocol, ovarian response, reproductive potential, FSH & LH

Introduction

The optimization and individualization of controlled ovarian stimulation (COS) is important to improve the success rate of *in vitro* fertilization-embryo transfer (IVF-ET) (1). Clinicians usually select the appropriate protocols and gonadotrophin doses or types according to the patients' basic characteristics, such as age, anti-Müllerian hormone (AMH), antral follicles count (AFC), basal serum follicle stimulating hormone (FSH), body mass index (BMI) and the ovarian response in previous treatment cycles (2–6). Compared with the use of basal characteristics alone, integrated evaluation of multiple sensitive markers during the entire COS process will provide more accurate prediction.

Recently, concerns have been raised regarding the accuracy of serum FSH level, luteinizing hormone (LH) level and FSH/LH ratio for predicting the ovarian response and oocyte quality in IVF. FSH and LH are secreted by the pituitary gland (7). The gonadotropin FSH plays a central role in stimulating follicular growth by binding to its receptors located in the granulosa cells of the follicles (8). LH acts synergistically with FSH to stimulate follicle recruitment and promote oocyte maturation, and ovulation is triggered by an LH surge (9). Multiple studies have shown that the basal FSH and LH levels, as well as the basal FSH/LH ratio, reflect the ovarian reserve, and allow early prediction of mature oocyte yield during the GnRH agonist protocol (10–13). For instance, FSH/LH ratio inversion is a characteristic of polycystic ovary syndrome with increased LH concentrations (14), while a high basal FSH/LH ratio is predictive of higher rates of cycle cancellation, poorer ovarian response to COS or lower pregnancy rates, even with different cutoff values (2, 3, and 3.6) in different studies (10–13). During COS, the serum delta FSH levels between the starting day (basal level) and stimulation day 6 (SD6) were higher in normal responders than in hyper-responders during the GnRH-agonist protocol (15). Decreased LH concentrations during ovarian stimulation using the GnRH-agonist long protocol with rec-FSH had a negative effect on ART outcomes (16).

In recent years, the GnRH-ant protocol has become the favored choice because of its effectiveness and convenience (17). Without the prolonged suppression of pituitary FSH and LH secretion in this protocol, the potential of the FSH/LH ratio at the basal level, SD6 and trigger day to predict outcomes should be different from that for the GnRH agonist protocol. Therefore, evaluation of the ovarian response and IVF outcomes based on FSH/LH ratios during COS is important for the clinical application of the GnRH-ant protocol.

In this study, we retrospectively assessed valuable indicators to explore the potential of serum FSH/LH ratios at three representative times (basal day, SD6 and trigger day) during the COS with the GnRH-ant protocol for prediction of ovarian response and reproductive potential. This information is important to guide regimen adjustment for the GnRH-ant protocol during the entire COS to improve outcomes.

Methods

Patients

This retrospective cohort study was performed at the Reproductive Medical Center of Ruijin Hospital (China) from June 2017 to October 2021. A total of 2,013 patients who underwent their first ovarian stimulation following the GnRH-ant protocol were selected for eligibility according to the following inclusion criteria: (a) aged 20–40 years old with a regular menstrual cycle; (b) received fixed GnRH-ant protocol; (c) signed informed consent. The following exclusion criteria were applied: (a) known chromosomal aberration among the patients; (b) endometriosis; (c) adenomyosis; (d) submucosal myoma; (e) intramural myoma close to the endometrium or > 5 cm in size, (f) polycystic ovary syndrome. After exclusion of the patients who did not meet the inclusion criteria, a total of 1,681 patients were enrolled in this study. After exclusion due to missing basal serum FSH or LH data, 1,673 patients were included in the data analysis. After exclusion due to missing SD6 serum FSH or LH data, 1,489 patients were included in the data analysis. After exclusion due to missing trigger day FSH or LH data, 1,449 patients were included in the final data analysis (Figure 1).

The patients with ≤ 5 retrieved oocytes were defined as poor responders, while patients with > 5 retrieved oocytes were defined as normal responders (18). Because no consensus exists on the number of available embryos, ≤ 3 available embryos was defined as poor reproductive potential, while > 3 was defined as normal reproductive potential. The study protocol was approved by Institutional Ethics Committee of Ruijin Hospital and informed consent was obtained from all participants. The study was conducted in accordance with the Declaration of Helsinki (revised in 2013).

Stimulation protocol

Patients' basal FSH, basal LH, estradiol (E2), progesterone (P4) levels and AFC were measured on menstrual day 2 of the stimulation

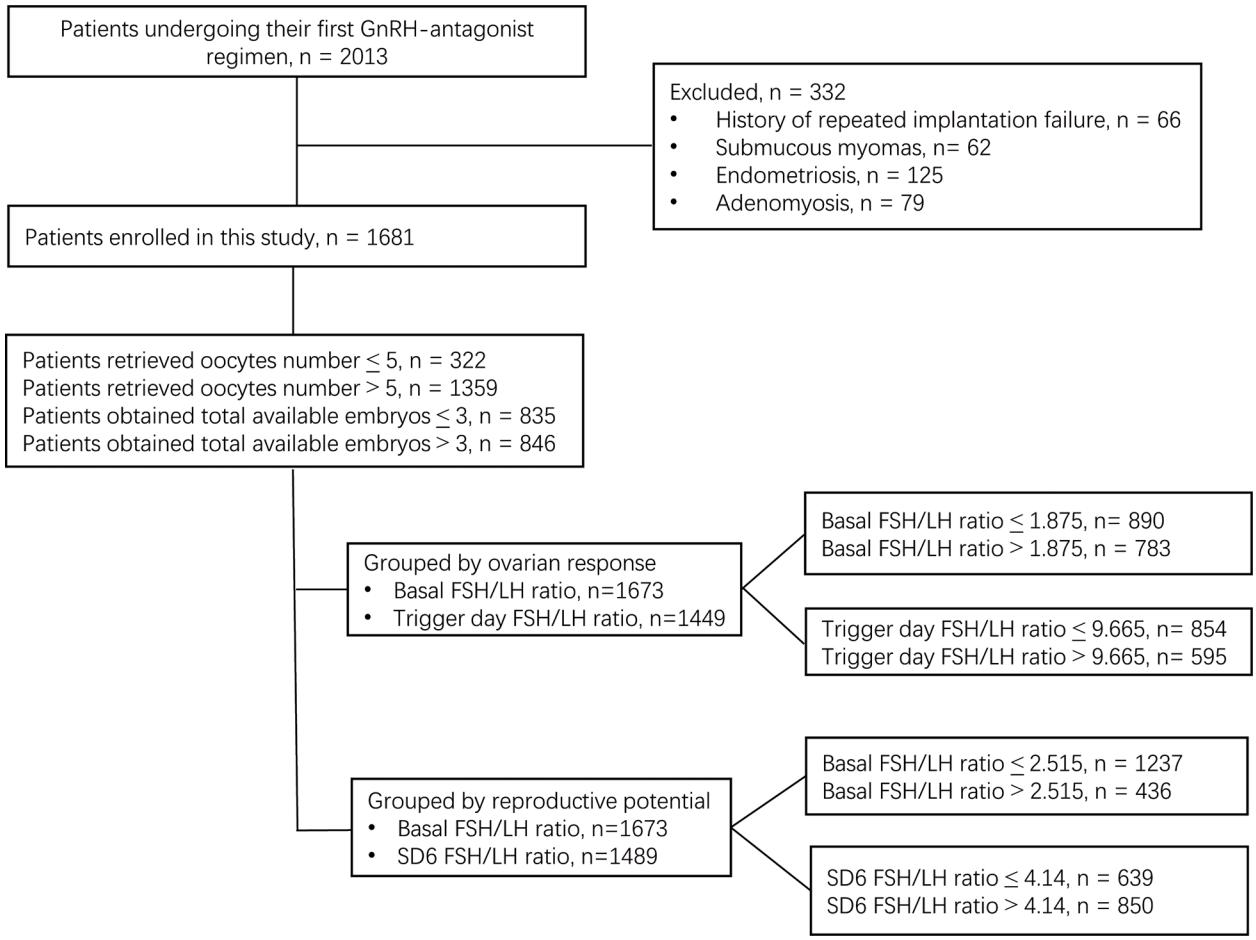

FIGURE 1
Flowchart of this study.

cycle. On the same day, recombinant FSH (Gonal-F, Merck-Serono SA, Switzerland) was administered, according to the baseline characteristics, with doses ranging from 112.5 to 300 IU per day. Ovarian response was monitored by routine measurement of serum FSH, serum LH, E2, P4 level and follicle scanning every 2–3 days during COS. Cetrorelix acetate (0.25 mg per day, Cetrotide, Merck-Serono, SA, Switzerland) was administered from SD6 until the day before trigger day (19). The final oocyte maturation trigger consisting of either 5,000–7,000 u hCG (Lizhu, Zhuhai, China) or 0.2 mg GnRH agonist (triptorelin acetate; France) was administered when three follicles reached a mean diameter of 17 mm. Oocyte aspiration was performed 35–36 h after the trigger day.

IVF and embryo quality assessment

Oocytes were fertilized on the day of oocyte aspiration. Fertilization was then assessed approximately 16–18 h after insemination. Normal fertilization was confirmed by the presence of two pronuclei (PN). All fertilized oocytes were cultured in sequential media (Vitrolife, Sweden), and incubated at an atmosphere of 6% CO_2, 5% O_2 and 89% N_2 at 37°C. Cleavage stage embryos at day 3 were assessed based on morphological characteristics using a standardized scoring system (19). Embryos with scores ≥ 8 were regarded as good quality embryos (20). The top two available cleavage embryos at day 3 were transferred or frozen. The surplus embryos were cultured until day 5 or 6 and the available blastocysts were transferred or frozen according to the Gardner grading system (21). The maturation rate was calculated as the percentage of metaphase II oocytes among the total number of oocytes for ICSI cycles. Fertilization rate for IVF cycle was calculated as the percentage of normal fertilized oocytes of the inseminated oocytes. The fertilization rate for ICSI cycles was calculated as the percentage of normal fertilized oocytes among the MII oocytes. Embryological outcomes were assessed based on four parameters: the number of oocytes retrieved, fertilized oocytes, good quality embryos at day 3 and total available embryos.

Embryo transfer

One or two cleavage embryos with scores ≥ 5 on day 3, or one blastocyst with available ranking on day 5 following ovum retrieval, were transferred under transabdominal ultrasound guidance. The luteal phase was supported by 90 mg of sustained-release progesterone gel (8% Crinone; Merck-Serono, Switzerland), which was administered vaginally starting on the first day after oocyte retrieval. Biochemical pregnancy was defined as serum β-HCG > 5

mIU/ml measured 11 days after cleavage embryo transfer and 9 days after blastocyst transfer. Clinical pregnancy was defined as visualization of a gestational sac and fetal cardiac activity on transvaginal ultrasound 6 weeks after embryo transfer. All follow-up data were recorded until live birth.

Statistical analysis

Pearson's correlation coefficient was calculated to analyze the association of FSH/LH ratio (at basal day, SD6 and trigger day) with the embryological outcomes. A Poisson regression model was used to confirm whether the above indicators were independent determinants of the oocyte retrieval number or embryo quality. The Poisson regression model were presented as odds ratio (OR) and 95% confidence intervals (CIs). By plotting sensitivity and 1-specificity, the receiver operating characteristic (ROC) curve was constructed and the area under the ROC curve (AUC) was calculated to assess the predictive power of FSH/LH ratio for differentiating poor responders or poor reproductive potential. The cutoff value was calculated according to the maximum Youden index value, and the Youden index was calculated using the formula sensitivity + specificity -1. Analysis of variance was used to compare the differences between the subgroups according to the cutoff value. Nomogram models were constructed on the basis of determinants identified in the multivariate logistic model to predict the risk of poor ovarian responders and low ovarian potential during the cycle.

A two-sided *P*-value < 0.05 was considered to indicate statistical significance. Continuous data were reported as the mean ± standard deviation (SD) and categorical data were presented as frequencies and percentages. Mann-Whitney U-tests and Student's *t*-tests were used to compare means for continuous data. Chi-square tests of Fisher's exact tests were used to determine the differences between percentages for categorical data. All statistical analyses were performed using SAS software (v. 9.4) (SAS Institute Inc., USA) or R Project v.3.5.2 (The R Foundation for Statistical Computing, Vienna, Austria. http://www.r-project.org).

Results

Among the 2,013 patients included, 1,681 patients were enrolled in this study. Of these patients, 322 had ≤ 5 retrieved oocytes, while 1,359 had > 5 retrieved oocytes. In addition, 835 patients had ≤ 3 total available embryos, while 846 patients had > 3 total available embryos (Figure 1).

Relationship between FSH/LH ratios and embryological outcomes

Table 1 shows the relationships between FSH/LH ratios during the entire COS and the embryological outcomes. After adjusting for the potential confounding factors (age, AMH, AFC, starting dose of Gn and total Gn), the basal FSH/LH ratio showed significant negative correlations with all embryological outcomes, including the number of oocytes retrieved (OR: 0.94; 95% CI: 0.93–0.96, $P < 0.05$), the number of fertilized oocytes (OR: 0.93; 95% CI: 0.91–0.95, $P < 0.05$), the number of good quality day 3 embryos (OR: 0.93; 95% CI: 0.88–0.98, $P < 0.05$) and the total number of available embryos (OR: 0.94;

TABLE 1 Correlation analysis and Poisson regression analysis of factors associated with embryological outcomes.

Variable	Pearson's correlation coefficient(γ)	Unadjusted *P*	Adjusted OR (95% CI)*	Adjusted *P**
No. of oocytes retrieved				
Basal FSH/LH	-0.340	0.000	0.94 (0.93-0.96)	0.000
SD6 FSH/LH	-0.228	0.000	1.00 (1.00-1.00)	0.409
Trigger day FSH/LH	0.115	0.000	1.00 (1.00-1.00)	0.000
No. of fertilized oocytes				
Basal FSH/LH	-0.320	0.000	0.93 (0.91-0.95)	0.000
SD6 FSH/LH	-0.224	0.000	1.00 (0.99-1.00)	0.032
Trigger day FSH/LH	0.098	0.000	1.00 (1.00-1.00)	0.000
No. of day 3 good quality embryos				
Basal FSH/LH	-0.111	0.000	0.93 (0.88-0.98)	0.007
SD6 FSH/LH	-0.073	0.005	1.00 (0.99-1.01)	0.945
Trigger day FSH/LH	0.024	0.371	–	–
No. of total available embryos				
Basal FSH/LH	-0.247	0.000	0.94 (0.91-0.96)	0.000
SD6 FSH/LH	-0.209	0.000	0.99 (0.99-1.00)	0.003
Trigger day FSH/LH	0.030	0.261	–	–

OR, odds ratio; CI, confidence interval; No., Number; SD6, Stimulation Day six.
*Adjusted for confounding factors (AMH, age, AFC, starting dose of Gn and Total Gn).

95% CI: 0.91–0.96, $P < 0.05$). In addition, the SD6 FSH/LH ratio showed a significant negative association with the number of fertilized oocytes (OR:1.00; 95% CI: 0.99–1.00, $P < 0.05$) and the number of total available embryos (OR:0.99; 95% CI:0.99–1.00, $P < 0.05$). The FSH/LH ratio at trigger day showed a significant positive correlation with the number of oocytes retrieved (OR: 1.00; 95% CI:1.00–1.00, $P < 0.05$) and the number of fertilized oocytes (OR: 1.00; 95% CI:1.00–1.00, $P < 0.05$).

Analysis of diagnostic accuracy of serum FSH/LH ratios

The basal FSH/LH ratio demonstrated significant accuracy in distinguishing poor responders from normal responders (AUC = 72.3%, $P < 0.05$) (Figure 2A) with a cutoff value of 1.875. The trigger day FSH/LH ratio also showed significant accuracy in distinguishing poor responders from normal responders (AUC = 63.1%, $P < 0.05$) (Figure 2B) with a cutoff value of 9.665. The multivariable model of the FSH/LH ratio at basal and trigger day showed a higher level of confidence in the accuracy of distinguishing poor responders from normal responders (AUC = 78.1%, $P < 0.05$) (Figure 2C). The AUC-ROC curve showed significant accuracy in discriminating poor reproductive potential from normal reproductive potential, with a cutoff value of 2.515 for the basal FSH/LH ratio (AUC = 66.3%, $P < 0.05$) (Figure 2D) and 4.14 for the SD6 FSH/LH ratio (AUC = 63.8%, $P < 0.05$) (Figure 2E). The multivariable model of the FSH/LH ratio at basal day and SD6 showed a higher level of confidence in the accuracy of distinguishing poor reproductive potential from normal reproductive potential (AUC = 67.4%, $P < 0.05$) (Figure 2F).

Characteristics and outcomes of subgroups based on the basal FSH/LH ratio

Table 2 shows the basic characteristics, embryological outcomes and clinical outcomes after dividing groups according to their basal FSH/LH ratio at a cutoff value of 1.875. The groups with a higher basal FSH/LH ratio (> 1.875) showed inferior basic characteristics, and the number of oocytes retrieved (8.73 ± 5.02 vs. 12.79 ± 5.58, $P < 0.05$), the number of fertilized oocytes (6.82 ± 4.22 vs. 10.07 ± 4.80, $P < 0.05$) and the number of total available embryos (3.29 ± 2.49 vs. 4.89 ± 3.32, $P < 0.05$) were significantly lower after adjusting for the confounding factors (AMH, age, AFC, starting dose of Gn and total Gn). The proportion of poor responders was also significantly higher (30.7% vs. 8.99%, $P < 0.05$) when the basal FSH/LH ratio was > 1.875. There were no significant differences in the clinical outcomes in terms of implantation rate, biochemical pregnancy rate, clinical pregnancy

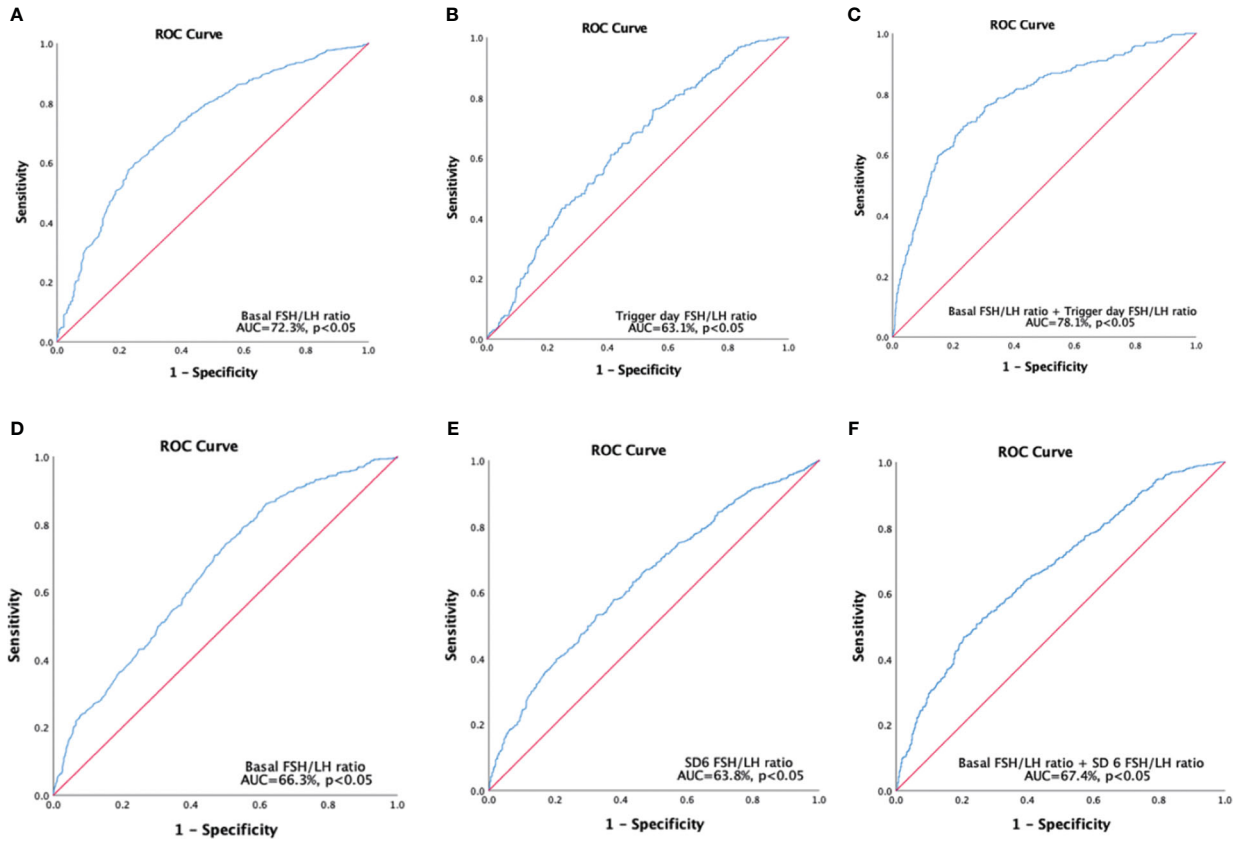

FIGURE 2
Receiver operating characteristics curves were constructed to predict poor responders **(A-C)**, and poor reproductive potential **(D-F)**. **(A)** Single variable for basal FSH/LH ratio, cutoff value=1.875. **(B)** Single variable for trigger, day FSH/LH ratio cutoff value =9.665. **(C)** Multivariable models for basal FSH/LH ratio + trigger day FSH/LH ratio; **(D)** Single variable for basal FSH/LH ratio, cutoff value=2.515. **(E)** Single variable for SD6 FSH/LH ratio, cutoff value=4.14. **(F)** Multivariable models for basal FSH/LH ratio + SD6 FSH/LH ratio. Poor responders refer to patients who retrieved oocytes ≤ 5; Poor reproductive potential refers to patients who obtained total available embryos ≤ 3.SD6: Stimulation Day six.

rate, ongoing pregnancy rate, multiple pregnancy rate, live birth rate, abortion rate and ectopic pregnancy rate between the two groups.

As shown in Table 3, the basic characteristics of the basal FSH/LH ratio > 2.515 group were inferior compared with the group with FSH/LH ratio ≤ 2.515, and thus, the four embryological parameters were all significantly lower after adjusting for the confounding factors ($P < 0.05$), while the proportion of low reproductive potential patients was significantly higher (72.9% vs. 41.6%, $P < 0.05$). There were no significant differences in the clinical outcomes in terms of implantation rate, biochemical pregnancy rate, clinical pregnancy

TABLE 2 Basic characteristic, embryological outcomes and clinical outcomes of subgroups based on the basal FSH/LH ratio cutoff value for poor responders.

	Basal FSH/LH ≤1.875	Basal FSH/LH > 1.875	*P*
N	890	783	
AMH (ng/ml)	5.32 ± 4.07	2.65 ± 2.17	0.000[a]
Age (years)	30.96 ± 3.78	32.72 ± 4.08	0.000[a]
AFC	14.46 ± 5.78	10.09 ± 4.73	0.000[a]
BMI (kg/m^2)	22.18 ± 2.92	22.13 ± 2.88	0.583[a]
Basal FSH (IU/L)	6.98 ± 2.04	8.63 ± 3.12	0.000[a]
Basal LH (IU/L)	6.66 ± 5.57	3.18 ± 1.21	0.000[a]
Basal E2 (pg/ml)	43.92 ± 33.90	42.42 ± 37.96	0.133[a]
Basal P4 (pg/ml)	0.74 ± 1.12	0.60 ± 0.46	0.020[a]
Basal FSH/LH ratio	1.24 ± 0.41	2.95 ± 1.54	0.000[a]
Starting dose of Gn (IU)	205.65 ± 55.16	231.83 ± 56.31	0.000[a]
Stimulation duration of Gn (days)	9.76 ± 1.60	9.79 ± 1.74	0.652[a]
Total Gn (IU)	2113.91 ± 748.14	2388.91 ± 749.71	0.000[a]
EMBRYOLOGICAL OUTCOMES			
No. of oocytes retrieved	12.79 ± 5.58	8.73 ± 5.02	0.000[b]
No. of fertilized oocytes	10.07 ± 4.80	6.82 ± 4.22	0.000[b]
No. of day 3 good quality embryos	1.06 ± 1.56	0.79 ± 1.25	0.685[b]
No. of total available embryos	4.89 ± 3.32	3.29 ± 2.49	0.005[b]
Maturation rate (%)	2278/2682 (84.9%)	1711/2060 (83.1%)	0.079[c]
Fertilization rate of IVF (2PN) (%)	6531/8700 (75.1%)	3602/4772 (75.5%)	0.595[c]
Fertilization rate of ICSI (2PN) (%)	1937/2278 (85.0%)	1427/1711 (83.4%)	0.161[c]
Proportion of poor responders	80/890 (8.99%)	240/783 (30.7%)	0.000[c]
FRESH EMBRYO TRANSFER			
No. of cycles transferred	258	278	
No. of embryos transferred	410	460	
Endometrium thickness on trigger day (cm)	1.06 ± 0.16	1.04 ± 0.16	0.237[a]
P4 level on trigger day (pg/ml)	0.97 ± 0.42	0.91 ± 0.36	0.281[a]
Average number of embryos transferred	1.59 ± 0.49	1.65 ± 0.48	0.118[a]
Average score of cleavage embryos transferred	7.34 ± 1.11	7.14 ± 1.18	0.061[a]
Proportion of blastocyst embryo transfer (%)	31/258 (12.0%)	21/278 (7.60%)	0.081[c]
CLINICAL OUTCOMES			
Implantation rate (%)	141/410 (34.4%)	130/460 (28.3%)	0.051[c]
Biochemical pregnancy rate (%)	137/258 (53.1%)	131/278 (47.1%)	0.167[c]
Clinical pregnancy rate (%)	113/258 (43.8%)	110/278 (39.0%)	0.321[c]

(Continued)

TABLE 2 Continued

	Basal FSH/LH ≤1.875	Basal FSH/LH > 1.875	*P*
Ongoing pregnancy rate (%)	92/258 (35.7%)	92/278 (33.1%)	0.532[c]
Multiple pregnancy rate (%)	31/258 (12.0%)	26/278 (9.40%)	0.318[c]
Live birth rate (%)	92/258 (35.7%)	92/278 (33.1%)	0.532[c]
Abortion rate (%)	16/113 (14.2%)	14/110 (12.7%)	0.754[c]
Ectopic pregnancy rate (%)	5/113 (4.40%)	4/110 (2.80%)	0.381[c]

Data is expressed as mean ± SD, or number (percentage); AMH, anti-Müllerian hormone; AFC, antral follicles count; P4, progesterone; No., Number.
[a]Student's t-test or Mann-Whitney U test.
[b]Adjusted for confounding factors (AMH, age, AFC, starting dose of Gn and Total Gn).
[c]Chi-test or Fisher's exact test.

TABLE 3 Basic characteristic, embryological outcomes and clinical outcomes of subgroups based on the basal FSH/LH ratio cutoff value for poor reproductive potential.

	Basal FSH/LH ≤ 2.515	Basal FSH/LH > 2.515	*P*
N	1237	436	
AMH (ng/ml)	4.73 ± 3.80	2.20 ± 1.84	0.000[a]
Age (years)	31.34 ± 3.90	33.04 ± 4.08	0.000[a]
AFC	13.57 ± 5.73	9.12 ± 4.34	0.000[a]
BMI (kg/m^2)	22.10 ± 2.86	22.31 ± 3.01	0.327[a]
Basal FSH (IU/L)	7.25 ± 2.13	9.17 ± 3.62	0.000[a]
Basal LH (IU/L)	5.83 ± 4.94	2.76 ± 1.15	0.000[a]
Basal E2 (pg/ml)	42.90 ± 30.70	44.11 ± 47.58	0.345[a]
Basal P4 (pg/ml)	0.71 ± 0.99	0.59 ± 0.39	0.009[a]
Basal FSH/LH ratio	1.50 ± 0.55	3.58 ± 1.83	0.000[a]
Starting dose of Gn (IU)	210.02 ± 56.20	240.25 ± 54.06	0.000[a]
Stimulation duration of Gn (days)	9.74 ± 1.61	9.87 ± 1.82	0.170[a]
Total Gn (IU)	2153.90 ± 740.00	2494.42 ± 764.72	0.000[a]
EMBRYOLOGICAL OUTCOMES			
No. of oocytes retrieved	12.08 ± 5.60	7.51 ± 4.48	0.000[b]
No. of fertilized oocytes	9.51 ± 4.77	5.82 ± 3.80	0.000[b]
No. of day 3 good quality embryos	1.05 ± 1.53	0.59 ± 1.03	0.004[b]
No. of total available embryos	4.64 ± 3.17	2.71 ± 2.18	0.000[b]
Maturation rate (%)	3215/3808 (84.4%)	774/934 (82.9%)	0.243[c]
Fertilization rate of IVF (2PN) (%)	8407/11133 (75.5%)	1726/2339 (73.8%)	0.080[c]
Fertilization rate of ICSI (2PN) (%)	2724/3215 (84.7%)	640/774 (82.7%)	0.161[c]
Proportion poor reproductive potential	514/1237(41.6%)	318/436 (72.9%)	0.000[c]
FRESH EMBRYO TRANSFER			
No. of cycles transferred	390	146	
No. of embryos transferred	630	240	
Endometrium thickness on trigger day (cm)	1.06 ± 0.17	1.03 ± 0.15	0.167[a]
P4 level on trigger day (pg/ml)	0.95 ± 0.40	0.91 ± 0.37	0.569[a]
Average number of embryos transferred	1.62 ± 0.49	1.64 ± 0.48	0.545[a]

(Continued)

TABLE 3 Continued

	Basal FSH/LH ≤ 2.515	Basal FSH/LH > 2.515	*P*
Average score of cleavage embryos transferred	7.30 ± 1.16	7.09 ± 1.13	0.063[a]
Proportion of blastocyst embryo transfer (%)	42/390 (10.8%)	10/146 (6.80%)	0.172[a]
CLINICAL OUTCOMES			
Implantation rate (%)	205/630 (32.5%)	66/240 (27.5%)	0.151[c]
Biochemical pregnancy rate (%)	200/390 (51.3%)	68/146 (46.6%)	0.332[c]
Clinical pregnancy rate (%)	165/390 (42.3%)	58/146 (39.7%)	0.589[c]
Ongoing pregnancy rate (%)	137/390 (35.1%)	47/146 (32.2%)	0.524[c]
Multiple pregnancy rate (%)	47/390 (12.1%)	10/146 (6.80%)	0.082[c]
Live birth rate (%)	137/390 (35.1%)	47/146 (32.2%)	0.534[c]
Abortion rate (%)	20/165 (12.1%)	10/58 (17.2%)	0.326[c]
Ectopic pregnancy rate (%)	8/165 (4.80%)	1/58 (1.70%)	0.452[c]

Data is expressed as mean ± SD, or number (percentage); AMH, anti-Müllerian hormone; AFC, antral follicles count; P4, progesterone; No., Number.
[a]Student's t-test or Mann-Whitney U test.
[b]Adjusted for confounding factors (AMH, age, AFC, starting dose of Gn and Total Gn).
[c]Chi-test or Fisher's exact test.

rate, ongoing pregnancy rate, multiple pregnancy rate, live birth rate, abortion rate and ectopic pregnancy rate between the two groups ($P > 0.05$).

Characteristics and outcomes of subgroups based on the SD6 FSH/LH ratio

Compared with the SD6 FSH/LH ratio ≤ 4.14 group, the SD6 FSH/LH ratio > 4.14 group had significantly poorer basic characteristics, as AMH, age, and AFC were all disadvantaged in this group, and the number of retrieved oocytes, the number of fertilized oocytes, and the total number of available embryos were all significantly lower after adjusting for the confounding factors ($P < 0.05$) (Table 4). The proportion of low reproductive potential patients was also significantly higher (58.8% vs. 37.9%, $P < 0.05$). There were no significant differences in the clinical outcomes in terms of implantation rate, biochemical pregnancy rate, clinical pregnancy rate, ongoing pregnancy rate, multiple pregnancy rate, live birth rate, abortion rate and ectopic pregnancy rate between the two groups ($P > 0.05$).

TABLE 4 Basic characteristic, embryological outcomes and clinical outcomes of subgroups based on SD6 FSH/LH ratio cutoff value for poor reproductive potential.

	SD6 FSH/LH ≤ 4.14	SD6 FSH/LH > 4.14	*P*
N	639	850	
AMH (ng/ml)	5.37 ± 4.24	3.08 ± 2.68	0.000[a]
Age (years)	31.27 ± 3.85	32.16 ± 4.10	0.000[a]
AFC	14.85 ± 6.20	10.90 ± 4.93	0.000[a]
BMI (kg/m^2)	22.29 ± 3.11	22.01 ± 2.74	0.119[a]
Basal FSH (IU/L)	7.28 ± 2.31	8.11 ± 2.91	0.000[a]
Basal LH (IU/L)	6.20 ± 4.22	3.97 ± 1.99	0.000[a]
Basal E2 (pg/ml)	44.78 ± 35.09	41.41 ± 35.33	0.000[a]
Basal P4 (pg/ml)	0.67 ± 0.82	0.69 ± 0.86	0.701[a]
Basal FSH/LH ratio	1.52 ± 0.84	2.41 ± 1.51	0.000[a]
Starting dose of Gn (IU)	199.09 ± 53.53	226.35 ± 56.78	0.000[a]
Stimulation duration of Gn (days)	9.60 ± 1.76	9.86 ± 1.62	0.000[a]
Total Gn (IU)	2021.89 ± 760.11	2352.31 ± 743.66	0.000[a]
EMBRYOLOGICAL OUTCOMES			

(Continued)

TABLE 4 Continued

	SD6 FSH/LH ≤ 4.14	SD6 FSH/LH > 4.14	*P*
No. of oocytes retrieved	12.82 ± 5.99	9.40 ± 5.11	0.005[b]
No. of fertilized oocytes	10.07 ± 5.03	7.35 ± 4.34	0.002[b]
No. of day 3 good quality embryos	1.08 ± 1.59	0.82 ± 1.32	0.768[b]
No. of total available embryos	5.01 ± 3.42	3.48 ± 2.58	0.001[b]
Maturation rate (%)	1642/1930 (85.1%)	1937/2327 (83.2%)	0.103[c]
Fertilization rate of IVF (2PN) (%)	4680/6261 (74.7%)	4258/5666 (75.2%)	0.613[c]
Fertilization rate of ICSI (2PN) (%)	1402/1642 (85.4%)	1616/1937 (83.4%)	0.109[c]
Proportion poor reproductive potential	242/639 (37.9%)	500/850 (58.8%)	0.000[c]
FRESH EMBRYO TRANSFER			
No. of cycles transferred	179	330	
No. of embryos transferred	287	541	
Endometrium thickness on trigger day (cm)	1.03 ± 0.15	1.06 ± 0.17	0.123[a]
P4 level on trigger day (pg/ml)	0.98 ± 0.42	0.93 ± 0.38	0.293[a]
Average number of embryos transferred	1.60 ± 0.49	1.64 ± 0.48	0.423[a]
Average score of cleavage embryos transferred	7.32 ± 1.11	7.18 ± 1.18	0.172[a]
Proportion of blastocyst embryo transfer (%)	27/179 (15.1%)	23/330 (7.00%)	0.003[c]
CLINICAL OUTCOMES			
Implantation rate (%)	87/287 (30.3%)	162/541 (29.9%)	0.912[c]
Biochemical pregnancy rate (%)	91/179 (50.8%)	161/330 (48.8%)	0.659[c]
Clinical pregnancy rate (%)	72/179 (40.2%)	136/330 (41.2%)	0.828[c]
Ongoing pregnancy rate (%)	58/179 (32.4%)	113/330 (34.1%)	0.692[c]
Multiple pregnancy rate (%)	18/179 (10.1%)	32/330 (9.70%)	0.897[c]
Live birth rate (%)	58/179 (32.4%)	113/330 (34.1%)	0.692[c]
Abortion rate (%)	10/72 (13.9%)	18/136 (13.2%)	0.895[c]
Ectopic pregnancy rate (%)	4/72 (5.60%)	5/136 (3.70%)	0.500[c]

Data is expressed as mean ± SD, or number (percentage); AMH, anti-Müllerian hormone; AFC, antral follicles count; P4, progesterone; No., Number.
[a]Student's t-test or Mann-Whitney U test.
[b]Adjusted for confounding factors (AMH, age, AFC, starting dose of Gn and Total Gn).
[c]Chi-test or Fisher's exact test.

Characteristics and outcomes of subgroups based on the trigger day FSH/LH ratio

As shown in Table 5, there were no differences in the age and AFC of the trigger day FSH/LH ratio > 9.665 group compared with the group of trigger day FSH/LH ratio ≤ 9.665 ($P > 0.05$), while the AMH and BMI were slightly lower, and the starting dose of Gn and total Gn were significantly higher ($P < 0.05$). Thus, after adjusting for the basic confounding factors (AMH, BMI, starting dose of Gn and total Gn), the number of oocytes retrieved (11.76 ± 5.29 vs. 10.44 ± 5.95, $P < 0.05$), the number of fertilized oocytes (9.20 ± 4.58 vs. 8.14 ± 4.94, $P < 0.05$) and the number of total available embryos (4.21 ± 2.86 vs. 4.13 ± 3.14, $P < 0.05$) were significantly higher in the group of trigger day FSH/LH ratio > 9.665. In addition, the fertilization rate of IVF was slightly higher (75.7% vs. 74.1%) and the proportion of poor responders was lower (10.9% vs. 24.0%) in the trigger day FSH/LH ratio > 9.665 group ($P < 0.05$). For clinical outcomes, the endometrium thickness on the trigger day was slightly thinner in the trigger day FSH/LH ratio > 9.665 group (1.03 ± 0.15 cm vs. 1.07 ± 0.17 cm, $P < 0.05$), while the P4 level was also higher (1.00 ± 0.41 ng/ml vs. 0.90 ± 0.38 ng/ml, $P < 0.05$) in the group of trigger day FSH/LH ratio > 9.665. Thus, compared with the group of trigger day FSH/LH ratio ≤ 9.665, the implantation rate (25.2% vs. 33.9%), biochemical pregnancy rate (43.0% vs. 53.9%), clinical pregnancy rate (33.0% vs. 45.8%), ongoing pregnancy rate (26.0% vs. 38.6%) and live birth rate (26.0% vs. 38.5%) were all significantly lower ($P < 0.05$).

Predictive nomogram for poor ovarian response or poor reproductive potential

As shown in Figure 3, nomogram models were constructed to predict the risk of poor response or poor reproductive potential, with the following variables entered into the model: age (< 35 y, 35–40 y),

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TABLE 5 Basic characteristic, embryological outcomes and clinical outcomes of subgroups based on the trigger day FSH/LH ratio cutoff value for poor responders.

	Trigger day FSH/LH≤9.665	Trigger day FSH/LH > 9.665	*P*
N	854	595	
AMH (ng/ml)	4.43 ± 4.05	3.70 ± 3.01	0.041[a]
Age (years)	31.88 ± 4.03	31.66 ± 4.06	0.264[a]
AFC	12.98 ± 6.38	12.22 ± 4.92	0.140[a]
BMI (kg/m^2)	22.60 ± 3.00	21.52 ± 2.64	0.000[a]
Basal FSH (IU/L)	7.99 ± 2.93	7.36 ± 2.19	0.001[a]
Basal LH (IU/L)	5.51 ± 3.89	4.07 ± 2.05	0.000[a]
Basal E2 (pg/ml)	43.39 ± 38.09	41.08 ± 29.40	0.355[a]
Basal P4 (pg/ml)	0.66 ± 0.87	0.70 ± 0.81	0.001[a]
Basal FSH/LH ratio	1.94 ± 1.47	2.16 ± 1.13	0.000[a]
Trigger day FSH (IU/L)	16.46 ± 6.00	19.97 ± 6.45	0.000[a]
Trigger day LH (IU/L)	4.09 ± 3.27	1.17 ± 0.57	0.000[a]
Trigger day E2 (pg/ml)	3835.08 ± 2933.23	4209.53 ± 2590.50	0.000[a]
Trigger day P (pg/ml)	1.04 ± 0.70	1.32 ± 1.02	0.000[a]
Starting dose of Gn (IU)	208.41 ± 57.28	223.03 ± 55.66	0.000[a]
Stimulation duration of Gn (days)	9.63 ± 1.79	9.98 ± 1.43	0.000[a]
Total Gn (IU)	2123.07 ± 786.24	2357.98 ± 713.68	0.000[a]
EMBRYOLOGICAL OUTCOMES			
No. of oocytes retrieved	10.44 ± 5.95	11.76 ± 5.29	0.000[b]
No. of fertilized oocytes	8.14 ± 4.94	9.20 ± 4.58	0.000[b]
No. of day 3 good quality embryos	0.95 ± 1.49	0.90 ± 1.37	0.852[b]
No. of total available embryos	4.13 ± 3.14	4.21 ± 2.86	0.000[b]
Maturation rate (%)	1963/2341 (83.9%)	1467/1759 (83.4%)	0.698[c]
Fertilization rate of IVF (2PN) (%)	4873/6573(74.1%)	3968/5240 (75.7%)	0.048[c]
Fertilization rate of ICSI (2PN) (%)	1654/1963 (84.3%)	1226/1467 (83.6%)	0.588[c]
Proportion of poor responders	205/854 (24.0%)	65/595(10.9%)	0.000[c]
FRESH EMBRYO TRANSFER			
Number of cycles transferred	306	200	
Number of embryos transferred	495	329	
Endometrium thickness on trigger day (cm)	1.07 ± 0.17	1.03 ± 0.15	0.007[a]
P4 level on trigger day (pg/ml)	0.90 ± 0.38	1.00 ± 0.41	0.003[a]
Average number of embryos transferred	1.62 ± 0.49	1.65 ± 0.48	0.534[a]
Average score of cleavage embryos transferred	7.23 ± 1.16	7.24 ± 1.18	0.877[a]
Proportion of blastocyst embryo transfer (%)	29/306 (9.50%)	22/200 (11.0%)	0.578[c]
CLINICAL OUTCOMES			
Implantation rate (%)	168/495 (33.9%)	83/329 (25.2%)	0.008[c]
Biochemical pregnancy rate (%)	165/306 (53.9%)	86/200 (43.0%)	0.016[c]
Clinical pregnancy rate (%)	140/306 (45.8%)	66/200 (33.0%)	0.004[c]
Ongoing pregnancy rate (%)	118/306 (38.6%)	52/200 (26.0%)	0.003[c]

(Continued)

TABLE 5 Continued

	Trigger day FSH/LH≤9.665	Trigger day FSH/LH > 9.665	*P*
Multiple pregnancy rate (%)	35/306 (11.4%)	17/200 (8.50%)	0.287[c]
Live birth rate (%)	118/306 (38.6%)	52/200 (26.0%)	0.004[c]
Abortion rate (%)	17/140 (12.1%)	12/66 (18.2%)	0.245[c]
Ectopic pregnancy rate (%)	5/140 (3.60%)	2/66 (3.00%)	0.841[c]

Data is expressed as mean ± SD, or number (percentage); AMH, anti-Müllerian hormone; AFC, antral follicles count; P4, progesterone; No., Number.
[a]Student's t-test or Mann-Whitney U test.
[b]Adjusted for confounding factors (AMH, BMI, starting dose of Gn and Total Gn).
[c]Chi-test or Fisher's exact test.

AMH (< 1.1 ng/ml, 1.1–4.7 ng/ml, > 4.7 ng/ml), BMI (< 20 kg/m^2, 20–25 kg/m^2, > 25 kg/m^2), AFC (< 6, 7-15, >16) and FSH/LH ratio at basal, SD6 and the trigger day of COS. Using this model, the risk is calculated as the sum of the individual points identified on the point scale for each variable. The total points for each variable projected onto the lower scale indicate the risk of poor response (Figure 3A), and the risk for poor reproductive potential (Figure 3B) during this cycle.

Discussion

In this study, we investigated the association of the FSH/LH ratios during the entire COS with ovarian response and reproductive potential at three key stages (the starting day, the sixth day and the trigger day) during treatment with the GnRH-ant protocol. For the fixed GnRH-ant protocol, patients' hormone levels at the start of the stimulation reflect the real levels without prolonged suppression of pituitary FSH and LH secretion, while the hormone levels at SD6 reflect the levels after ovarian stimulation without downregulation. In addition, the hormone levels on the trigger day reflect the levels after short-term suppression by GnRH-ant. These three stages, which correspond to the early, middle and late stages of follicular development, represent important time-points for evaluating ovarian response during GnRH-ant protocol.

As shown in Table 1, the basal FSH/LH ratio showed a significant negative correlation with all embryological outcomes. In contrast, the trigger day FSH/LH ratio was positively correlated only with the number of oocytes retrieved and fertilized oocytes, and showed no correlation with the embryo quality. The SD6 FSH/LH ratio was related only to the number of fertilized oocytes and total available embryos, and showed no correlation with the number of oocytes retrieved. These findings indicated that the basal FSH/LH ratio is the most important predictor of ovarian reserve, while the FSH/LH ratios at the other two time-points (SD6 and trigger day) can be used as secondary indicators for guiding the regimen. Previous studies showed that elevated day 3 FSH/LH ratio was associated with reduced ovarian response and pregnancy rates in patients undergoing IVF with the GnRH agonist protocol (10, 11, 22, 23). Therefore, we first evaluated the potential of basal FSH/LH for prediction of ovarian response and embryo quality using the GnRH-ant protocol. Furthermore, our results suggested that the basal FSH/LH ratio is a potential indicator to predict poor responders with a cutoff value of 1.875 (AUC = 72.3%, $P < 0.05$). The basal FSH/LH ratio also showed significant ability to discriminate poor reproductive potential from normal groups with a cutoff value of 2.515 (AUC = 66.3%, $P < 0.05$).

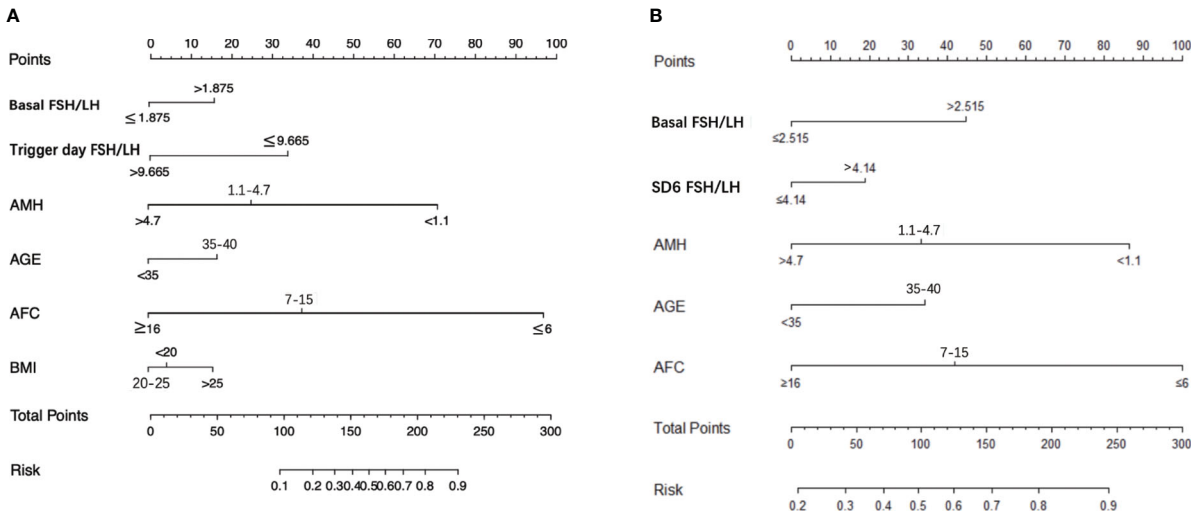

FIGURE 3
Nomogram graph to predict poor responder or poor reproductive potential for patients underwent IVF-ET. **(A)** obtained ≤ 5 oocytes, **(B)** obtained ≤ 3 available embryos during the cycle. AMH, anti-Mullerian hormone; BMI, body mass index; AFC, antral follicles count; SD6, Stimulation Day six.

The patients were then further divided according to the basal FSH/LH ratio cutoff value (1.875) for poor responders. The ovarian reserve parameters (age, AMH, AFC, basal FSH and basal LH) were apparently inferior in the basal FSH/LH > 1.875 subgroup, with a relatively higher starting and total dose of Gn. After correction for these confounding factors, the number of oocytes retrieved, fertilized oocytes and the number of total available embryos showed significant disadvantages in this subgroup and the incidence of poor responders remained higher. The differences in the ovarian reserve and embryo quality were more apparent between subgroups divided by the basal FSH/LH ratio (2.515). All of the results showed that the ovarian response or reproductive potential was inferior in the subgroups with higher basal FSH/LH ratio (> 1.875, or > 2.515) and that the higher basal FSH level and lower basal LH level lead to this change.

Furthermore, the SD6 FSH/LH ratio is a potential indicator to predict poor reproductive potential with a cutoff value of 4.14 (AUC = 63.8%, $P < 0.05$). The combination of the basal FSH/LH ratio and the SD6 FSH/LH ratio provided higher confidence in the prediction of poor reproductive potential with a higher AUC at 67.4%, and showing a moderate complementary value. The subgroup with a higher SD6 FSH/LH ratio (> 4.14) showed poor ovarian reserve, with a lower AMH, advanced age, higher FSH level, lower LH level and higher basal FSH/LH ratio. Compared with the starting day of the Gn stimulation, the FSH/LH ratio was higher at SD6 after 5 days of Gn treatment. This could be accounted for in several ways. First, the high FSH level at SD6 might benefit from the high starting and total dose of Gn. Second, population differences in pharmacokinetics might lead to higher FSH levels (24). Third, polymorphisms of the FSH receptor (FSHR), which is located in ovarian granulosa cells and is sensitive to exogenous rFSH (25), might play a role since such genetic variations may reduce the affinity of the receptor for serum FSH (26, 27). Therefore, patients with such polymorphisms have higher serum FSH levels due to the lower affinity of the FSH receptor. The proportion of patients with poor reproductive potential was also higher in the group of SD6 FSH/LH ratio > 4.14. Despite the higher starting and total doses of Gn, embryological outcomes were still disadvantaged after adjusting for confounding factors. Our results were consistent with a previous report that a high FSH/LH ratio in the early phase of the COS had a negative effect on oocyte quality (10).

These results provide evidence that the FSH/LH ratio at the starting day or SD6 are significant predictors of ovarian response and reproductive potential. It can be speculated that recombinant human follicle stimulating hormone (rFSH) adjustment and effective LH supplementation at the two important time-point might improve the embryological outcomes for poor ovarian responders. LH plays an important role during folliculogenesis by regulating both granulosa and theca cells. It has been hypothesized that carriers of a less bioactive LH may require higher Gn doses and/or benefit from LH activity supplementation during ovarian stimulation (28, 29). A recent study showed a higher incidence of top-quality preimplantation embryos when LH activity was supplemented in women undergoing IVF (30). It has also been demonstrated that LH supplementation rescues the ovarian response in patients with an initial poor response to rFSH and increases the number of oocytes retrieved or the rate of clinical pregnancy (29, 31). However, the optimal LH supplementation dosage and treatment regimen remains to be established. Our study suggested that embryological outcomes using the GnRH-ant protocol can be improved by LH supplementation based on the cutoff value.

The trigger day FSH/LH ratio is also a potential indicator to predict poor responders with a cutoff value of 9.665 (AUC = 63.1%, $P < 0.05$). The combination of the trigger day FSH/LH ratio with the basal FSH/LH ratio provided higher confidence in the prediction of poor responders, with a higher AUC (AUC = 78.1%, $P < 0.05$). The subgroup with a higher FSH/LH ratio (> 9.665) showed lower ovarian reserve, with a significantly lower AMH. However, the FSH level on the trigger day was significantly higher in this subgroup, which might result from the higher starting and total doses of Gn. This could be accounted for by a higher dose of rFSH leading to higher serum FSH levels and a slightly greater oocyte yield (32, 33). Therefore, the number of oocytes retrieved, fertilized oocytes and the number of total available embryos were higher in this subgroup. The proportion of poor responders was also smaller. These results indicated that appropriately increased amount of Gn for poor responders can improve the number of oocytes retrieved and total number of available embryos. Thus, our findings indicate that the trigger day FSH/LH ratio shows complementary value for predicting the poor responders.

Based on the changes in the FSH/LH ratio during COS, we then constructed a nomogram model to predict the risk of a poor response or poor reproductive potential in women undergoing GnRH-ant protocol (Figure 3). For instance, for a 30-year-old woman with AMH 1.5 ng/ml, BMI 22.5, total AFC 5, basal FSH/LH ratio > 1.875, SD6 FSH/LH ratio > 4.14; trigger day FSH/LH ratio < 9.665, the risk of poor ovarian response was approximately 50%, and the risk of poor reproductive potential was approximately 75%. Thus, further studies are warranted to verify and optimize this model for accurate prediction of outcomes in women receiving the GnRH-ant regimen.

In terms of clinical outcomes, there were no significant differences in the basal and SD6 FSH/LH ratio cutoff values among the subgroups, suggesting that a higher FSH/LH ratio in the early follicle phase does not affect endometrial receptivity. However, all the clinical outcomes in the subgroup with a higher FSH/LH ratio on the trigger day were significantly inferior compared with those of the subgroup with a lower FSH/LH ratio at the same time-point, most likely due to the significantly higher P4 level and lower endometrium thickness on the trigger day. Thus, for those patients, we do not recommend fresh embryo transfer in that cycle.

The limitations of this study should be noted. First, the potential bias of the study design cannot be excluded. Second, only a small number of patients underwent fresh embryo transfer, which might affect the clinical outcomes. Therefore, a prospective study of a larger number of patients is required to confirm our findings.

In conclusion, our findings indicate that the basal FSH/LH ratio provides the most prediction of the ovarian response and embryological outcomes in women undergoing the GnRH-ant regimen. The FSH/LH ratio at SD6 and the trigger day of COS can be used as secondary indicators for guiding the regimen. The cutoff value of the FSH/LH ratio at the starting day and SD6 can be used as guide to adjust the regimen and improve clinical outcomes.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Ethics Committee of Ruijin Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

BX, AZ and DZ conception and design, review and final approval of the version to be published. JL analyses the data. SZ and HX draft and revise the article. XW and LX collect and analyze the data. 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

Supplementary material

The The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1093954/full#supplementary-material

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Long-acting recombinant human follicle-stimulating hormone (SAFA-FSH) enhances spermatogenesis

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Introduction: Administration of follicle-stimulating hormone (FSH) has been recommended to stimulate spermatogenesis in infertile men with hypogonadotropic hypogonadism, whose sperm counts do not respond to human chorionic gonadotropin alone. However, FSH has a short serum half-life requiring frequent administration to maintain its therapeutic efficacy. To improve its pharmacokinetic properties, we developed a unique albumin-binder technology, termed "anti-serum albumin Fab-associated" (SAFA) technology. We tested the feasibility of applying SAFA technology to create long-acting FSH as a therapeutic candidate for patients with hypogonadotropic hypogonadism.

Methods: SAFA-FSH was produced using a Chinese hamster ovary expression system. To confirm the biological function, the production of cyclic AMP and phosphorylation of ERK and CREB were measured in TM4-FSHR cells. The effect of gonadotropin-releasing hormone agonists on spermatogenesis in a hypogonadal rat model was investigated.

Results: In in vitro experiments, SAFA-FSH treatment increased the production of cyclic AMP and increased the phosphorylation of ERK and CREB in a dose-dependent manner. In animal experiments, sperm production was not restored by human chorionic gonadotropin treatment alone, but was restored after additional recombinant FSH treatment thrice per week or once every 5 days. Sperm production was restored even after additional SAFA-FSH treatment at intervals of once every 5 or 10 days.

Discussion: Long-acting FSH with bioactivity was successfully created using SAFA technology. These data support further development of SAFA-FSH in a clinical setting, potentially representing an important advancement in the treatment of patients with hypogonadotropic hypogonadism.

KEYWORDS

FSH, gonadotrophin replacement therapy, hypogonadotropic hypogonadism, infertility, testis

1 Introduction

Hypogonadotropic hypogonadism (HH) is associated with decreased secretion of the gonadotropins, luteinizing hormone and follicle-stimulating hormone (FSH), resulting in deficiency in both testosterone and spermatogenesis (1). A logical approach for fertility induction in men with HH is to replace gonadotropin-releasing hormone (GnRH) in a pulsatile manner (2). However, this approach is not effective in patients with primary pituitary disease and is limited by the availability of drugs and suitable infusion devices capable of delivering pulsatile GnRH (3). Importantly, fertility outcomes are similar between pulsatile GnRH and gonadotrophin replacement therapies (4). Therefore, gonadotrophin replacement therapy is the most common approach for fertility induction in men with HH.

In practice, monotherapy with human chorionic gonadotropin (hCG) (2000 IU thrice per week), which has the biological activity of luteinizing hormone but a longer half-life in circulation, may be sufficient for the stimulation of spermatogenesis. However, hCG monotherapy is less successful in men who lack testicular development (5). If sperm counts do not respond to hCG alone, FSH (75–300 IU thrice per week) should be added to the regimen in the form of highly purified urinary gonadotropins or recombinant FSH (2, 6). FSH pretreatment followed by combination treatment with hCG and FSH appears to improve fertility in a subset of men with severe HH (7).

Owing to its short half-life, FSH needs to be injected multiple times per week for extended periods, from several months to years. The inconvenience of repeated injections is a burden to patients and hence treatment with fewer injections may be more favorable and result in fewer medication errors and improved adherence (8). Therefore, over the past decades, several different approaches have been used to generate FSH preparations with different pharmacokinetic profiles, particularly to reduce the frequency of administration (9, 10). Corifollitropin alfa, the longest-acting FSH available in clinics, has an approximately 1.5- to 2-fold longer elimination half-life than FSH (9). Previous studies have shown that long-acting FSH can effectively and safely replace FSH in the treatment regimen of adult men with HH desiring improved fertility (8, 11).

We previously developed a unique albumin-binder technology that profoundly extended the half-life of a therapeutic protein, termed “anti-serum albumin Fab-associated” (SAFA) technology (12, 13). Using this technology, a long-acting recombinant human interferon beta (at least 2-fold longer serum half-life in rats and monkeys) for multiple sclerosis and a long-acting feline granulocyte colony-stimulating factor (approximately 5-fold longer serum half-life in cats) were developed (14, 15). In the present study, we tested the feasibility of applying SAFA technology to develop long-acting FSH as a therapeutic candidate for patients with HH.

2 Materials and methods

2.1 SAFA-FSH production and purification

SAFA-FSH was produced by CHO glutamine synthetase null$^{-/-}$ K1 cell (Horizon Discovery, Waterbeach, UK) and purified using a three step purification protocol—capture for affinity chromatography, intermediate purification for multimodal chromatography, and polishing for cation exchange chromatography, as described previously (14, 15). After purification, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and size-exclusion high-performance liquid chromatography (SE-HPLC) under native conditions were used to determine the apparent molecular weight and purity.

2.2 Cell culture

TM4 cells, a mouse Sertoli cell line, were purchased from the Korean Cell Line Bank (KLCB No. 21715; Seoul, South Korea). 293FT cells were purchased from Invitrogen (Carlsbad, CA, USA). Cells were cultured in Dulbecco’s Modified Eagle’s medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 1% penicillin/streptomycin (Hyclone) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

2.3 Plasmid construction and stable cell line generation

The human FSH receptor gene was inserted into the lentiviral vector pLECE3-Green Fluorescent Protein (GFP) using the HpaI/NotI restriction sites to generate pLECE3-hFSHR-GFP. Lentiviral particles were generated using three plasmids, VSVG, RSV-REV, and PMDLg/pPRE, in HEK293FT cells cotransfected with pLECE3-hFSHR-GFP. Cells were transfected using polyjet transfection reagent (SignaGen Laboratories, Frederick, MD, USA). Two days after transfection, the culture medium was harvested and sterilized using a 0.45 µm syringe filter. Purified lentiviral particles were used to infect TM4 cells. Three days after infection, cells were separated from GFP-positive single cells into a 96-well plate using BD LSRFortessa (Becton Dickinson, Franklin Lakes, NJ, USA).

2.4 Measurement of cyclic AMP (cAMP) production

TM4 and TM4-FSHR cells were seeded (0.3×10^6 cells/plate) and cultured in 6 cm plates. The next day, after starvation for 4 h, the cells were stimulated with recombinant FSH (Follitrope, LG Chem, Seoul, South Korea) or SAFA-FSH at different concentrations in 0.5 mM isobutylmethylxanthine (Sigma-Aldrich, St. Louis, MO, USA) for 15 min at 37°C. The reaction was stopped by aspirating the medium and washing twice with cold Dulbecco’s phosphate-buffered saline (DPBS). Cells were lysed with 80 µL of lysis buffer and incubated on ice for 30 min. Cell lysates were obtained by centrifugation at 14000 ×g for 10 min at 4°C. cAMP concentrations were measured using a cAMP XP assay kit (Cell Signaling Technology, Danvers, MA, USA). In each experiment, a standard curve was generated and used to calculate the cAMP concentration.

2.5 Western blotting

Whole cell lysates were prepared, and the assay was carried out according to standard procedures. Cells were washed twice with cold DPBS and lysed in lysis buffer containing 1 mM phenylmethylsulfonyl fluoride and a 1× protease inhibitor cocktail (Sigma-Aldrich). The lysates were centrifuged at 12000 rpm for 15 min at 4°C, and the supernatants obtained were used for analysis. The protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Equal quantities of protein were loaded onto Bolt 4–12% Bis-Tris Plus Gels (Invitrogen) and separated at 200 V for 35 min. Proteins were transferred onto polyvinylidene fluoride membranes (Invitrogen) using a Power Blotter (Invitrogen). The membranes were blocked with EveryBlot blocking buffer (Bio-Rad, Hercules, CA, USA) for 15 min at 24°C. Membranes were incubated with the following primary antibodies for 1 h at 24°C: rabbit anti-CREB (1:1000), rabbit anti-phospho CREB (1:1000), mouse anti-ERK (1:2000), and rabbit anti-phospho ERK (1:2000). Primary antibodies were purchased from Cell Signaling Technology. Blots were washed six times for 5 min with Tris-buffered saline containing 0.05% Tween 20 and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG or anti-rabbit IgG secondary antibodies (1:2000, Thermo Fisher Scientific) for 1 h at 24°C. Immunoreactivity was detected with Amersham ECL (Cytiva, Marlborough, MA, USA) using an iBright 1500 (Invitrogen). The intensity of the protein bands was quantified using iBright analysis and normalized to β-actin in each sample.

2.6 Animal experimental design

Male Sprague-Dawley (SD) rats were purchased from Orient Bio (Seoul, South Korea). The animals were maintained under controlled conditions (22°C, 12 h light, 12 h dark cycle) and received rodent chow and tap water. The animals were acclimatized for 1 week prior to the study.

For the pharmacokinetic study, recombinant FSH (88 μg/kg) (Group 1), SAFA-FSH (200 μg/kg) (Group 2), and SAFA-FSH (600 μg/kg) (Group 3) were injected intravenously into 8-week-old male SD rats (n = 5 in each group). The dosing concentration for Group 2 (SAFA-FSH, 200 μg/kg) was determined to adjust the approximate equivalent molar ratio compared to recombinant FSH, whereas that for Group 3 (SAFA-FSH, 600 μg/kg) was determined to confirm the dose-dependent behavior of SAFA-FSH. Blood samples were collected at a predetermined time, and the plasma concentrations of recombinant FSH and SAFA-FSH were determined by quantitative enzyme-linked immunosorbent assay (ELISA). Briefly, an ELISA plate (Greiner Bio-One, Kremsmünster, Austria) was coated with a mouse anti-human FSH beta Antibody (100 ng/well) for 16 h at 4°C using a carbonated coating buffer (pH 9.6), followed by treating with a Starting Block buffer (Thermo Fisher Scientific) at 25°C for 3 h. After washing the plates, the appropriately diluted serum samples and standards were aliquoted into each well of the plate, followed by incubation at 25°C for 2 h. After washing the plates, a mouse anti-human FSH alpha-biotinylated Antibody (Bio-Rad) was aliquoted and incubated at 25°C for 1 h. After washing again, a Pierce™ high sensitivity streptavidin-HRP was added to each well, and the TMB substrate (Surmodics, Eden Prairie, MN, USA) was added to react with HRP. Finally, 1 N hydrochloric acid (Daejung Chemicals, Siheung, South Korea) was added into each well to quench the reaction and the absorbance at 450 nm was measured using a microplate reader (BMG LABTECH, Ortenberg, Germany). The pharmacokinetic parameters were evaluated Phoenix® WinNonlin® (Ver. 8.1, Certara, Princeton, NJ, USA). Meanwhile, the pharmacokinetic study was performed by KNOTUS Co., Ltd. (Incheon, South Korea).

For the pharmacodynamic study, 5-week-old male SD rats were randomly divided into seven groups (n = 5–6 in each group) based on body weight and two rats were housed per cage. Body weight was measured twice per week to record weight gain. Rats received subcutaneous injections of Diphereline (5 mg/kg, Ipsen, Paris, France) at the start of the experiment and 3 weeks later to ensure suppressed production of gonadotropin in all groups, except for Group 1 (control) (Figure 1). Subsequently, hCG (20 IU/kg, LG Chem) was injected subcutaneously thrice per week in all groups, except for Groups 1 and 2 (Diphereline only) for approximately 9 weeks. Groups 1 and 2 were injected with saline instead of drugs. Additional recombinant FSH (25 IU/kg = 1.85 μg/kg) was injected subcutaneously thrice per week (Group 4) or once every 5 days (Group 5). Additional SAFA-FSH (9.87 μg/kg, thrice more than recombinant FSH when considering the molecular weight) was injected subcutaneously once every 5 days (Group 6) or once every 10 days (Group 7). The rats were sacrificed after 9 weeks of hCG and FSH injections. The Institutional Animal Care and Use Committee (IACUC, Yonsei University Health System) approved the study protocol (approval number 2021-0087).

2.7 Testosterone measurement

Serum testosterone levels were measured weekly from 1 week after the first Diphereline injection. Blood samples were collected from the orbital sinus using capillary tubes after anesthesia at the same time every week, and only serum was isolated by centrifugation at 3000 rpm for 10 min at 4°C. Testosterone levels were measured by Seoul Clinical Laboratories (SCL, Yongin, South Korea) using an electrochemiluminescence assay (ECLIA, Roche Diagnostics, Indianapolis, IN, USA).

2.8 Sperm count

Sperm was collected from the epididymis cauda. The cauda was placed in a 6 cm plate with 10 mL DPBS and minced with a scalpel, allowing sperm to be dispersed in saline and incubated for 15 min at 37°C under 5% CO_2. The mixture was heated at 60°C for 1 min and then cooled to 24°C. The dispersed sperm solution was diluted appropriately to increase the accuracy of the sperm count. Sperms

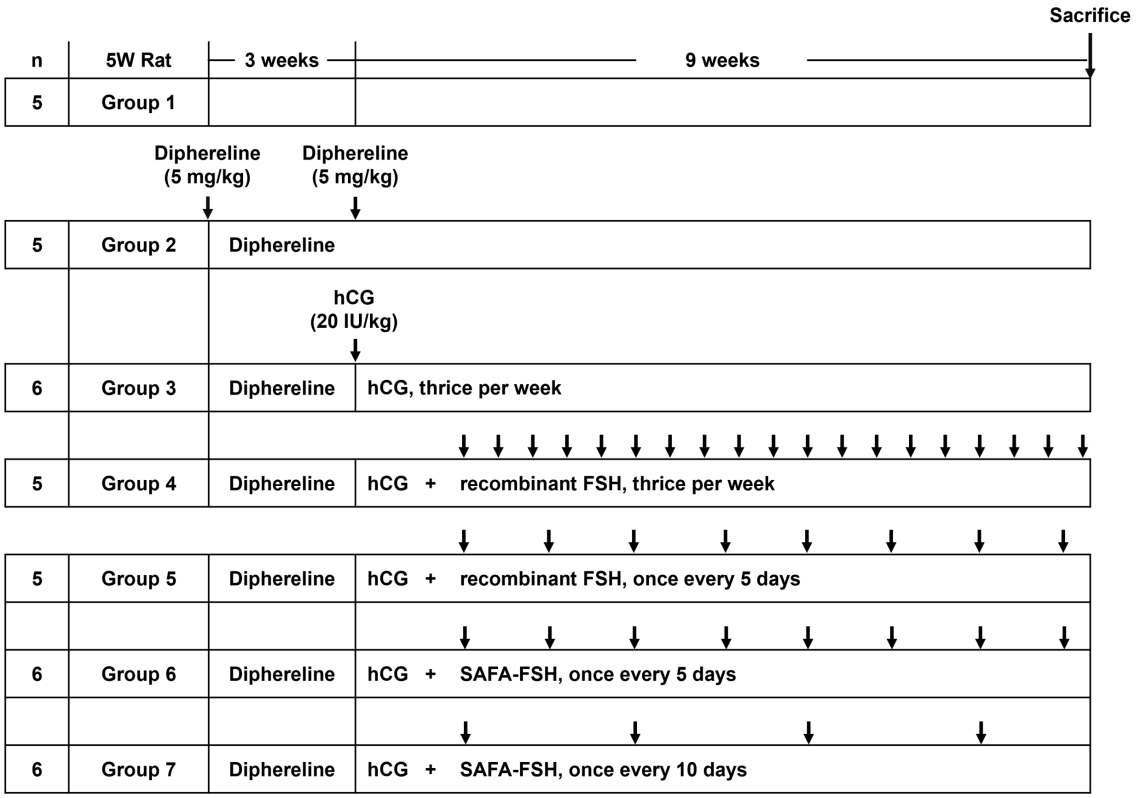

FIGURE 1
Time course of Sprague-Dawley (SD) rat *in vivo* experiments per group. Arrows indicate the injection cycle of each drug. FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; SAFA, anti-serum albumin Fab-associated.

were counted and evaluated using a Neubauer hemocytometer (INCYTO, Cheonan, South Korea).

2.9 Statistical analysis

IBM SPSS Statistics version 25 (IBM, Armonk, NY, USA) was used for all statistical analyses. Data are presented as mean ± standard error of the mean. Statistical significance was determined using the Mann–Whitney U test. Differences were considered statistically significant at $P < 0.05$.

3 Results

3.1 SAFA-FSH characterization

SAFA-FSH, a long-acting recombinant human FSH, is shown in Figure 2A. SAFA-FSH is a protein composed of two subunits, alpha and beta subunits, which are bound to the light and heavy chain in the Fab, respectively. Using SDS–PAGE under non-reducing conditions, SAFA-FSH appeared as a single band by Coomassie Blue staining, with a molecular weight between 60 and 75 kDa (Figure 2B). The average molecular mass of SAFA-FSH was approximately 1.78-fold greater than that of recombinant FSH (non-reduced 71 versus 40 kDa) as the molecular weight of the SAFA region is approximately 50 kDa. The purity of the SAFA-FSH used in our study was > 95%, as determined by SE-HPLC (Figure 2C).

3.2 *In vitro* functional assays

To assess the bioactivity of SAFA-FSH versus recombinant FSH *in vitro*, TM4 cells were treated with different doses of recombinant FSH, and intracellular cAMP levels were measured by ELISA. However, the concentration of cAMP did not increase, regardless of recombinant FSH concentration (Figure 3A). Additional FSH treatment experiments could not be performed in TM4 Cells. Therefore, TM4-FSHR cells were generated by overexpression of the *FSHR* gene using lentivirus in TM4 cells and were treated with recombinant FSH or SAFA-FSH at different concentrations. Unlike in TM4 cells, as the concentration of FSH increased, cAMP concentration increased in a dose-dependent manner (Figures 3B, C). The effect of SAFA-FSH was lower than that of recombinant FSH when treated with similar concentrations of FSH. Calculating the dose needed to achieve a similar cAMP level indicated that thrice more SAFA-FSH than recombinant FSH was required, demonstrating a considerable difference in the relative bioactivity of the two compounds.

Moreover, the molecular responses stimulated by recombinant FSH or SAFA-FSH in TM4-FSHR cells were analyzed by detecting

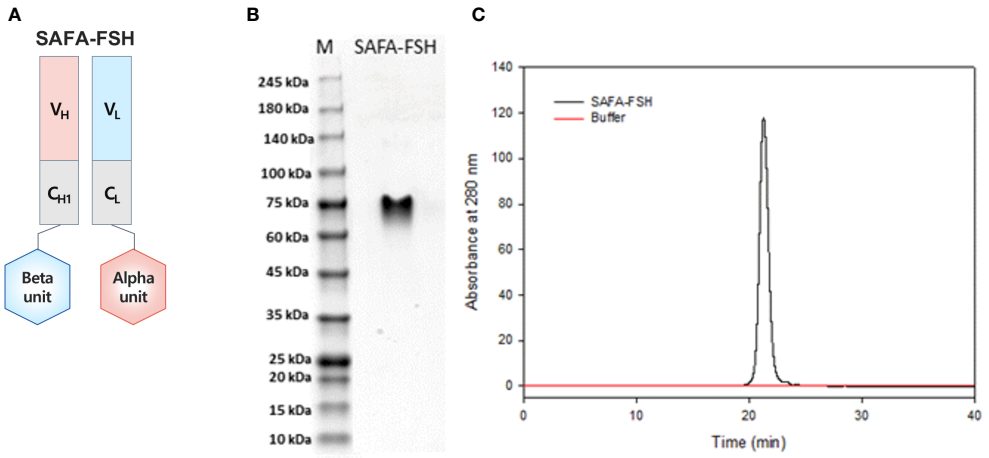

FIGURE 2
Anti-serum albumin Fab-associated (SAFA)-follicle-stimulating hormone (FSH) characterization. **(A)** Illustration of SAFA-FSH. **(B)** Sodium dodecyl sulfate–polyacrylamide gel electrophoresis under the non-reducing condition. The protein bands were visualized by using Coomassie Blue staining. **(C)** Size-exclusion high-performance liquid chromatography under the native condition. Elution was monitored using UV absorption at 280 nm. V, variable region; C, constant region; H, heavy chain; L, light chain.

cAMP-related signaling molecules by assessing ERK and CREB activation, i.e., phosphorylation. When TM4-FSHR cells were treated with recombinant FSH or SAFA-FSH at different concentrations of two points, the phosphorylation level of ERK was higher than that of the negative control (Figures 4A, B). Likewise, when TM4-FSHR cells were treated with recombinant FSH or SAFA-FSH, CREB phosphorylation increased expectedly (Figures 4C, D). Both recombinant FSH and SAFA-FSH increased phosphorylation compared to the control; however, the increase noted with recombinant FSH was not concentration dependent. These results suggest that SAFA-FSH has the same effect on the signaling pathway as recombinant FSH.

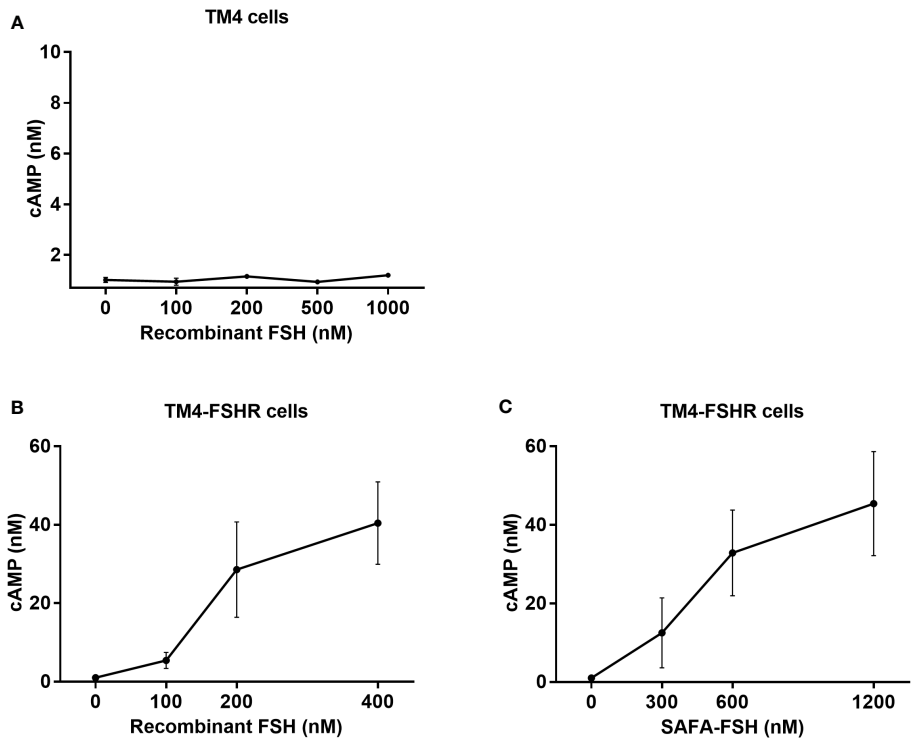

FIGURE 3
Cyclic adenosine monophosphate (cAMP) production for recombinant follicle-stimulating hormone (FSH) or anti-serum albumin Fab-associated (SAFA)-FSH *in vitro*. **(A)** cAMP production of TM4 cells treated with recombinant FSH at indicated dose. **(B, C)** cAMP production of TM4-FSHR cells treated with recombinant FSH or SAFA-FSH at indicated doses. Line plots indicate the mean ± SEM of three independent experiments.

3.3 *In vivo* pharmacokinetic assays

The pharmacokinetic profiles of SAFA-FSH were studied using a rat model that was administered a single intravenous injection. Recombinant FSH was used in this study as a reference molecule. As shown in Figure 5, the high concentration of SAFA-FSH was maintained for much longer than that of recombinant FSH, and the pharmacokinetic parameters revealed that SAFA-FSH had an approximately 2.7-fold longer serum half-life than recombinant FSH ($t_{1/2}$ = 27.1–29.3 h vs. 10.4 h) (Table 1). In the case of T_{max} values, SAFA-FSH showed T_{max} = 0.08–0.12 h and recombinant FSH showed T_{max} = 0.08 h, suggesting that SAFA-FSH enters the blood circulation similar to recombinant FSH. C_{max} values were 68292.8–252519.4 pM for SAFA-FSH and 57431.6 pM for recombinant FSH, and renal clearance (CL) rates were 0.00028–0.00041 μg/(h × pM)/kg for SAFA-FSH and 0.00078 μg/(h × pM)/kg for recombinant FSH. In the case of AUC_{inf}, SAFA-FSH showed 4.5- to 19-fold higher value than recombinant FSH (505688.6–2142357.8 h × pM vs. 113565.0 h × pM), indicating the sustained bioavailability of SAFA-FSH.

3.4 *In vivo* functional assays

To confirm the function of SAFA-FSH as a long-acting hormone, the GnRH agonist Diphereline was injected into male rats to suppress the production of gonadotropin and create an animal model of hypogonadism. Rats were then treated with recombinant FSH or SAFA-FSH. There was no difference in body weight between the groups during the study period. There was no difference in mean serum testosterone levels in all groups during weeks 1–4 because the rats were adolescent (Figure 6A). At weeks 5–8, the mean serum

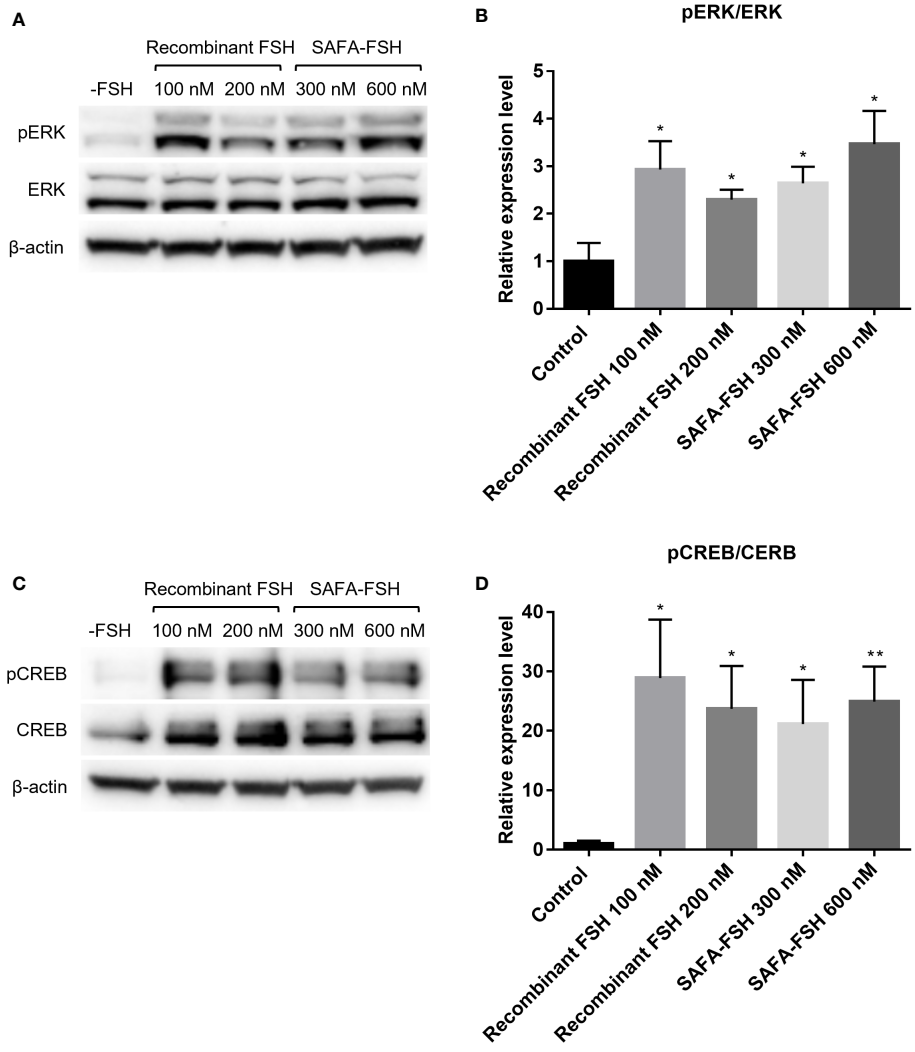

FIGURE 4
Protein expression for recombinant follicle-stimulating hormone (FSH) or anti-serum albumin Fab-associated (SAFA)-FSH *in vitro*. **(A)** Representative western blot analysis of protein extracts from TM4-FSHR cells showing the levels of ERK and pERK. **(B)** Relative ERK activity was derived as pERK normalized to ERK. **(C)** Representative western blot analysis of protein extracts from TM4-FSHR cell showing the levels of CREB and pCREB. **(D)** Relative CREB activity was derived as pCREB normalized to CREB. Each sample was normalized with β-actin. Data are representative of four independent experiments and are presented as mean ± standard error of mean (SEM). *P < 0.05, **P < 0.01 versus control.

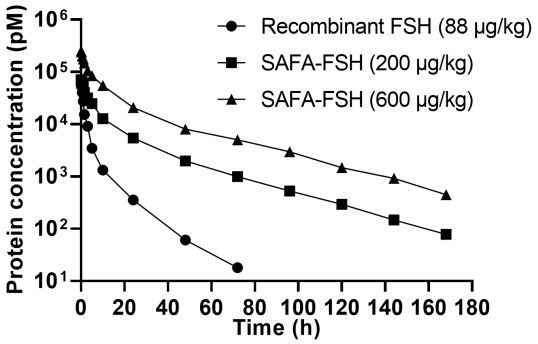

FIGURE 5
Pharmacokinetics of anti-serum albumin Fab-associated (SAFA)-follicle-stimulating hormone (FSH) in rats.

testosterone level was elevated in Group 1 (Figure 6B). However, the mean serum testosterone level in Group 2 was lower than that in Group 1, implying that the hypogonadism model was well established in male SD rats. Compared with Group 2, Groups 3–7, which received hCG, showed an increase in the mean serum testosterone level. In weeks 9–12, the mean serum testosterone level in Group 2 remained low when compared with that in Group 1. This suggests that the hypogonadism animal model continued to be well-maintained. Compared to Group 2, Groups 3–7 still showed an increase in mean serum testosterone levels. The mean serum testosterone levels in Groups 5 and 6 were higher than those in Group 1. In weeks 9–12, the mean serum testosterone level in Group 2 remained low when compared with that in Group 1 (Figure 6C).

After 9 weeks of hCG and FSH injection, the rats were sacrificed, and testis were weighed. The testis weights were lower in Groups 2, 3, and 7 than in Group 1 (Figure 7A) and were higher in Groups 3–7 than in Group 2. The testis coefficient (testis/body weight ratio) values also showed similar results (Figure 7B). The total number of sperms in Group 2 was reduced by 68% compared with that in Group 1 (Figures 7C, D). Group 3 did not show restoration compared to Group 2. However, sperm production was restored after additional recombinant FSH treatment at intervals of thrice per week (Group 4) or once every 5 days (Group 5). Sperm production was restored even after additional SAFA-FSH treatment at intervals of once every 5 days (Group 6) or once every 10 days (Group 7). These results suggest that even when SAFA-FSH was injected over a longer cycle than recombinant FSH, sperm production was restored in a rat model of hypogonadism.

4 Discussion

Peptide hormones typically have short circulatory half-lives due to their rapid clearance from circulation. At the clinical level, frequent peptide hormone injections are required, which cause considerable discomfort to the patient (16). Thus, there is a need for technologies that can prolong the half-life of peptide hormones while maintaining high pharmacological efficacy. Long-acting growth hormones, a leader in the development of long-acting hormones, have been or are currently being developed in various ways to improve compliance (17). According to GlobalData's recent report (Report Code: GDHCOA002), long-acting growth hormones will account for over 90% of the growth hormone deficiency market share across the US, Germany, and Japan by 2030. However, for peptide hormones other than growth hormones, continuous developmental research is not being actively conducted.

Corifollitropin alfa, a long-acting FSH that is currently available clinically, is indicated for controlled ovarian stimulation in combination with a GnRH antagonist for the development of multiple follicles in women undergoing fertility treatment and for treatment in adolescent men with HH in combination with hCG (18). Although corifollitropin alfa has a long half-life, 7 days after its injection, daily recombinant FSH injections should be continued until the criterion for triggering final oocyte maturation in women has been met. Its half-life is not long enough to sustain the entire therapeutic period. Corifollitropin alfa should be administered once every 2 weeks in combination with hCG injections twice weekly in men with HH (11). We attempted to apply SAFA technology to invent long-acting biologics using FSH as a model. It is expected to have a half-life of approximately 2 weeks in humans, and if development is successful, it is expected that additional daily recombinant FSH administration will not be required in women

TABLE 1 Pharmacokinetic parameters for subcutaneous administration of recombinant FSH and SAFA-FSH in SD rats.

Parameters	Recombinant FSH (88 μg/kg)	SAFA-FSH (200 μg/kg)	SAFA-FSH (600 μg/kg)
C_{max} (pM)	57431.6	68292.8	252519.4
T_{max} (h)	0.08	0.08	0.12
AUC_{last} (h × pM)	113291.1	502616.4	2123006.7
AUC_{inf} (h × pM)	113565.0	505688.6	2142357.8
$T_{1/2}$ (h)	10.4	27.1	29.3
CL (μg/(h × pM)/kg)	0.00078	0.00041	0.00028
AUC_{ext} (%)	0.2	0.6	0.9

SD, Sprague-Dawley; FSH, follicle-stimulating hormone; SAFA, anti-serum albumin Fab-associated; C_{max}, maximum concentration; T_{max}, time to reach C_{max}; AUC_{last}, area under the curve to last measurable concentration; AUC_{inf}, area under the curve to time infinity; $T_{1/2}$, biological half-life; CL, clearance rate; AUC_{ext}, extrapolated area under the curve, $[(AUC_{inf} - AUC_{last})/AUC_{inf}] \times 100$.

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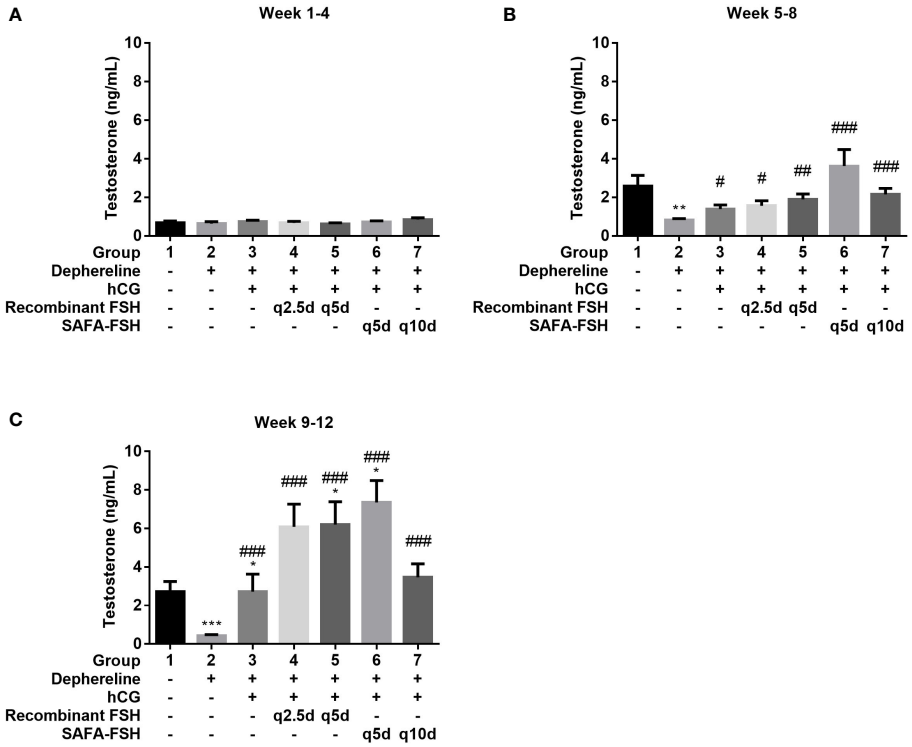

FIGURE 6
Mean serum testosterone level. **(A)** Week 1–4, **(B)** Week 5–8, and **(C)** Week 9–12. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 versus Group 1. #P < 0.05, ##P < 0.01, and ###P < 0.001 versus Group 2. FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; SAFA, anti-serum albumin Fab-associated.

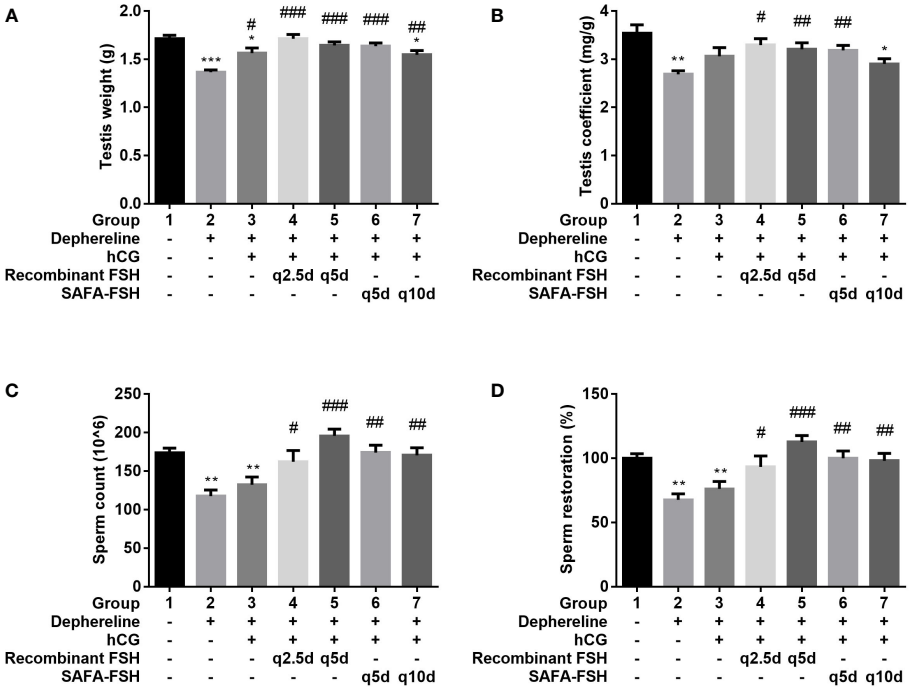

FIGURE 7
Effect of recombinant follicle-stimulating hormone (FSH) or anti-serum albumin Fab-associated (SAFA)-FSH on spermatogenesis in hypogonadism rats. **(A)** Testis weight, **(B)** Testis coefficient, **(C)** Sperm count, and **(D)** Sperm restoration. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 versus Group 1. #P < 0.05, ##P < 0.01, and ###P < 0.001 versus Group 2. hCG, human chorionic gonadotropin.

and longer dosing intervals are expected in men. SAFA can cross-react with serum albumin from various species, including cynomolgus monkeys and rats, implying that SAFA or its derivatives can be assessed using animal models in a preclinical setting (12).

In the present study, we generated SAFA-FSH, a long-acting recombinant human FSH. We also investigated the bioactivity of SAFA-FSH, both *in vitro* and *in vivo*. To confirm the applicability of SAFA-FSH, we investigated *in vitro* cell activation using recombinant FSH or SAFA-FSH in FSH receptor-expressing cells; the FSH receptor is expressed in TM4 cells (19). As the FSH receptor is a G protein-coupled receptor, it was expected that cAMP would increase when FSH was administered (20). cAMP-related signaling involves ERK and CREB activation (21). In Sertoli cells, cAMP binds to protein kinase A and mediates ERK phosphorylation (22). However, TM4 cells did not respond well to recombinant FSH stimulation. TM4 cells are reported to respond to FSH with an increase in cAMP production, but the FSH responsiveness is much reduced compared to primary Sertoli cell cultures (23). Unlike freshly prepared Sertoli cells, which expressed abundant FSH receptors, both primary cultured Sertoli cells and Sertoli cell lines express very low levels of FSHR (24). Likewise, when the expression level of FSHR in TM4 cells was confirmed by quantitative PCR, the expression level of FSHR was very low. Therefore, we created TM4-FSHR cells by overexpressing the *FSHR* gene and confirmed that cAMP levels and phosphorylation of ERK and CREB were increased by treatment with recombinant FSH or SAFA-FSH, respectively. These results clearly indicated that recombinant FSH and SAFA-FSH had the same effect on the signaling pathway. The bioactivity of SAFA-FSH seemed to be approximately 3-fold lower than that of recombinant FSH when considering the molecular weight, implying that the fusion of FSH to SAFA might slightly interfere with the biological activity of FSH, probably due to steric hindrance.

In our pharmacokinetic experiments using a rat model, the $t_{1/2}$ of SAFA-FSH in rats after a single dose was approximately 2.7-fold greater at 27.1–29.3 h. SAFA-FSH displays a prolonged kinetic profile due to the presence of human anti-serum albumin Fab by utilizing the neonatal Fc receptor recycling mechanism similar to other albumin binders (12, 25, 26). However, since SAFA technology combines the Fab antibody fragment that binds to human serum albumin with high affinity, the half-life in rats is inevitably lower than that in humans. Of course, its half-life is longer than that of recombinant FSH, but direct comparison with humans is likely to be difficult. Considering that the serum half-life of endogenous rat serum albumin is approximately 46 h and a previous unpublished pharmacokinetic study using a non-human primate model showed that the serum half-life of SAFA-anti-CD40L is approximately 10 days, SAFA may have a serum half-life of approximately 2 weeks in humans, although this calculation is only an assumption (12, 27).

In vivo, we investigated the effect SAFA-FSH on spermatogenesis in a rat model of hypogonadism. Through several preliminary experiments, we successfully created a hypogonadism model in male SD rats, whose sperm counts did not respond to hCG alone (28). We confirmed that sperm production could be restored when SAFA-FSH was injected over a longer cycle than that when recombinant FSH was injected. The injection cycle of SAFA-FSH could be extended to once every 10 days, which is approximately four times longer than that of recombinant FSH treatment at intervals of thrice per week. The increase in testis weight and sperm count observed in this study suggests that SAFA-FSH could effectively replace recombinant FSH in the treatment regimen of adult men with HH desiring fertility (8). However, sperm production was unexpectedly restored after recombinant FSH treatment at intervals of once every 5 days. Although we showed that SAFA-FSH was effective when administered once every 10 days, comparative experiments with a wider injection cycle are needed in the future. The assays in this study were performed only on animals. Clinical trials are needed to determine whether these results can be extrapolated to humans. SAFA-FSH is a valuable alternative to recombinant FSH and may have great potential for therapeutic applications.

SAFA-FSH is intended to replicate the mechanism of the current thrice per week recombinant FSH treatments used to enhance spermatogenesis, but with a reduced number of administrations. It is clear from our study that this newly developed sustained FSH exerts pharmacological and physiological effects similar to those of recombinant FSH at the FSH receptor. SAFA-FSH can also be applied to women undergoing infertility treatment through comparative experimental analysis with recombinant FSH for ovarian weight gain and ovulation. The half-life of SAFA-FSH is expected to be sufficiently long to sustain the entire therapeutic period (29, 30).

This study had several limitations. First, the bioactivity of SAFA-FSH was lower than that of recombinant FSH, probably because of steric hindrance. Although recombinant FSH was used as a commercial material, SAFA-FSH was tested with an experimental material with a purity of > 95%. The manufacturing of high-purity materials and optimal formulation design for long-term storage are required. Second, we used genetically modified TM4-FSHR cells which may have altered their responsiveness to stimuli. Primary cultures of Sertoli cells provide an interesting model to study how signaling pathways induced (22, 31–33). However, primary cultured Sertoli cells frequently do not maintain their functions for prolonged periods of time in culture. The purpose of our *in vitro* study was to assess the bioactivity of SAFA-FSH versus recombinant FSH, and showed SAFA-FSH can activate cells similar to recombinant FSH. Third, we could not show that the half-life was very long, as expected from the pharmacokinetic study in rats. Considering the half-life of human serum albumin (3 weeks), the expected half-life of SAFA-FSH in humans is much longer because the half-life of SAFA-FSH in our study was slightly shorter than that of endogenous rat serum albumin (34). Fourth, safety tests were not conducted. SAFA is a substance of human origin and is expected to have no side effects, such as platelet activation, due to the absence of an Fc domain. However, further studies are required to confirm this. Fifth, unexpectedly, we observed that recombinant FSH, which was used as a reference, restored sperm count at intervals of once every 5 days and hence, should be compared at intervals of once every 10 days. Although further comparative studies are needed for confirmation, we

showed that the injection cycle of SAFA-FSH can be extended to once every 10 days. Owing to its different kinetic profile, SAFA-FSH can replace multiple injections of FSH by promoting sustained sperm production.

In conclusion, SAFA-FSH can activate cells similar to recombinant FSH, and because it has a longer half-life, the drug concentration is maintained without frequent injections compared to recombinant FSH, thereby increasing the utility of long-acting hormone preparations. In this study, we demonstrated the successful development of SAFA-FSH using SAFA technology and believe that our approach can provide many benefits to patients in the future by generating highly effective and long-acting therapeutic biologics at reasonably affordable prices.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC, Yonsei University Health System, approval number 2021-0087).

Author contributions

DK, CK, MS, SH-C, and EL contributed to the study conception and design. SL, YC, MK, HC, JA, KL, JH, and SC organized the database. SL performed the statistical analyses. DK wrote the first draft of the manuscript. DK and SL wrote sections of the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

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Conflict of interest

At the time of publication, HC, JA, KL, JH, SC, MS, and S-HC were employees of AprilBio Co., Ltd Chuncheon, South Korea.

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

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Letrozole cotreatment improves the follicular output rate in high-body-mass-index women with polycystic ovary syndrome undergoing IVF treatment

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Background: Women who have polycystic ovary syndrome (PCOS) with high body mass index (BMI) typically have an attenuated ovarian response and decreased follicular size, which are linked to unfavourable clinical outcomes following *in vitro* fertilization (IVF) therapy. The follicular output rate (FORT), a qualitative indicator of follicular response, seems to be positively linked to the clinical outcomes of IVF. Progestin-primed ovarian stimulation (PPOS) has become an alternative to gonadotropin-releasing hormone (GnRH) analogues to inhibit the premature luteinizing hormone (LH) surge. As letrozole (LE) shows promise in enhancing ovarian response, we compared PPOS with and without LE for PCOS in high BMI women with a focus on the FORT and associated clinical and pregnancy outcomes.

Methods: For the recruited 1508 women, ten variables including AFC; age; basal sex hormone level; BMI; infertility type; period of infertility and number of previous IVF attempts were chosen in the propensity score matching (PSM) model to match 1374 women who taken the MPA+ hMG protocol with 134 women who received the MPA+ hMG+ LE treatment at a 1:1 ratio. FORT was selected as the primary outcome measure. The number of oocytes retrieved, viable embryos, hMG dosage, duration, oocyte maturity rate, fertilization rate, and implantation rate were established as secondary outcomes.

Results: FORT was substantially elevated in the MPA+hMG+LE group compared with the MPA+hMG group (61% [35%, 86%] vs. 40% [25%, 60%], P <.001). Interestingly, the LE cotreatment group had a considerably lower mature oocyte rate despite having a similar number of mature oocytes and embryos recovered. The average hMG dosages and durations in the study group were similar to those in the control group. The implantation rate in the study group was numerically higher but without statistic significant than that in the control groups (43.15% (107/248) vs. 38.59% (115/298), OR 1.008, 95% CI 0.901-1.127; P >.05).

Conclusion: The effect of LE combined with PPOS on FORT is better than the effect of the standard PPOS treatment in women with PCOS and a high BMI, but

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there is no substantially beneficial impact on pregnancy outcomes or the cycle features of COS, including consumption of hMG.

KEYWORDS

letrozole, polycystic ovarian syndrome, high body mass index, progestin-primed ovarian stimulation, follicular output rate

Introduction

Polycystic ovary syndrome (PCOS) is a prevailing form of endocrinopathy that affects women of reproductive age (1). The rates of hyperandrogenism, obesity, and primary infertility have increased dramatically among women with PCOS over the last decade, resulting in a more severe phenotype among this population (2). Infertile women with PCOS may be treated with *in vitro* fertilization (IVF), laparoscopic ovarian surgery, and behavioural, and pharmaceutical interventions (including gonadotropins, metformin, aromatase inhibitors, and clomiphene citrate (CC)) (3). IVF is regarded as a third-line therapy and is often used in cases in which tubal factors and male factors exist (3).

As an alternative to standard GnRH analogues, progestin-primed ovarian stimulation (PPOS) by administering human menopausal gonadotropin (hMG) and medroxyprogesterone acetate (MPA) simultaneously from the early follicular phase successfully inhibits the oestradiol (E2)-induced LH surge (4). The PPOS protocol could achieve similar numbers of oocytes, viable embryos, and pregnancy outcomes (5); moreover, it is more patient-friendly, as it can further reduce the injection burden compare with the conventional GnRH analogue protocol (6).

Previous studies found that increased body mass index (BMI) is probably linked to an increased risk of insufficient follicle development as well as an increased follicle-stimulating hormone (FSH) requirement in the process of ovarian stimulation for IVF (7, 8) or dysregulation of meiotic spindle formation (9) and, consequently, developmental ability (10). Preliminary data showed that in normo-cycling women, the ratio of the preovulatory follicle count (PFC) to the antral follicle count (AFC), widely recognised as the follicular output rate (FORT), is positively linked to IVF outcomes (11, 12) and is regarded as a qualitative indicator of the follicular response. For women with PCOS, especially those who are obese, letrozole (LE) is recommended as a first-line treatment option for the induction of ovulation (13). LE can improve the follicular response to FSH by elevating intrafollicular androgen levels and reducing circulating oestrogen concentrations (14). At present, LE is extensively utilised as an adjunct for IVF treatment (15). Therefore, the current retrospective cohort study was conducted to evaluate the impact of combining LE with the PPOS protocol on FORT, as well as the features of the frozen embryo transfer (FET) cycle and oocyte pick-up cycle in high-BMI women with PCOS receiving IVF treatment.

Abbreviations: PPOS, Progestin-primed ovarian stimulation; IVF, *In vitro* fertilization; ICSI, Intracytoplasmic sperm injection; FET, Frozen embryo transfer; AFC, Antral follicle count; BMI, Body mass index; hCG, Human chorionic gonadotropin; P, Progestin; LH, Luteinizing hormone; FSH, Follicle stimulating hormone; MPA, Medroxyprogesterone acetate; LE, Letrozole; PCOS, Polycystic ovarian syndrome.

Materials and methods

Patients and study setting

The research protocol for this study was approved by the Shanghai Ninth People's Hospital Ethics Committee (Institutional Review Board). Women with PCOS who completed IVF/ICSI cycles between January 2017 and September 2022 were recruited to the control group (hMG+MPA) and the study group (hMG+MPA +LE). For patients who received more than one cycle of COS within this time frame, only the first cycle was considered to prevent repeated inclusion. The patients satisfied the following conditions: 1. BMI between 25 and 37 kg/m2; 2. basal FSH level < 10 mIU/ml; 3. Age between 21 and 40 years; and 4. at most 1 previous cycle with no available embryo. According to the 2003 Rotterdam consensus (16), at least two of the following symptoms were required for women to be diagnosed with PCOS. 1) oligo- and/or anovulation; 2) ultrasonography appearance of polycystic ovaries; or 3) biochemical and/or clinical indicators of hyperandrogenism. Ultrasonography was unnecessary in cases where both hyperandrogenism and oligo- or anovulation existed. Reproductive, metabolic, and psychological factors were all considered in the assessment and management of the condition once diagnosed with PCOS. The exclusion criteria included the presence of the following disorders: hyperandrogenaemia and ovulatory dysfunction due to other aetiologies, such as thyroid disease, hyperprolactinaemia, androgen-secreting tumours, and congenital adrenal hyperplasia. Women who were currently receiving treatment for a clinical condition, for example diabetes or high blood pressure, were also eliminated. Figure 1 depicts the study process.

Controlled ovarian stimulation

Patients were given 150-225 IU/d hMG intramuscularly (Anhui Fengyuan Pharmaceutical Co., Ltd.) and 4 mg/d MPA orally

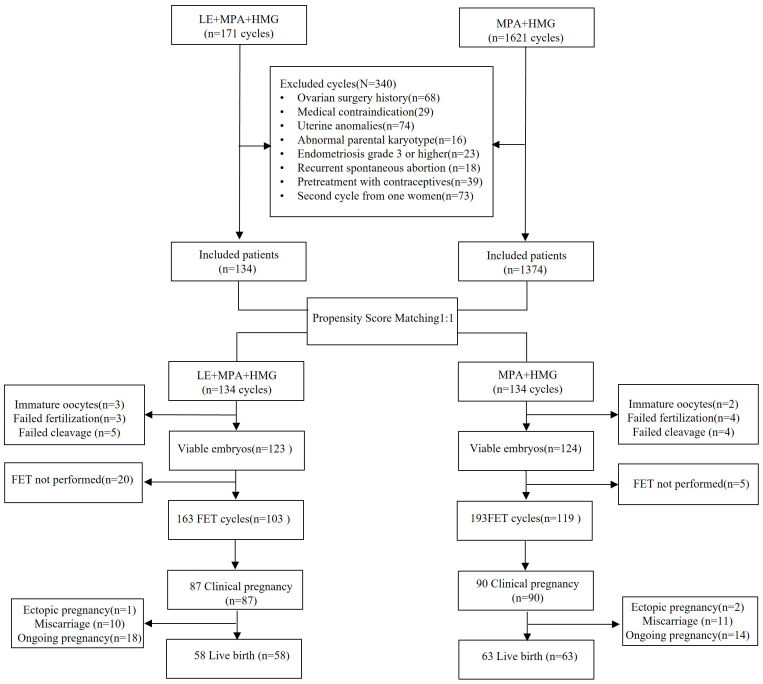

FIGURE 1
Flow chart of the study. IVF, *In vitro* fertilization; ICSI, Intracytoplasmic sperm injection; FET, Frozen embryo transfer; LE, Letrozole; MPA, Medroxyprogesterone acetate; hMG, Human menopausal gonadotropin.

(Shanghai Xinyi Pharmaceutical Co., Ltd.) from the third day of the menstrual cycle (MC3) until the trigger day. The study group was given oral LE (Jiangsu Hengrui Pharmaceutical Co., Ltd., 2.5 mg/day) starting on MC3 and continued this treatment for 5 days. Our research objects were women who have PCOS with high BMI, the median number of AFC was 20 and the mean BMI was 28 kg/m^2. Except the number of AFC and BMI, the basal FSH value also been suggested as one of the influencing factors to select the initial Gn doses in IVF/ICSI treatment (17, 18). Women with basal FSH <7 mIU/ml were administered hMG 225 IU/day, for individuals with mildly increased basal FSH (7-10 mIU/ml) were administered hMG at a beginning dosage of 150 IU/day. From MC8 forwards, hMG dosages were modified for both groups every 2–4 days. If there were more than 20 follicles with a diameter >10 mm, we decreased the dosage of hMG from 225 to 150 IU. And if the FSH level was lower than 10 mIU/ml, we would add 75IU to the original dose. The hMG dose was adjust every 2–4 days according to the above principles.

When the dominant follicle reached a size exceeding 20 mm or more than three follicles reached a size exceeding 18 mm, 2000 IU or 5000 IU human chorionic gonadotropin (Lizhu Pharmaceutical Trading Co., China) in combination with 0.1 mg triptorelin (Decapeptyl; Ferring Pharmaceuticals, Germany) was employed to trigger the final phase of oocyte maturation. However, or 5000 IU human chorionic gonadotropin (hCG (500 IU hCG combined with 0.2 mg triptorelin or only 0.2 mg triptorelin was used if the patient was at risk of developing hyperstimulation. Under transvaginal ultrasound (TVS) guidance, oocytes were retrieved 34-38 hours after triggering. Aspiration was performed on all follicles measuring >10 mm in diameter (4).

Oocytes were then fertilised using conventional IVF or intracytoplasmic sperm injection (ICSI), depending on the quality of the semen and the success rate of previous fertilization attempts (19). The quantity and distribution of blastomeres, as well as the extent of fragmentation, were measured in the embryos. Within three days following oocyte retrieval, high-quality embryos (grade-1 and grade-2 6-cell embryos and above) were vitrified and frozen using the procedures stipulated by Cummins et al. (4, 20). Low-quality embryos were subjected to culture for a longer period, whereas blastocysts that were well-formed were frozen on day 5 or 6 (4).

Measurement of *hormones*

On MC3, MC8, MC10-12, the day of the trigger and the day following the trigger, serum levels of LH, FSH, E2, and P4 were measured. Chemiluminescence (Abbott Biologicals B.V., the Netherlands) was used to analyse the hormone levels. The maximum E2 value that could be measured was 5000 pg/ml. Samples with an E2 concentration over 5000 pg/ml were recorded at a value of 5000 pg/ml. The sensitivity thresholds were as follows: P4 0.1 ng/ml; E2, 10 pg/ml; LH, 0.09 mIU/ml; and FSH, 0.06 mIU/ml.

Preparation of endometrium and frozen embryo transfer

Following the procedures outlined previously (4, 21), the preparation of endometrium and FET were scheduled in the

second cycle following oocyte retrieval. LE was initially prescribed for mild stimulation of the endometrium as our data showed superiority of this protocol over hormone replacement therapy (HRT) (22). Those who had trouble conceiving after undergoing mild stimulation cycles or who had a history of an abnormally thin endometrium (≤ 6 mm) were then subjected to HRT. The women who participated in the mild stimulation cycle received 2.5 or 5 mg of LE for 5 days, starting at MC3. Ovulation was induced by injecting urine hCG (5000 IU) under the following conditions: dominant follicle diameter ≥17 mm, endometrium lining ≥8 mm, P4 level ≤1 ng/ml, and E2 level ≥150 pg/ml. Two or three days later, progesterone was started, and then five days or seven days after ovulation induction, abdominal ultrasound was used to guide the transfer of day-3 embryos or day-7 blastocysts. We used the "freeze-all" strategy and there was a waiting period between oocyte retrieval and embryo transfer. Our recruitment period was from January 2017 to September 2022 and patients were followed up to January 9, 2023.

Outcome measures

In this investigation, FORT was used as the primary outcome. The number of retrieved oocytes and viable embryos, the oocyte retrieval rate, the oocyte maturity and fertilization rates, the hMG dosage and duration, and the implantation rate were established as secondary outcomes. FORT was computed by using the ratio between the number of preovulatory follicles (PFCs) on hCG day × 100 and the number of AFCs at baseline. A prior study (12) concluded that only follicles between 16 and 22 mm in diameter should be included in the computation of FORT, to establish small antral follicles that responded best to FSH. The rate of oocyte retrieval was computed by dividing the total number of ruptured follicles by the sum of recovered oocytes. The rate of oocyte maturation was computed by dividing the sum of mature oocytes by the sum of all retrieved oocytes; To determine the fertilization rate, we divided the sum of fertilized oocytes by the sum of mature oocytes; the sum of fertilized oocytes was divided by cleaved embryos to obtain the cleavage rate. The rate of cycle cancellation was calculated as the sum of the number of patients whose oocyte retrieval resulted in zero viable embryos. At 4 weeks following FET, ultrasound detection of a gestational sac with or without foetal heart activity indicated the diagnosis of clinical pregnancy. The clinical pregnancy rate was determined by dividing the total number of clinical pregnancies by the sum of FET cycles. The implantation rate was computed by dividing the sum of embryo transfers by the total number of gestational sacs. The miscarriage rate was determined by the percentage of pregnancies that ended early due to therapeutic or spontaneous abortions.

Statistical *analysis*

To account for inherent disparities in the baseline characteristics of the two groups, we developed a propensity score matching (PSM) model. Ten variables were chosen for use in the propensity score estimation, including, AFC; age; basal levels of P4, E2, LH, and FSH; BMI; infertility type (primary or secondary); period of infertility and number of previous IVF attempts (0, 1–2 or ≥3). We used the nearest neighbour random matching technique to match patients receiving MPA+hMG+LE protocol with those receiving MPA+hMG treatment in a 1: 1 ratio. PSM was completed utilising R software (version 4.0.3; R Foundation for Statistical Computing, Vienna, Austria).

The mean ± standard deviation (SD) and Student's t test were used to display and evaluate normally distributed continuous data. The median [25th percentile, 75th percentile] and Mann–Whitney U tests were used to express and assess nonnormally distributed continuous data. Categorical data are displayed as n (percentage) and compared with Fisher's exact test or Pearson's chi-squared test. The SPSS statistical package (version 24, SPSS Inc.) was utilised for data analysis. Two-sided P <.05 was the criterion for statistical significance.

As a patient may have more than one FET cycle, we used the generalised estimating equation (GEE) modelling to reduce the potential bias of repeated cycles and compared the different treatment protocols with the odds ratio (OR) and corresponding 95% confidence interval (CI). P <.05 was the criterion for statistical significance. Variables as the clinical pregnancy rate, implantation rate, miscarriage rate, ectopic pregnancy rate and live birth rate were included in the regression equation.

Results

Patient *features*

The study's flow chart is summarised in Figure 1. Specifically, we enrolled 1792 high-BMI (BMI>25) women with PCOS who were candidates for assisted reproductive technology treatment in our clinical centre between January 2017 and September 2022. Based on the criteria outlined in the Methods and Materials, 340 cycles were eliminated. For the remaining 1508 women, we used the nearest neighbour random matching method to match 1374 women who received the MPA+hMG+LE protocol with 134 women who received the MPA+hMG treatment at a 1:1 ratio. The baseline and outcome parameters before PSM are presented in Table 1. Although our patients were recruited from January 2017 to September 2022, but the number of patients enrolled in 2022 only accounted for less than 7% of the total number due to the COVID-19 epidemic in China. Women who received the MPA+hMG+LE protocol were concentrated in the year of 2020 and 2021, while those received the MPA+hMG treatment were evenly distributed during the enrolment period. Our follow-up time was up to January 9, 2023, hence 93% of the patients had at least one year of follow-up after recruitment. Recruitment trend of patients in different years before and after PSM are provided in Supplemental Figure 1. All patients postmatching finished their oocyte retrieval cycles and were successful in obtaining oocytes (ranging from 1 to 52), however, 21 of these patients did not have any viable embryos. In addition, 222 of the remaining 247 patients finished 356 FET cycles. Postmatching analysis showed no significant variations in any baseline characteristics across the groups (all P >.05) (Table 1).

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TABLE 1 The baseline parameters of the two groups before and after PSM.

Parameters	Pre-match		P value	Post-match		P value
	Study group: hMG+MPA+LE (n=134)	Control group: hMG+MPA (n=1374)		Study group: hMG+MPA+LE (n=134)	Control group: hMG+MPA (n=134)	
Age (years)	32.43 ± 3.58	35.36 ± 4.08	<0.001	32.43 ± 3.58	32.35 ± 3.7	0.854
Duration of infertility (years)	4.04 ± 2.33	4.2 ± 2.42	0.52	3.7 ± 2.7	3.93 ± 2.91	0.501
Primary infertility, n (%)	64.93% (87/134)	22.56% (310/1374)	<0.001	64.93% (87/134)	62.69% (84/134)	0.703
Indication, n (%)			<0.001			0.884
Male factor	14.93% (20/134)	19.65% (270/1374)		14.93% (20/134)	13.43% (18/134)	
Tubal factor	65.67% (88/134)	50.15% (689/1374)		65.67% (88/134)	67.91% (91/134)	
Combination of factors	8.21% (11/134)	21.11% (290/1374)		8.21% (11/134)	9.70% (13/134)	
Unknown factor	11.19% (15/134)	9.1% (125/1374)		11.19% (15/134)	8.96% (12/134)	
Previous IVF failure			<0.001			0.905
0	88.80% (119/134)	65.28% (897/1374)		88.80% (119/134)	90.30% (121/134)	
1–2	8.96% 12/134)	21.83% (300/1374)		8.96% (12/134)	7.46% (10/134)	
> 3	2.24% (3/134)	12.88% (177/1374)		2.24% (3/134)	2.24% (3/134)	
BMI	28.44 ± 2.65	30.12 ± 90.97	<0.001	28.44 ± 2.65	28.18 ± 2.58	0.411
Basal hormone concentrations						
FSH (IU/L)	5.23 ± 1.28	5.23 ± 1.38	0.768	5.23 ± 1.28	5.26 ± 1.5	0.848
LH (IU/L)	4.12 ± 2.18	4.01 ± 2.43	0.132	4.24 ± 2.6	4.07 ± 2.29	0.583
E2 (pg/ml)	33.34 ± 11.79	32.26 ± 12.56	0.253	33.34 ± 11.79	33.25 ± 12.35	0.991
P (ng/ml)	0.23 ± 0.1	0.23 ± 0.11	0.682	0.23 ± 0.13	0.22 ± 0.13	0.217
AFC	19.64 ± 6.99	17.82 ± 6.49	0.002	20 [15,22]	20 [16,20.25]	0.774

Data are presented as mean ± standard deviation and median [25th percentile, 75th percentile]. BMI, Body Mass Index; FSH, Follicle stimulating Hormone; LH, Luteinizing Hormone; E2, Estrogen; P, Progesterone; AFC, Antral Follicle Counting.

Ovarian stimulation, follicle development, and oocyte performance

The study and control groups exhibited similar numbers of retrieved oocytes (13.5 [8.75, 20.25] vs. 15 [9, 21], P >.05) and viable embryos (5 [2, 8] vs. 5 [2.75, 8], P >.05). Neither the hMG doses nor the period of ovarian stimulation were significantly different between the two groups (P >.05). The sums of follicles with diameters of 10-12 mm and 12-14 mm were comparable across the two groups. The study group had a substantial reduction in the sum of follicles with a diameter of 14-16 mm (2 [0, 6] vs. 3 [1, 6], P <.05) but significantly higher number of follicles larger than 16 mm when compared with the control group (10.5 [7, 18] vs. 8 [4, 11], P <.05) (Table 2). Consistent with the above results, FORT was substantially elevated in the study group compared with the control group (61% [35%, 86%] vs. 40% [25%, 60%], P <.01). Although the mature oocyte rate was meaningfully reduced in the LE cotreatment group (81% ± 18% vs. 86% ± 12%, P <.05), the oocyte retrieval rate was similar in the two groups. Additionally, the number of aspirated follicles and mature oocytes were not significantly different. Of the 134 women receiving LE+MPA+HMG treatment, 3 had no mature oocytes, 3 failed fertilization, and 5 failed cleavage. Of the 134 women who underwent the MPA+HMG protocol, 2 had no mature oocytes, 4 failed fertilization, and 4 failed cleavage. The cycle cancellation rates for unviable embryos that were not significantly different between the two groups (8.2% (11/134) vs. 7.5% (10/134), P >.05) (Table 2).

Profiles of hormones during treatment

Figure 2 depicts the endocrine dynamics that occurred in response to ovarian stimulation, including those of P4, E2, LH, and FSH. After the hMG injection, FSH levels spiked considerably 5

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TABLE 2 COS characteristics and outcomes after PSM.

	Study group: hMG+MPA+LE (n=134)	Control group: hMG+MPA (n=134)	P value
hMG dose (IU)	2370.15 ± 738.98	2392.16 ± 789.52	0.841
hMG duration (d)	9.31 ± 1.86	9.63 ± 2.4	0.391
hCG Dose on trigger day (IU)	2082.09 ± 1255.36	2221.64 ± 1637.68	0.989
GnRHa Dose on trigger day (mg)	0.14 ± .05	0.12 ± 0.04	0.000
10-12-mm follicles on hCG day (n)	2 [0,4]	2 [0,5]	0.143
12-14-mm follicles on hCG day (n)	2 [0,5.25]	3 [1,6]	0.278
14-16-mm follicles on hCG day (n)	2 [0,6]	3 [1,6]	0.033
> 16-mm follicles on hCG day (n)	10.5 [7,18]	8 [4,11]	0.000
FORT (%)	61 [35,86]	40 [25,60]	0.000
Punctured follicles (n)	18 [12,29]	20 [13,27.25]	0.555
Oocyte retrieved (n)	13.5 [8.75,20.25]	15 [9,21]	0.777
Mature oocytes (n)	11 [7,16]	12.5 [7,17]	0.149
Fertilized oocytes (n)	9 [5,13]	10 [6,14]	0.290
Cleaved embryos (n)	9 [5,12.25]	9.5 [5.75,13]	0.310
High-quality embryos (n)	3 [2,6]	4 [2,7]	0.370
Blastocyst embryos (n)	1 [0,3]	1 [0,2]	0.178
All cryopreserved embryos (n)	5 [2,8]	5 [2.75,8]	0.756
Oocyte retrieval rate (%)	75% ± 19%	74% ± 22%	0.904
Mature oocyte rate (%)	81% ± 18%	86% ± 12%	0.040
Fertilization rate (%)	79% ± 18%	79% ± 16%	0.749
Cleavage rate (%)	96% ± 11%	97% ± 7%	0.907
Cycle cancellation rate (%)	8.2% (11/134)	7.5% (10/134)	0.820

Data are presented as mean ± standard deviation and median [25th percentile, 75th percentile] or number (percentage). All the value of (n) were calculated per cycle.

days later and then remained constant until the trigger day. Rapid elevation of FSH to over 15 mIU/ml was observed following the dual trigger. No remarkable differences were observed in FSH levels between the two groups at any time point (Figure 2A).

LH remained low in both groups throughout the COS. There was a declining trend in LH levels in the control group. On the other hand, the LH concentration in the study group was rather stable for the initial five days. During COS, LH levels at MC8, MC9-11 and the trigger day were remarkably higher in the study group than in the control group(P <.01). Neither group had any cases of premature LH surge (Figure 2B).

As several follicles matured, there was a continuous rise in serum E2. Due to the use of LE, the oestrogen values in the study group were lower than those in the control group during COS, and the variation was statistically significant at all time points (P <.01) (Figure 2C).

P4 levels in both groups gradually increased during ovulation stimulation. Additionally, the P4 levels in the study group were substantially elevated compared with those in the control group at MC9-11(P <.01) (Figure 2D).

Outcomes of pregnancies following FET procedures

There were 247 women with viable embryos that developed successfully. A total of 546 embryos were thawed, and all (100%) were viable after the thawing procedure. Ultimately, 356 FET cycles were completed by 222 women. In the LE cotreatment group, 103 women finished 163 FET cycles in total: 63 women had accomplished one FET cycle, 26 women had completed two FET cycles, 14 women had accomplished more than or equal to three FET cycles. Whereas 119 women in the PPOS group finished 193 FET cycles: including 65 women with one FET cycle, 38 women with two FET cycles, 16 women finished greater than or equal to three FET cycles. In total, 86% of patients in both groups had fewer than three FET cycles. However, 46 women failed to start their FET cycles for numerous reasons in the two groups. There was no significant difference on the stage and number of embryos transferred per cycle between the two groups, and the neonatal status between the two groups were similar (P >.05) (Table 3).

Our results showed that after controlling for the potential bias of repeated cycles from one patient, the different treatment

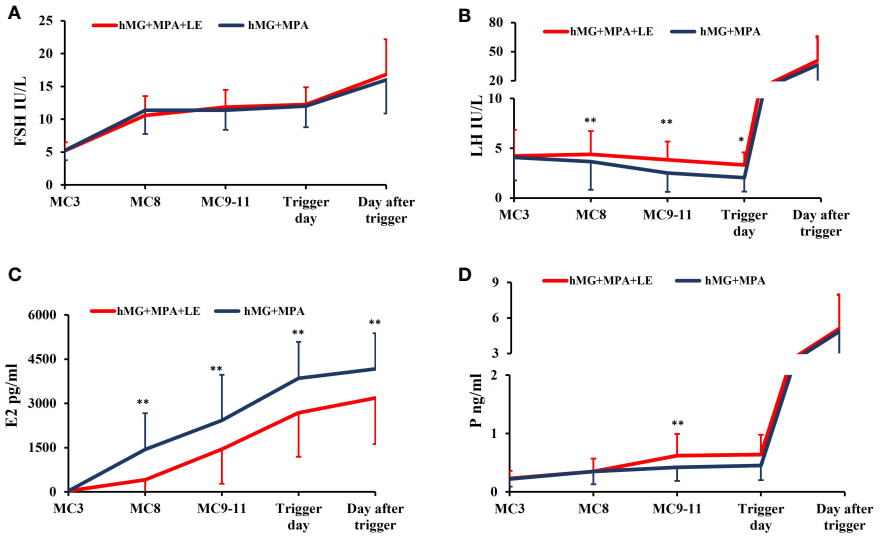

FIGURE 2
The dynamic changes in hormones during ovarian stimulation in the two groups. **(A)**. Serum FSH levels in the two groups during COS. **(B)**. Serum LH concentration in the two groups during COS. **(C)**. Serum E2 level in the two groups during COS. **(D)** Serum P levels in the two groups during the COS. The solid red lines represent the study group (hMG+MPA+LE) and the solid heavy blue lines represent the control group (hMG+MPA). The asterisks denote significant changes in hormone levels at the indicated time points (*P <.05, **P <.01).

protocols in the LE cotreatment group and the PPOS group were not associated with significant differences in the indicators as implantation rates (43.15% (107/248) vs. 38.59% (115/298), OR 1.008, 95% CI 0.901-1.127; P >.05), clinical pregnancy rate, miscarriage rate, and live births rate (P >.05). (Table 3). All pregnancy data were followed up until January 9, 2023 (Table 3).

Discussion

This research showed that LE may play a role as an adjuvant medicine to enhance the FORT of the PPOS regimen in PCOS patients with a high BMI. Nevertheless, the number of retrieved oocytes and mature oocytes, the dose of gonadotropin consumption and gonadotropin days, and the number of embryos in the combined group were similar to those in the group that received the PPOS protocol alone. The implantation rate was elevated in the combined group compared with the PPOS group, but the difference was not significant, and the rates of clinical pregnancies, miscarriages and live births were comparable between the two groups.

Increased BMI in PCOS is linked to elevated androgen levels, which could block dominant follicle development and cause follicular degeneration (23). In our study, the FORT in the LE cotreatment group was significantly greater than that in the control group. The higher FORT after LE combination therapy may be the result of endocrine alterations. Coadministration of LE causes an acute hypoestrogenic condition, which relieves the hypothalamic-pituitary axis of oestrogenic negative responses and enhance gonadotropins production (24), these changes may explain the increased follicle diameter at oocyte retrieval time in the LE cotreatment group. We found that the number of larger follicles (>16 mm) was substantially elevated in the study group compared with the control group. After diameter deviation, increased LH levels tend to promote dominant follicle selection and enhance the development of dominant follicles (25, 26). Notably, the dose of GnRH-a for triggering was markedly enhanced and the dose of hCG was decreased without statistical difference in the LE cotreatment group compared with the PPOS-only group. An increased number of large preovulatory follicles was observed in the LE cotreatment group, suggesting that women in that group had a higher likelihood of receiving a single GnRHa trigger rather than a dual trigger. The number of oocytes retrieved and the oocyte retrieval rate were similar between the two groups, indicating that the ovulation trigger method in the present study did not affect oocyte retrieval.

Although LH values were meaningfully increased in letrozole cotreatment group compared with the standard PPOS group, FSH levels were similar between the two group. Women with PCOS would have a partial pituitary desensitization and relative decline of FSH responsiveness (27, 28) which might owe to the hyperactive GnRH neurons (29). Higher LH values in the study group might induced by LE through blocking oestrogen production (24). Progestogen was one of the precursors of estrogens and transformed into estrogens by aromatase (30). LE, an aromatase inhibitor, can inhibit the production of estrogens, thereby reducing estrogens level and accumulating progesterone (15, 24, 30). Hence, lower serum E2 levels and higher P levels were observed after cotreatment with LE in the PPOS protocol.

Notably, oocyte maturity rates were significantly decreased in the LE cotreatment group compared with the PPOS-only group, although mature oocyte yields were comparable. The influence of LE on oocyte maturity remains controversial in the literature (31–33). LE, an aromatase inhibitor, is usually used in patients who need fertility preservation, such as those with breast cancer, to reduce oestrogen levels (33). Our research is consistent with Quinn's study showing that GnRH antagonist protocol cotreatment with LE in breast cancer patients decreased the oocyte maturity rate (33). LE coadministration

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TABLE 3 Pregnancy outcomes after FET.

Variable	Study group: hMG+MPA+LE	Control group: hMG+MPA			P value
Patients (n)	103	119			
FET cycles (n)	163	193			
Thawed embryos (n)	248	298			
Viable embryos after thawed (n)	248	298			
The number of embryos per transfer (n)	1.53 ± 0.50	1.54 ± 0.50			0.758
Indication, n (%)					0.497
cleavage-stage embryo	56.85% (141/248)	59.73% (178/298)			
blastocyst embryo	43.15% (107/248)	40.27% (120/298)			
Endometrial preparation n (%)					0.605
Mild stimulation	63.80% (104/163)	61.14% (118/193)			
Hormone therapy	36.20% (59/163)	38.86% (75/193)			
Endometrial thickness (mm)	10.44 ± 2.56	10.09 ± 2.04			0.249
Newborn					
Single birth (n)	47	56			
Single birthweight (g)	3399.57 ± 565.19	3304.17 ± 531.81			0.403
Twin birth (n)	11	9			
Twin birthweight (g)	2535 ± 392.22	2310 ± 495.71			0.117
Variable adjusted in GEE models			OR	95% CI	
Clinical pregnancy rate per transfer (%)	53.37% (87/163)	52.85% (102/193)	1.008	0.901-1.127	0.891
Implantation rate (%)	43.15% (107/248)	38.59% (115/298)	1.065	0.918-1.235	0.405
Miscarriage rate (%)	13.79% (12/87)	15.69% (16/102)	0.982	0.891-1.083	0.714
Ectopic pregnancy rate (%)	1.15% (1/87)	1.96% (2/102)	0.987	0.95-1.026	0.514
Live birth rate per cycle (%)	35.58% (58/163)	34.20% (66/193)	0.991	0.838-1.171	0.913
Live birth rate per patient (%)	56.31% (58/103)	60.55% (66/119)	0.991	0.838-1.171	0.913

Data display as mean ± SD or number (percentage). Pregnant data were followed up until January 9, 2023.

decreased oestrogen levels and resulted in the accumulation of progesterone, 17α-progesterone and testosterone (15, 24, 30), These changes in the endocrine microenvironment affect meiotic maturation probably by inhibiting oocyte cytoplasmic maturation, contributing to reduced oocyte maturation. There is also evidence that LE does not increase the risk of spindle assembly and preimplantation developmental arrest (34). Hu's study of mouse oocytes showed that the antral space formed earlier if they were cultured in the presence of aromatase inhibitor, while the oocyte competency was not reduced (35). In the current retrospective study, lead follicles were trigger at diameters of 18 mm, leading to lower oocyte maturity rates in the LE cotreatment group. Hence, Oktay (32) suggested that instead of triggering lead follicles at diameters of 17–18 mm, they should trigger at 19.5–20.5 mm under LE-containing stimulation, as LE cotreatment requires a different trigger criterion. In the present research, the lower oocyte maturity rates in the LE cotreatment group did not affect the number of mature oocytes as the number of large follicles on the trigger day was significantly higher. Prospective randomised controlled studies with different trigger criteria for the PPOS- LE cotreatment protocol are needed in the future.

In our present study, FORT didn't convert into significant higher implantation rate here. Although previous studies illustrated that patients with an elevated FORT can achieve improved clinical outcomes (36) and good pregnancy outcomes (37) in IVF cycles and that embryos generated from oocytes from the dominant follicle group could have enhanced implantation potential since they are less fragmented (38, 39), opposing studies have linked an abundance of dominant follicles to impaired oocyte growth performance and a poor pregnancy rate, since excessive follicular development during ovarian stimulation might cause oocyte overmaturation, leading to unsuccessful pregnancies (40). We have showed that oocyte maturity rates were significantly decreased in the LE cotreatment group compared with the PPOS-only group in the previous statement, and LE-containing stimulation should trigger lead follicles at larger diameters than the original standard (32). Therefore, we speculated that although the proportion of FORT increased significantly after adding LE in PPOS

protocol, its oocyte development potential did not increase significantly. However, this needs to be confirmed by large-scale prospective randomized controlled studies.

A higher BMI has been linked to a greater need for gonadotrophins during stimulation (7). PCOS may be accompanied by an altered ovarian response to gonadotropins, which would reduce early ovarian responsiveness compared with that in ovulatory controls (41). FORT is an objective method for determining the real effect of external FSH on follicles since it is not impaired by preexisting antral follicle population size (11). Although concurrent LE administration enhanced FORT in the PPOS protocol, no significant difference in gonadotropin consumption was observed. This was surprising given that previous studies have suggested that LE lowers the overall gonadotropin intake for ovarian stimulation (24, 42, 43). In contrast to low responders, high responders with PCOS exhibit FSH receptor upregulation in their follicular granulosa cells (44), which could explain why LE did not work to lower the overall gonadotropin dosage necessary for ovarian stimulation in hyperresponders (45).

Our study's retrospective nature and limited sample size are notable drawbacks. As a retrospective study, there existed selectivity bias in this research. Most Chinese patients with PCOS (80%) are within the healthy weight range (2). Additionally, our data were acquired from a single centre; as a result, it was challenging to gather sufficient patient data to detect statistically significant variations. There is also the possibility of unmeasured or unidentified confounders in this retrospective analysis, which might result in less-than-perfect matching and weaken the reliability of the results. In addition, despite having embryos of high quality, some patients were unable to finish their FET cycles for a variety of reasons. Thus, the present research should be considered a preliminary effort, and the development of additional evidence requires further by more prospective studies to validate the impact of combine LE with PPOS for women with PCOS women and a high BMI. Further research on the application of LE in women with PCOS and different BMIs is needed to help physicians to develop individualised treatment plans for each patient.

Conclusion

Our findings show that the addition of LE to the PPOS regimen increased FORT. In patients with PCOS and high BMI who were receiving IVF therapy, there was not a statistically favourable impact of LE addition on the cycle parameters of COS, including hMG consumption, or on pregnancy outcomes.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by The Ethics Committee of Shanghai Ninth People's Hospital (Institutional Review Board) (Number: SH9H-2021-T294–1). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

YK and QZ conceived and direct this study. XS and JL contribute to data collection and analysis. YL dedicated to draft and revise the manuscript. All authors have made a corresponding contribution to this article. The authors have nothing to declare on the final 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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1072170/full#supplementary-material

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Effect of acupuncture on the opening time of implantation window and endometrial receptivity in controlled ovarian hyperstimulation rats during peri-implantation period

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Purpose: To investigate the effect of acupuncture for improving the pregnancy rate of COH rats from the viewpoint of regulating the opening time of the implantation window and endometrial receptivity.

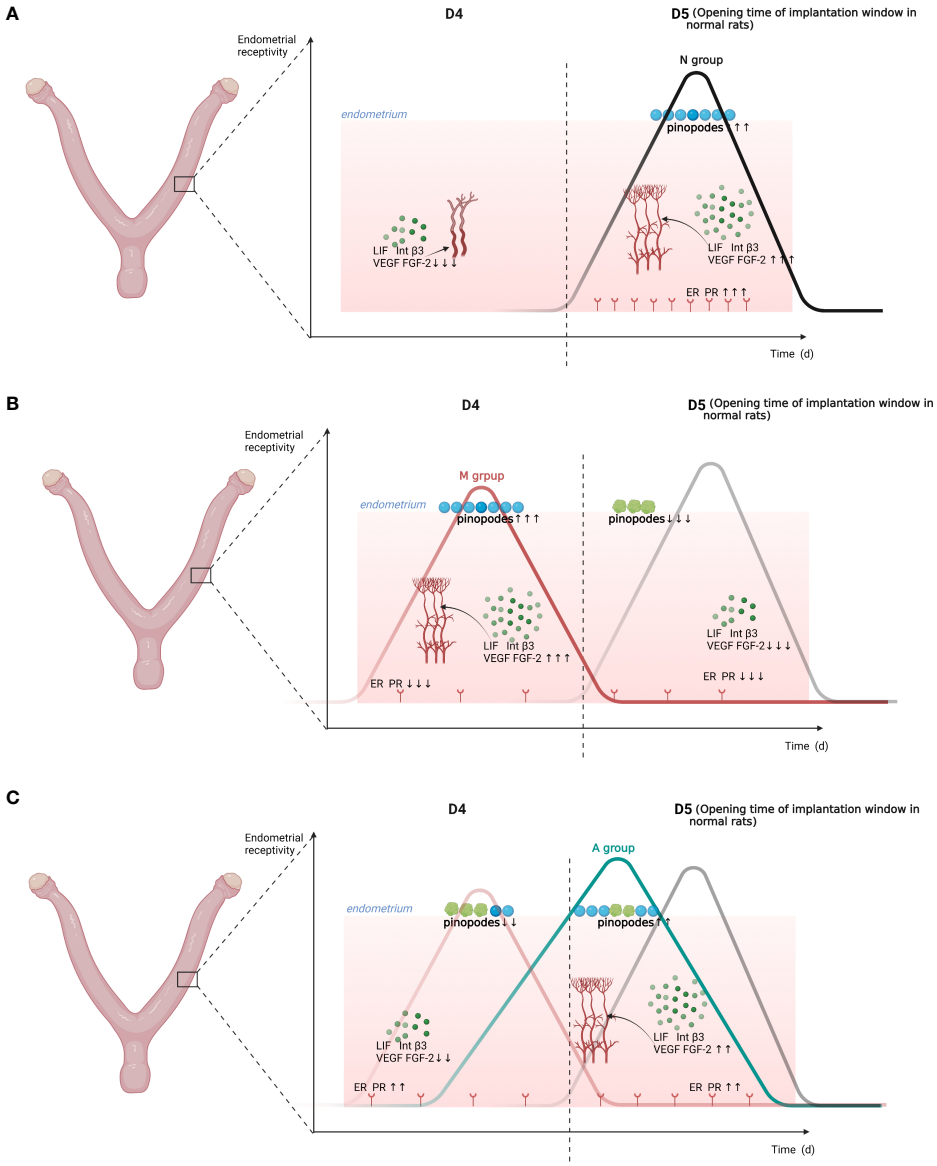
Methods: Experimental rats were randomly divided into normal group (N), model group (M) and acupuncture group(A), and samples were collected on Day 4, 5 and 6 after mating. COH rats were treated with acupuncture at SP6, LR3, and ST36 once a day for 7 times. The pinopodes were observed under a scanning electron microscope. Serum estrogen and progesterone levels were measured *via* ELISA. The protein and mRNA levels of estrogen receptor (ER), progesterone receptor (PR), leukemia inhibitory factor (LIF), integrin β3, vascular endothelial growth factor (VEGF), and fibroblast growth factor 2 (FGF-2) in the endometrium were evaluated *via* West-blot, immunohistochemistry, and PCR.

Results: Compared with group N, the pregnancy rate of group M was significantly decreased ($P<0.05$), and the abnormal serum hormone levels and implantation window advancement were observed. Compared with group M, the pregnancy rate of group A was significantly increased ($P<0.05$), the supraphysiological serum progesterone levels were restored to normalcy ($P<0.05$), and the advanced implantation window was restored to a certain extent. Further, the abnormal ER, PR, LIF, integrin β3, VEGF, and FGF-2 expression levels of the endometrium got recovered to varying degrees.

Conclusion: Acupuncture may restore the estrogen and progesterone balance in COH rats and the forward shift of the implantation window to a certain extent, improving the endometrial receptivity and finally improving the pregnancy rate of COH rats.

KEYWORDS

acupuncture, controlled ovarian hyperstimulation, sex steroid hormones, implantation window, endometrial receptivity



GRAPHICAL ABSTRACT
The embryo implantation window moving forward after COH and asynchrony with the development of the endometrium may be important factors associated with a reduced pregnancy rate in COH rats. Acupuncture may restore the estrogen and progesterone balance in COH rats and the forward shift of the implantation window to a certain extent, finally improving the pregnancy rate of COH rats. D, The embryo day; LIF, Leukemia inhibitory factor; Int b3,Integrin b3; VEGF, Vascular endothelial growth factor; FGF-2, Fibroblast growth factor 2; PR, Progesterone receptor; ER, Estrogen receptor; N, Normal group; M, Model group; A, Acupuncture group.

1 Introduction

Infertility affects up to 15% of couples worldwide (1), and more and more infertile couples are choosing *in vitro* fertilization-embryo transfer (IVF-ET) as the last resort for pregnancy. Controlled ovarian hyperstimulation (COH) is one of the most commonly used and important treatments for obtaining a large number of high-quality oocytes during IVF-ET. However, there always exit some bottlenecks in COH cycles, such as low implantation rate (only 20%–30%), high miscarriage rate, and high incidence of ovarian hyperstimulation syndrome (OHSS) (2).

Studies have shown that the steps of embryo attachment and implantation are strictly controlled by steroid hormones (3), and the super-physiological levels of estrogen, progesterone, high progesterone to estrogen ratio, unbalanced glycosylic conjugation, or human chorionic gonadotropin (HCG) induced by COH may be associated with endometrial dysplasia and unsynchronized endometrial and embryonic development, leading to implantation failure (4–6). In recent years, researchers have tried using drugs such as aspirin, heparin, and sildenafil to improve the microcirculation and trophoblast invasion during the peri-implantation period, thereby helping to enhance endometrial

receptivity, but their effectiveness and safety need to be further studied (7–10).

Acupuncture has a long history in the treatment of infertility. In recent years, it has been increasingly used in ART and an increasing number of clinical studies have proved its effectiveness and safety in improving the clinical pregnancy rate (11–14). Based on the dynamic analysis of VEGF mRNA in the endometrium during the peri-implantation period in previous studies and the close relationship between endometrial angiogenesis and embryo implantation (15), we proposed a hypothesis that "COH leads to the advancement of the implantation window, the asynchrony of which with endometrial development is a key factor leading to a reduced pregnancy rate, and the mechanism of acupuncture improving the pregnancy rate of COH rats may be *via* restoring the advanced implantation window by improving the progesterone to estrogen ratio." To verify the abovementioned hypothesis, we designed this study to further explore the curative effect of acupuncture for improving the pregnancy rate of COH rats.

2 Materials and methods

2.1 Animals and grouping

170 SPF-grade adult female virgin Wistar rats (220–250 g) and 45 healthy male Wistar rats (approximately 300 g) were purchased from the mouse Laibao Biotech Co., Ltd. and raised in the central barrier system of the Experimental Animal Center of Tongji Hospital, Tongji Medical College, Hua Zhong University of Science and Technology. Experimental rats were raised in a pathogen-free environment (20 ± 2 °C, 60 ± 5% humidity, 12 h: 12 h light/dark cycle), and were given free access to water and food (2 rats/cage). The males and females were reared in separate cages. This study was approved by the Animal Experiment Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China (prove number: TJH-202009008).

After one week of adaptive feeding, the vaginal smear method was used to observe their estrus cycle every day. The 156 rats with two consecutive regular estrus cycles were randomly divided into nine groups: D4 normal group (D4N), D4 model group (D4M), and D4 acupuncture group(D4A) (n = 12 each); D5 normal group (D5N), D5 model group (D5M), and D5 acupuncture group (D5A) (n = 12 each); and D6 normal group (D6N), D6 model group (D6M), and D6 acupuncture group (D6A) (n = 28 each).

2.2 Reagents and main devices

Pregnant mare serum gonadotropin (PMSG) was purchased from the Hangzhou Animal Medicine Factory, China. Human chorionic gonadotrophin (HCG) was provided by Livzon Pharmaceutical Factory, Zhuhai, China. Other materials included anti-vascular endothelial growth factor (VEGF) (Santa Cruz, SC-7269), anti-fibroblast growth factor 2 (FGF-2) (Immunoway, Cat NO.YT5549), anti-estrogen receptor α (Santa Cruz, SC-787); anti-progesterone receptor A (Proteintech, Cat NO.25871-1-AP); anti-LIF, (Gentex, Cat NO.GTX11940), ITG β-3(Abclonal, Cat NO.A2542) and Evans Blue (Cat. number E8010). Main reagents and devices included quantitative real-time PCR (qRT-PCR) equipment (Applied Biosystems, USA), SYBR Green qPCR Kit (Yesen, Cat NO.11201-11203), nucleic acid protein analyzer (DU730, Beckman Coulter, USA), Mastercycler gradient PCR apparatus (Eppendorf, Germany), Nikon microimaging system (TE2000-U, Tokyo, Japan), Step-One Real-Time PCR (Applied Biosystems, California, USA), scanning electron microscope (HITACHI, SU8100, Japan), estrogen ELISA kits (No.501890, Cayman, USA), progesterone ELISA kits (Cat: ELK7894, ELK Biotechnology, China), and Odyssey infrared imaging system (licor Biosciences, USA).

2.3 Modeling and intervention

Our previously reported method and related literature were followed for modeling (15, 16); female rats in both model (M) and acupuncture (A) groups were intraperitoneal injected with 20 IU PMSG at 5 PM on the Day 2 of the estrus period, followed by an injection of 20 IU HCG approximately 48 h later; subsequently, the female rats were mated with male rats overnight in independent cages. At the same time, the same volume of 0.9% saline was injected into the rats in the Normal (N) group. At 8 a.m. on the day after mating, a vaginal smear was taken and a large number of sperms or vaginal plug on the vaginal smear was considered to indicate successful mating and the day was marked Day 1 (D1). Only female rats that mated successfully were included in the follow-up study. The rats in the A groups were fixed in a homemade cloth bag, and acupuncture was performed at the three acupoints of Sanyinjiao (SP6), Taichong (LR3), and Zusanli (ST36) on both sides using sterile acupuncture needles (201104, Hanyi, 0.18 × 13 mm, Beijing Hanyi Medical Instruments Co., Ltd., China) for 25 min from the day of PMSG injection to Day 4 after mating (D4). The rats in N and M groups were fixed in the same cloth bags for 25 min from the day of PMSG injection to D4 without acupuncture being performed.

2.4 Harvesting

At 5 PM on D4, 5, and 6, the rats were anesthetized *via* an intraperitoneal injection of 1 ml 2% sodium pentobarbital. Rats in each group were killed by an overdose of anesthesia directly after obtaining blood from the abdominal aorta in the D4 and D5 groups. After the rats were deeply anesthetized in the D6 group, the embryo implantation point was stained by a tail vein injection of 1.5 ml Evans Blue; blood was drawn and the rats were sacrificed after 10 min of dyeing; the number of implantation points was recorded in detail to calculate the pregnancy rate (number of pregnant rats/

number of successfully caged rats) and number of implanted embryos (total number of implanted embryos/total number of pregnant rats). The blood samples were centrifuged (3000 rpm × 15 min) and the supernatant was collected and stored in a refrigerator at −80°C for the detection of steroid hormone levels. The uterine tissues of each group were carefully separated and preserved; a small section was put into the electron microscope fixation solution for subsequent electron microscope experiments; another small section was fixed and embedded in 4% paraformaldehyde for histochemical evaluation; the rest was freezed to −80°C in a refrigerator for subsequent experiments.

2.5 Scanning electron microscopy

Fresh endometrial surface tissues (approximately 1 mm^3 in size) were quickly put into the electron microscope fixation solution, and fixed at 4°C for 2–4 h. After fixation, dehydration, infiltration, embedding, slicing, and staining, endometrial pinopodes was observed and an image was captured under a scanning electron microscope (HITACHI, SU8100, Japan).

2.6 Enzyme-linked immunosorbent assay

Levels of serum estrogen and progesterone were analyzed by ELISA. The sensitivity of the estrogen ELISA kits (No.501890, Cayman, USA) was approximately 20 pg/ml, and the assay has a range of approximately 0.61–10000 pg/ml. The sensitivity of progesterone ELISA kits (Cat: ELK7894, ELK Biotechnology, China) was approximately 0.55 ng/ml, and the assay has a range of 1.57–100 ng/ml. The ELISA steps were carried out in strict accordance with the instructions mentioned in the kits.

2.7 Immunohistochemical assay

Paraffin sections were dewaxed in xylene, placed in gradient concentrations of ethanol to recover the antigen, and then blocked with goat serum at room temperature for 20 min. Subsequently, the sections were incubated with a primary antibody (anti-estrogen receptor α, Santa Cruz Biotechnology, 1:50; anti-progesterone receptor A, Proteintech, 1:300; anti-VEGF, Santa Cruz, 1:50; anti-FGF-2, Immunoway, 1:100; anti-LIF, Gentex, 1:200; ITG β-3, Abclonal, 1:150) at 4°C overnight, rinsed with phosphate-buffered saline with Tween (PBST) five times for 5 min, and incubated with secondary antibodies (anti-estrogen receptor α, Santa Cruz Biotechnology, 1:50; anti-progesterone receptor A, Proteintech, 1:300; anti-VEGF, Proteintech, 1:50; anti-FGF-2, Bioswamp, 1:100; anti-LIF, Gentex, 1:200; ITG β-3, Absin, 1:150) at room temperature for 1 h. After washing with PBST five times for 5 min, the sections were developed using 3,3'-diaminobenzidine and stabilized using hematoxylin for approximately 3 min. Finally, the sections were dehydrated, made transparent, and sealed. The images were scanned using a nanozoomer slide scanner (Hamamastu, Japan) and observed using the NDP view2 system.

2.8 Western blot analysis

Endometrial tissues were homogenized and lysed in tissue protein extraction reagent, supplemented with a protease inhibitor cocktail, placed on ice for 30 min, and centrifuged at 4°C (12,000 rpm for 10 min). After the protein concentration was determined, the samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and transferred to a PVDF membrane. The membranes were sealed with 5% skim milk at room temperature for 0.5 h, and then incubated with a primary antibody at 4 °C for 24–48 h. Antibodies include anti-estrogen receptor α (Santa Cruz Biotechnology, USA, 1:50); anti-progesterone receptor A (Proteintech, China, 1:200; anti-VEGF, Santa Cruz, China, 1:200); anti-FGF-2 (Immunoway, China, 1:500); anti-LIF (Gentex, China, 1:300); ITGβ3 (Abclonal, China, 1:500); and anti-β-actin (Proteintech, China, 1:1500). PVDF membranes were incubated with fluorescent secondary antibodies (CST, USA) on a shaking table at room temperature for 1 h. Finally, the bands were scanned using Odyssey infrared imaging system (licor Biosciences, USA).

2.9 Real-time PCR

Total RNA was extracted from the endometrial tissue using Trizol reagent (Takara, Japan), according to the manufacturer's instructions. After the RNA concentration was determined, the cDNA was synthesized with a reverse transcription reagent (Yesen, China). Quantitative real-time PCR (qRT-PCR) (Applied Biosystems, USA) was performed using SYBR Green qPCR Kit (Yesen, China). The $2^{-\Delta\Delta CT}$ method was used for data analysis. The sequence is in the Table 1.

2.10 Statistical analysis

IBM SPSS 20.0 was used for statistical analysis. Continuous data for normal distribution are expressed as means ± SD and categorical data are expressed as percentages (%). Differences were compared using one-way analysis of variance. If the variance was uniform, the LSD test was used, whereas if it was uneven, Dunnett's T3 test was used. P value < 0.05 was considered to be statistically significant.

3 Results

3.1 Comparison between the mating rate and pregnancy rate of rats in each group

A large number of spermatozoa or vaginal suppositories on vaginal smears were regarded to indicate successful mating. In addition, only rats in the D6 group were included for calculating the pregnancy rate. In all, 42 rats were injected with Evans blue through the tail vein on Day 6 to determine whether they were

pregnant (Table 2). Compared with the N group, the M group showed a significantly lower pregnancy rate ($P < 0.01$). Further, compared with the M group, the A group showed a significantly higher pregnancy rate ($P < 0.05$). But the specific number of pregnant embryos in each rat could not be accurately counted. Accordingly, the number of embryos was not calculated. According to Evans blue staining, the M group had more embryos than the N group (Figure 1).

TABLE 1 Primer information of the LIF, ITGβ3, VEGF, FGF-2, ERα and PRα mRNA sequences.

Name	Primer Information	Base sequence	annealing temperature	GC%	Length
LIF	R-lif-S	GGGATTGTGCCCCTACTGCTC	62.8	61.9	152
	R-lif-A	CCGTTGAGTTGAGCCAGTTGAC	61.94	54.55	
ITGB3	R-itgB3(2)-S	ACCGTTTCTGCCGAGATGAC	60.39	55	322
	R-itgB3(2)-A	CATTTGGCTCTGGCTCGTTC	59.55	55	
VEGF	R-VEGF(1)-S	GCACTGGACCCTGGCTTTACT	62.34	57.14	102
	R-VEGF(1)-A	AACTTCACCACTTCATGGGCTTT	60.95	43.48	
FGF	R-FGF2-S	GAGAAGAGCGACCCACACGT	59.7	60	232
	R-FGF2-A	CAGTTCGTTTCAGTGCCACATAC	59.9	47.8	
ERα	R-ERα(1)-S	GTTTGCTCCTAACTTGCTCTTGG	60.06	47.83	191
	R-ERα(1)-A	TCAAGGTGCTGGATAGAAATGTG	58.74	43.48	
PRα	R-PRα-S	TAGTCAAATGGTCTAAGTCTCTGCC	60.11	44	215
	R-PRα-A	GGTAAGGCACAGCGAGTAGAATG	61.29	52.17	
GAPDH	R-GAPDH-S	CTGGAGAAACCTGCCAAGTATG	58.99	50	138
	R-GAPDH-A	GGTGGAAGAATGGGAGTTGCT	60.27	52.38	

LIF, Leukemia inhibitory factor; ITGβ3, Integrin β3; VEGF, Vascular endothelial growth factor; FGF-2, Fibroblast growth factor 2; ERα, Estrogen receptorα; PRα, Progesterone receptorα.

TABLE 2 Mating rate of rats in each group and pregnancy rate of D6 rats.

Group	Mating Rate (%)	Pregnancy Rate on D6 (%)
N	100 (52/52)	100 (14/14)
M	90.38 (47/52)	42.86 (6/14)**
A	94.23 (49/52)	64.29 (9/14)#

The value is expressed as mean ± SD. *or** represents that there is significant difference between M and N groups (P<0.05 or P<0.01), #or ## represents that there is significant difference between A and M groups (P<0.05 or P<0.01), D6: The embryo day 6; N: Normal group; M: Model group; A: Acupuncture group.

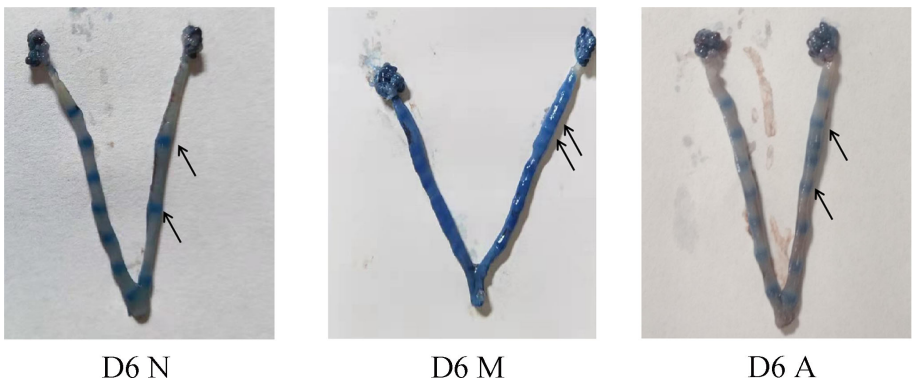


FIGURE 1
Uterus of embryo implantation in each group on D6. D, The embryo day; D6N, D6 normal group; D6M, D6 model group; D6A, D6 acupuncture group.

3.2 Comparison of pinopodes in rat endometrium

The ultrastructural results of endometrium on D4, D5, and D6 were observed under a scanning electron microscope (3000×). There was no pinopode or swollen microvilli on the surface of endometrium in the D4N group, a large number of pinopodes in the D4M group, and obvious pinopodes in the D4A group, but the number of pinopodes in the D4A group was less than that in the D4M group. There were a large number of mature pinopodes on the endometrial surface the rats in the D5N group, a small number of atrophic pinopodes in the D5M group, and a small number of mature or atrophic satiety pinopodes in the D5A group, and the number of pinopodes observed in the D5M group was less than that observed in the in D5A group. There were no pinopodes in the D6N, M, and A groups (Figure 2).

3.3 Comparison of protein and gene expressions of LIF, integrin β3, VEGF, and FGF-2 in the endometrium

Immunohistochemistry (IHC) results showed that LIF, integrin β3, VEGF, and FGF-2 proteins were mostly expressed in the luminal and glandular epithelia, stroma, and myometrium of the endometrium. The expression of endometrial LIF, integrin β3, VEGF, and FGF-2 protein by IHC (Figures 3–6) were consistent with the WB (Figure 7).

On D4, the expressions of LIF, integrin β3, VEGF, and FGF-2 protein in the endometrium of rats in the D4M group were significantly increased compared with those in the endometrium of rats in the D4N group ($P < 0.05$, $P < 0.01$), whereas the expressions in the D4A group were significantly decreased compared with those in the D4M group ($P < 0.05$, $P < 0.01$). On D5 and D6, the expressions of LIF, integrin β3, VEGF, and FGF-2 protein in the endometrium of rats in the M group were significantly decreased compared with those in the endometrium of rats in the N group ($P < 0.05$, $P < 0.01$), whereas the expressions in the A group were significantly increased compared with those in the M group($P < 0.05$, $P < 0.01$) (Figure 7).

PCR results showed that the mRNA expression of LIF and FGF in the D4M group was significantly higher than that in the D4N group ($P < 0.05$), and acupuncture could alleviate this change, but there was no significant difference between D4A and D4M groups ($P > 0.05$). In addition, there was no difference in the mRNA expression of integrin β3 and VEGF among D4N, D4M, and D4A groups ($P > 0.05$). On D5, the mRNA expression of integrin β3 and VEGF in the D5M group was significantly lower than that in the D5N group ($P < 0.01$), but acupuncture could significantly reverse this change ($P < 0.05$). At the same time, there was no significant difference of the mRNA expression of LIF and FGF among D5N, D5M, and D5A groups ($P > 0.05$). On D6, the mRNA expression of LIF, integrin β3, VEGF and FGF-2 in the D6M group was significantly decreased compared with that in the D6N group ($P < 0.05$ or $P < 0.01$), while acupuncture could increase the mRNA expression of LIF compared with that in the

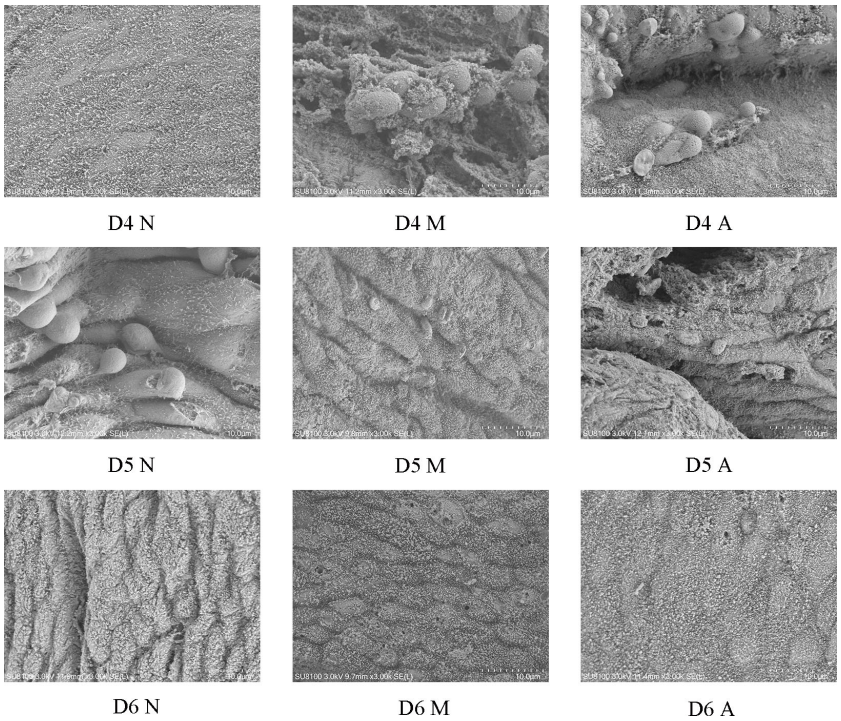


FIGURE 2
The expression of pinocytosis in endometrium of each group under scanning electron microscope (3000*). D, The embryo day; D4 N, D4 normal group; D4 M, D4 model group; D4 A, D4 acupuncture group; D5 N, D5 normal group; D5 M, D5 model group; D5 A, D5 acupuncture group; D6 N, D6 normal group; D6 M, D6 model group; D6 A, D6 acupuncture group.

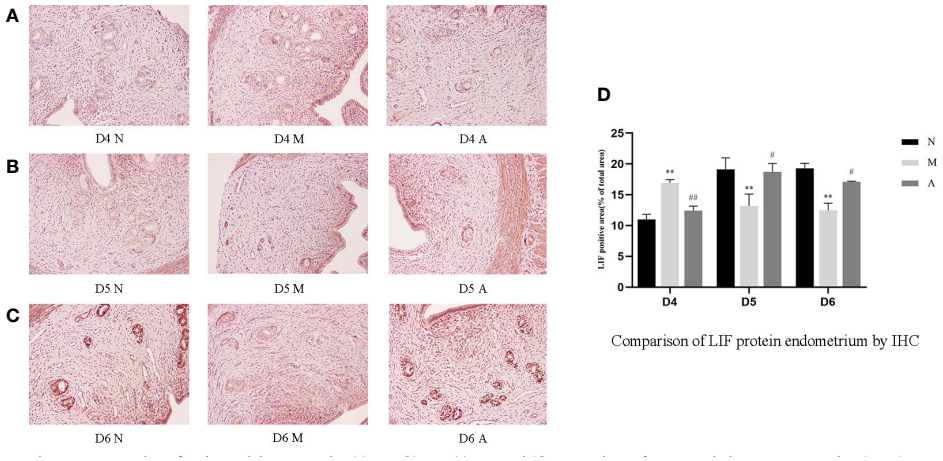


FIGURE 3
The expression of endometrial LIF protein: **(A)** D4; **(B)** D5; **(C)** D6; and **(D)** comparison of LIF protein by mean gray value (n=3). *or** represents that there is significant difference between M and N groups ($P < 0.05$ or $P < 0.01$), #or ## represents that there is significant difference between A and M groups ($P < 0.05$ or $P < 0.01$). Original magnification *200. LIF, Leukemia inhibitory factor; IHC, Immunohistochemistry; D, The embryo day; D4 N, D4 normal group; D4 M, D4 model group; D4 A, D4 acupuncture group; D5 N, D5 normal group; D5 M, D5 model group; D5 A, D5 acupuncture group; D6 N, D6 normal group; D6 M, D6 model group; D6 A, D6 acupuncture group; N, Normal group; M, Model group; A, Acupuncture group.

D6M group ($P < 0.05$). As for the mRNA expression of integrin β3, VEGF and FGF-2, although acupuncture could increase their expression, but no significant difference was observed between the D6A and D6M groups ($P > 0.05$) (Figure 8).

3.4 Comparison of serum estrogen and progesterone levels and endometrial estrogen and progesterone receptor protein and gene levels

There was no significant difference in D4, D5 and D6 estrogen levels among N, M, and A groups. Compared with the N group, the M group showed significantly higher levels of progesterone on D4, D5, and D6 ($P < 0.05$, $P < 0.01$). There were no significant differences in progesterone levels between the M and A groups on D4 and D5 ($P > 0.05$), whereas the levels were significantly lower on D6 in the A group compared with M group ($P < 0.05$) (Table 3, Figure 9).

IHC results showed that progesterone receptor (PR) and estrogen receptor α (ERα) mainly expressed in glandular epithelium and luminal epithelium of the endometrium, and a small amount was expressed in stromal cells. The epression of endometrial PR and Erα proteins by IHC (Figures 10, 11) were consistent with the WB (Figure 12).

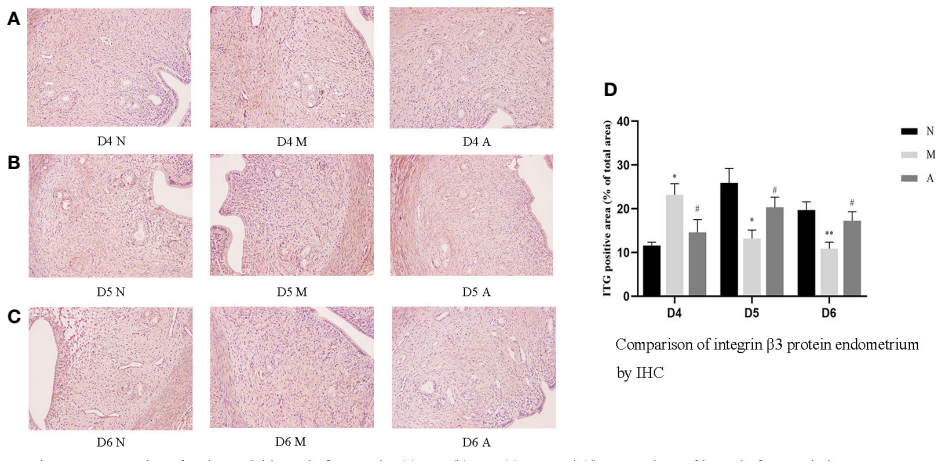


FIGURE 4
The expression of endometrial integrin β3 protein: **(A)** D4; **(B)** D5; **(C)** D6; and **(D)** comparison of integrin β3 protein by mean gray value (n=3). *or** represents that there is significant difference between M and N groups ($P < 0.05$ or $P < 0.01$), #or ## represents that there is significant difference between A and M groups ($P < 0.05$ or $P < 0.01$). Original magnification *200. IHC, Immunohistochemistry; D, The embryo day; D4 N, D4 normal group; D4 M, D4 model group; D4 A, D4 acupuncture group; D5 N, D5 normal group; D5 M, D5 model group; D5 A, D5 acupuncture group; D6 N, D6 normal group; D6 M, D6 model group; D6 A, D6 acupuncture group; N, Normal group; M, Model group; A, Acupuncture group.

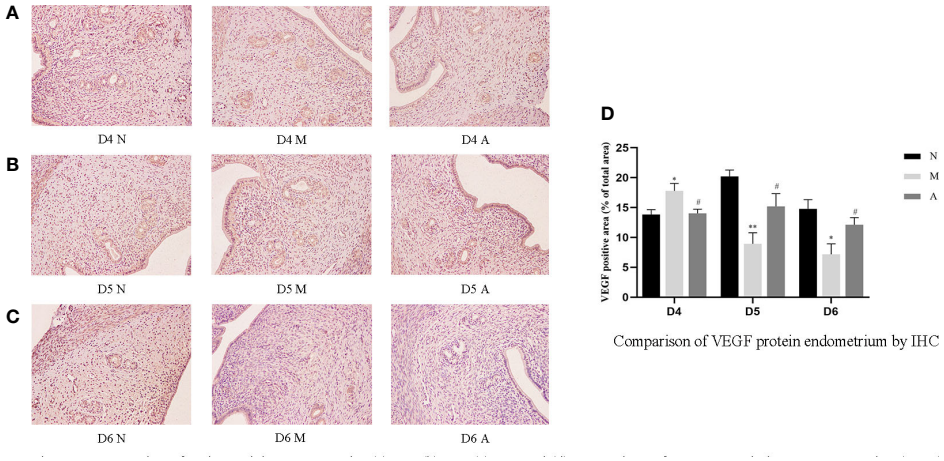


FIGURE 5
The expression of endometrial VEGF protein: **(A)** D4; **(B)** D5; **(C)** D6; and **(D)** comparison of VEGF protein by mean gray value (n=3). *or** represents that there is significant difference between M and N groups ($P < 0.05$ or $P < 0.01$), #or ## represents that there is significant difference between A and M groups ($P < 0.05$ or $P < 0.01$). Original magnification *200. VEGF, Vascular endothelial growth factor; IHC, Immunohistochemistry; D, The embryo day; D4 N, D4 normal group; D4 M, D4 model group; D4 A, D4 acupuncture group; D5 N, D5 normal group; D5 M, D5 model group; D5 A, D5 acupuncture group; D6 N, D6 normal group; D6 M, D6 model group; D6 A, D6 acupuncture group; N, Normal group; M, Model group; A, Acupuncture group.

PR protein levels in the D4M, D5M, and D6M groups were significantly lower than those in the corresponding N groups ($P < 0.01$). The PR protein levels in D5A and D6A groups were significantly higher than those in the corresponding M groups ($P < 0.01$). With regard to ERα, there was no significant difference between the M and N Group on D4 ($P > 0.05$). Compared with the D5N group, the D5M group showed significantly lower ERα protein levels ($P < 0.01$), whereas the D5A group showed significantly higher levels compared with the D5M group ($P < 0.01$). The expression trend for D6 ERα protein level was consistent with that observed for D5 (Figure 12).

PCR results showed that the mRNA level of ERα on the endometrium in the D4M group was increased compared with that in the D4N group ($P < 0.05$), whereas no significant difference was observed between D4A and D4M ($P > 0.05$). The expression trend for ERα protein levels in the D5M group was decreased compared with that in the D5N group ($P < 0.05$). There was no significant difference among the three D6 groups. The mRNA level of PR on the endometrium in D6M groups was decreased compared with that in the D6N group ($P < 0.01$); however, no significant difference was observed among other groups ($P > 0.05$) (Supplementary Image 1).

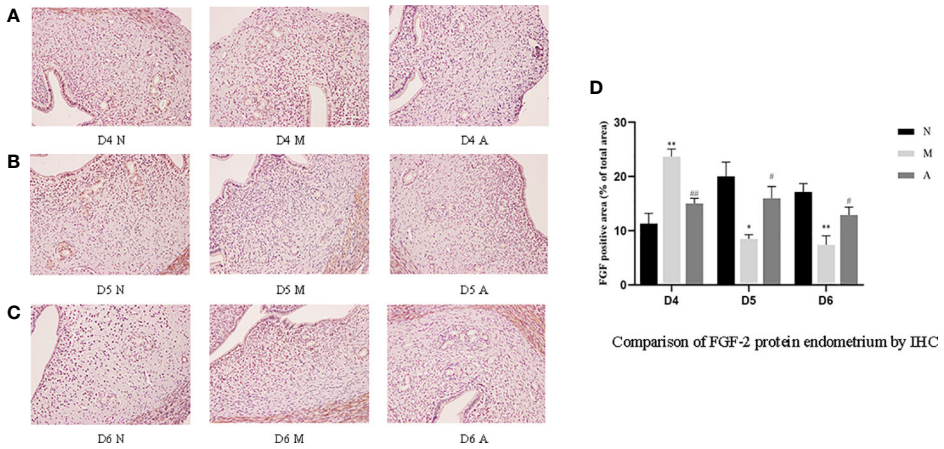


FIGURE 6
The expression of endometrial FGF-2 protein: **(A)** D4; **(B)** D5; **(C)** D6; and **(D)** comparison of VEGF protein by mean gray value (n=3). *or** represents that there is significant difference between M and N groups ($P < 0.05$ or $P < 0.01$), #or ## represents that there is significant difference between A and M groups ($P < 0.05$ or $P < 0.01$). Original magnification *200. FGF-2, Fibroblast growth factor 2; IHC, Immunohistochemistry; D, The embryo day; D4 N, D4 normal group; D4 M, D4 model group; D4 A, D4 acupuncture group; D5 N, D5 normal group; D5 M, D5 model group; D5 A, D5 acupuncture group; D6 N, D6 normal group; D6 M, D6 model group; D6 A, D6 acupuncture group; N, Normal group; M, Model group; A, Acupuncture group.

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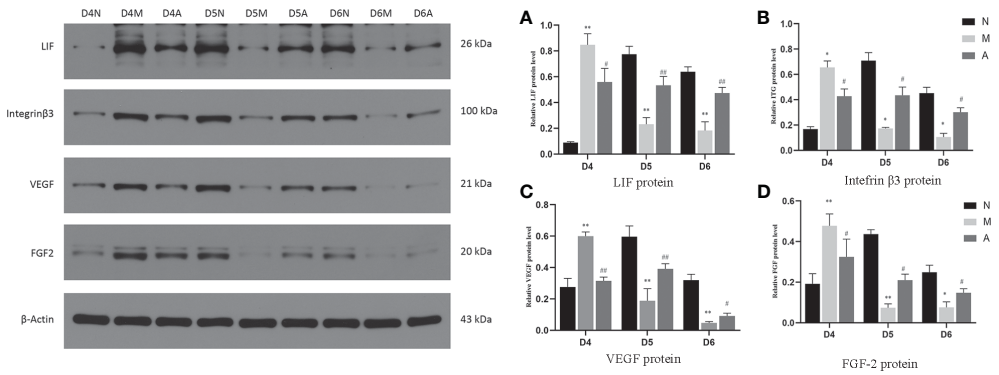

FIGURE 7
The expression of LIF **(A)**, integrin β3 protein **(B)**, VEGF **(C)**, and FGF-2 **(D)** protein in the endometrium by West-bolt. *or** represents that there is significant difference between M and N groups ($P < 0.05$ or $P < 0.01$), #or ## represents that there is significant difference between A and M groups ($P < 0.05$ or $P < 0.01$). LIF, Leukemia inhibitory factor; VEGF, Vascular endothelial growth factor; FGF-2, Fibroblast growth factor 2; D, The embryo day; N, Normal group; M, Model group; A, Acupuncture group.

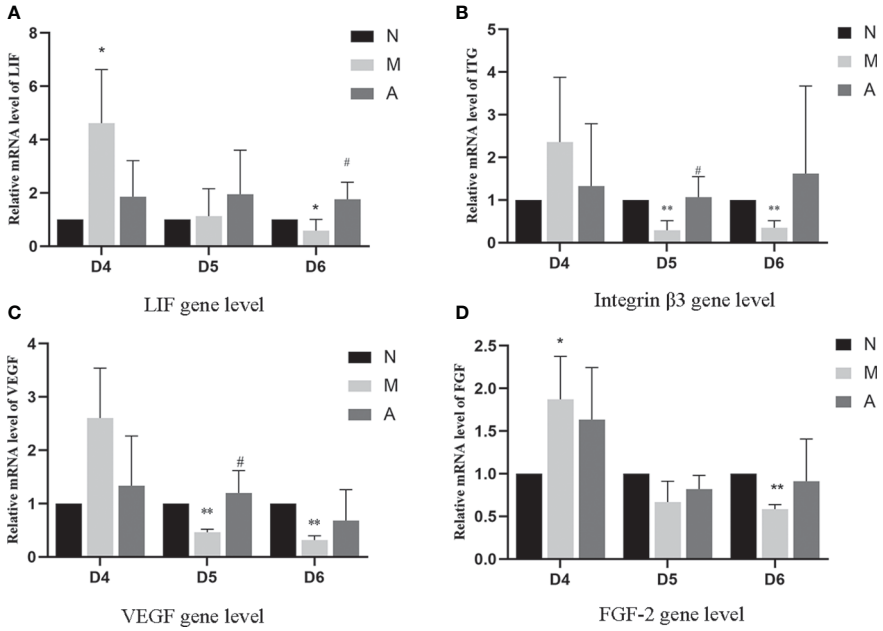

FIGURE 8
The expression of LIF **(A)**, integrin β3 protein **(B)**, VEGF **(C)**, and FGF-2 **(D)** mRNA levels in the endometrium (n=5). *or** represents that there is significant difference between M and N groups ($P < 0.05$ or $P < 0.01$), #or ## represents that there is significant difference between A and M groups ($P < 0.05$ or $P < 0.01$). LIF, Leukemia inhibitory factor; VEGF, Vascular endothelial growth factor; FGF-2, Fibroblast growth factor 2; mRNA, Messenger RNA; D, The embryo day; N, Normal group; M, Model group; A, Acupuncture group.

TABLE 3 The expression of serum progesterone and estrogen levels.

	Group	D4	D5	D6
Progesterone	N	34.99 ± 2.52 (n=8)	40.60± 3.55 (n=8)	48.35 ±3.19 (n=8)
	M	62.71± 6.72 (n=8)*	91.54 ± 9.48 (n=8) **	86.17± 8.70(n=8) **
	A	46.89 ±13.23 (n=8)	64.94± 3.43 (n=8)	55.05 ± 4.75 (n=8) #
Estrogen	N	67.84 ± 1.59 (n=8)	65.22 ± 1.75 (n=8)	60.80 ± 1.06(n=8)
	M	67.22 ± 2.66 (n=8)	61.22± 1.46 (n=8)	62.26 ± 1.28 (n=8)
	A	66.92 ± 2.37 (n=8)	60.94± 1.40 (n=8)	56.81 ± 2.21 (n=8)

The value is expressed as mean ± SD;*or** represents that there is significant difference between M and N groups (P < 0.05 or P < 0.01), #or ## represents that there is significant difference between A and M groups (P < 0.05 or P < 0.01), D4, The embryo day 4; D5, The embryo day 5; D6, The embryo day 6; N, Normal group; M, Model group; A, Acupuncture group.

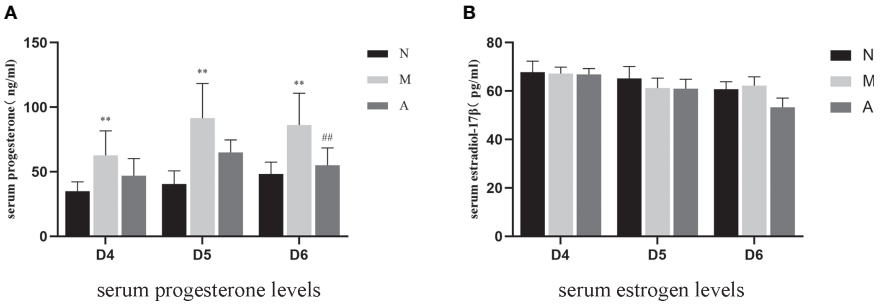

FIGURE 9
The expression of serum progesterone **(A)** and estrogen **(B)** levels (n=7). *or** represents that there is significant difference between M and N groups ($P < 0.05$ or $P < 0.01$), #or ## represents that there is significant difference between A and M groups ($P < 0.05$ or $P < 0.01$). D, The embryo day; N, Normal group; M, Model group; A, Acupuncture group.

4 Discussion

The results of pregnancy in this experiment are consistent with our previous findings (15). The overall pregnancy rate of COH rats is significantly reduced but the number of implanted embryos in pregnant rats is higher than normal rats, and acupuncture can increase the pregnancy rate and decrease the number of implanted embryos. Consistent with many previous studies, we speculated that the main reason for the decline in pregnancy rate after COH may be the decrease in the endometrial receptivity (17, 18). Our research results also confirm this viewpoint. However, interestingly, based on our research results, we believe that the damage caused by COH may be not only the ability of endometrium to withstand embryo implantation, which is generally considered and concerned by most researchers, but also the advance of embryo implantation window.

The endometrial receptivity and the quality of embryos are the key factors responsible for a successful pregnancy. The recent view is that endometrial receptivity is a complex process that provides the embryo with the opportunity to attach, invade, and develop, culminating in a new individual and continuation of the species (19). There are many factors affecting endometrial receptivity, and the opening of the implantation window is generally considered to be an important part of the complex process of endometrial receptivity (19). The implantation window refers to a period of close interaction between high-quality blastocysts required for embryo implantation and the endometrium that can accept embryo implantation and this time is usually very short (20). At present, the commonly used indicators for evaluating endometrial receptivity include ultrasound indicators (such as endometrial thickness, type, and subendometrial blood flow), cellular level indicators, and molecular level indicators (such as levels of integrated hormones, LIF, estrogen and progesterone) (15, 19–22). This study is to verify our hypothesis and the therapeutic mechanism of acupuncture through the dynamic detection of these cellular level indicators and molecular level indicators during the peri-implantation period.

The opening of the implantation window in normal rats occurs generally on about D5 after successful mating (23). Although

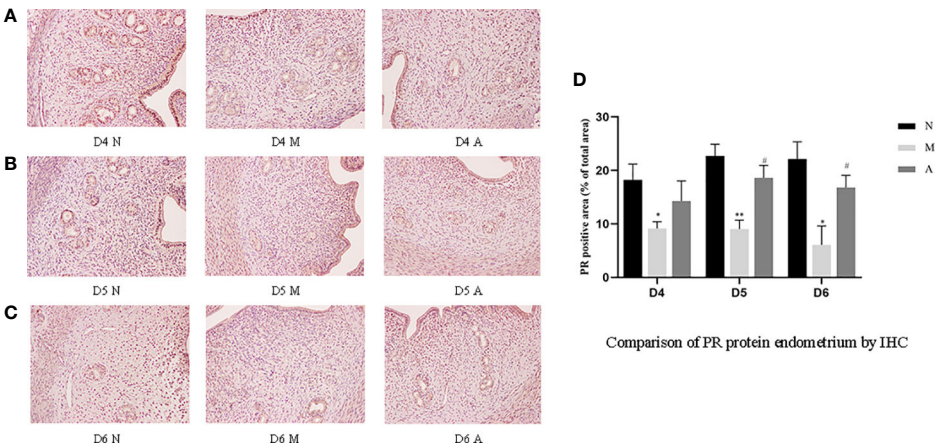

FIGURE 10
The expression of endometrial PR protein: **(A)** D4; **(B)** D5; **(C)** D6; and **(D)** comparison of PR protein by mean gray value (n=3). *or** represents that there is significant difference between M and N groups ($P < 0.05$ or $P < 0.01$), #or ## represents that there is significant difference between A and M groups ($P < 0.05$ or $P < 0.01$). Original magnification *200. PR: Progesterone receptor; IHC, Immunohistochemistry; D, The embryo day; D4 N, D4 normal group; D4 M, D4 model group; D4 A, D4 acupuncture group; D5 N, D5 normal group; D5 M, D5 model group; D5 A, D5 acupuncture group; D6 N, D6 normal group; D6 M, D6 model group; D6 A, D6 acupuncture group; N, Normal group; M, Model group; A, Acupuncture group.

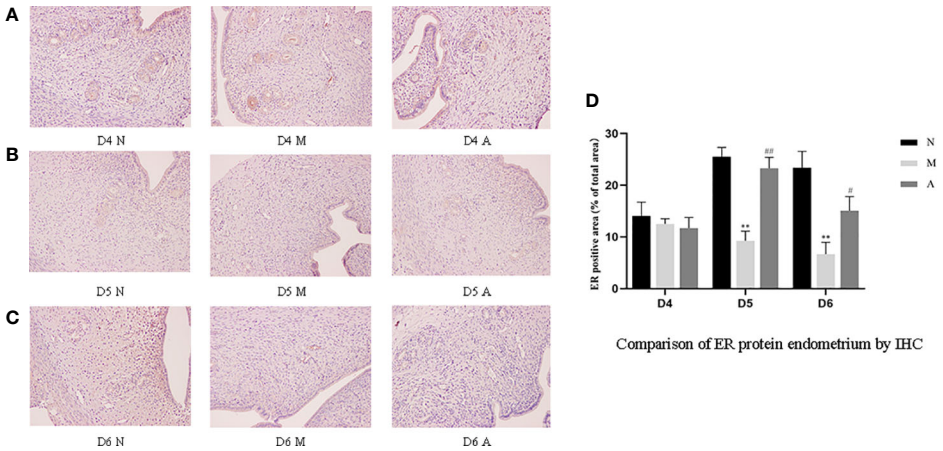

FIGURE 11
The expression of endometrial ER protein: **(A)** D4; **(B)** D5; **(C)** D6; and **(D)** comparison of ER protein by mean gray value (n=3). *or** represents that there is significant difference between M and N groups ($P < 0.05$ or $P < 0.01$), #or ## represents that there is significant difference between A and M groups ($P < 0.05$ or $P < 0.01$). Original magnification *200. ER, Estrogen receptor; IHC, Immunohistochemistry; D, The embryo day; D4 N, D4 normal group; D4 M, D4 model group; D4 A, D4 acupuncture group; D5 N, D5 normal group; D5 M, D5 model group; D5 A, D5 acupuncture group; D6 N, D6 normal group; D6 M, D6 model group; D6 A, D6 acupuncture group; N, Normal group; M, Model group; A, Acupuncture group.

pinopodes are rather disputed during the recent years, its number and shape are still closely associated with the endometrial receptivity and implantation window (24–26). In our experiment, it was observed that the normal rats showed no pinopodes in the endometrium on D4, there were a large number of mature pinopodes on D5, and the pinopodes was atrophied on D6. However, COH rats had a large number of mature pinopodes on D4, but they began to atrophy and subside on D5. In contrast, acupuncture could significantly pull back the forward movement of pinopodes, indicating that COH may lead to the forward movement of the implantation window, and acupuncture could restore the early implantation window.

Integrin β3 is an important molecule in the process of an embryo's initial attachment and cell adhesion, which can guide the

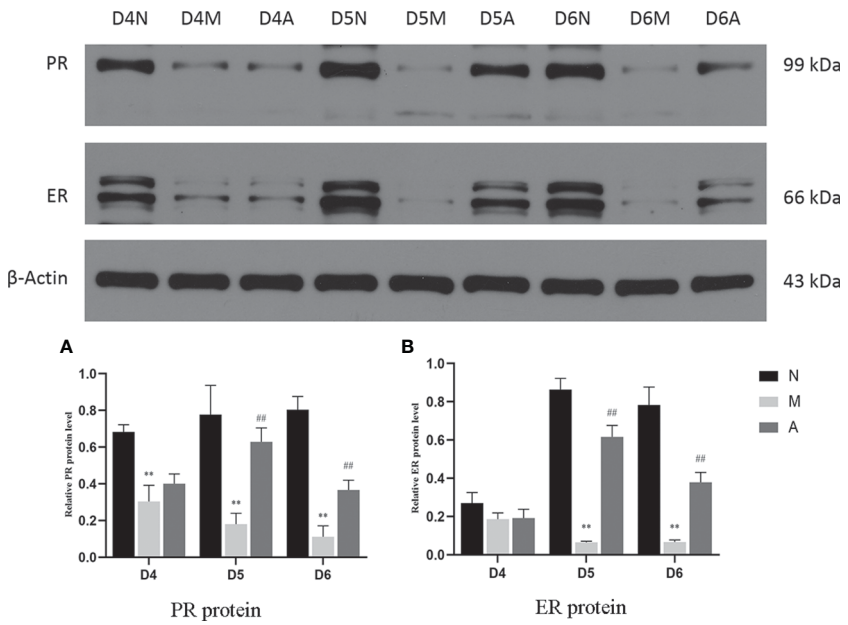

FIGURE 12
The expression of PR **(A)** and ER **(B)** protein in the endometrium by West-bolt. *or** represents that there is significant difference between M and N groups ($P < 0.05$ or $P < 0.01$), #or ## represents that there is significant difference between A and M groups ($P < 0.05$ or $P < 0.01$). PR, Progesterone receptor; ER, Estrogen receptor; D, The embryo day; N, Normal group; M, Model group; A, Acupuncture group.

adhesion of trophoblasts and subsequent implantation (21). In addition, integrin β3 is closely associated with the time order of endometrial receptivity and synchronized with the opening of the implantation window (27). Outcomes in IVF were poor in women that were in phase histologically but lacked the integrin β3 (19, 28). LIF is the first and most durable endometrial protein recognized as essential for implantation (29, 30). As a cytokine of the IL-6 family, LIF utilizes a receptor that consists of the LIF receptor β and gp130, and initiates the signal transduction cascades that phosphorylates STAT3 through Janus kinases (JAK) and signal transducer and activation of transcription protein (STAT) pathway in the uterus, and plays a very important role in implantation (21, 30–32). LIF is expressed on the endometrium throughout the menstrual cycle and increases significantly from mid-secretory to late-secretory which is a finite period defined as the implantation window (30, 33, 34). It has been reported that blastocysts in LIF-knockout mice cannot implant successfully (35). Studies have proved that acupuncture can increase Integrin β3 and LIF expression to improves pregnancy outcomes in rats with thin endometrium (25), in PCOS rats (26), and in rats of implantation failure (36). We further continuously and dynamically observed these indicators at three time points: before, during, and after implantation (D4, D5, and D6) to explore the dynamic changes of implantation window and the mechanism of acupuncture. Our results show that the levels of LIF and integrin β3 were first increase and then decrease in the normal group, reaching its peak level on D5. But in the model group, they were significantly higher than those in the normal group on D4, but decreased sharply on D5 and D6 days, reaching its peak level on D4. Acupuncture could restore the trend of early expression caused by COH to a certain extent. Our results is consistent with Fang's which showed that electroacupuncture improves endometrial receptivity through increasing LIF expression in COH rats (37). Although the results of LIF and integrin β3 at the gene level were not completely consistent those at the protein level, we got the same results at the protein level with regard to the expression of LIF mRNA on D4 and integrin β3 mRNA on D5 and D6. However, there was no statistical difference in the other comparison. The unknown cascade effect in gene-protein expression may be the reason.

During embryo implantation, angiogenesis is a key factor that determines the endometrial receptivity and the opening of the implantation window. Therefore, angiogenesis markers such as VEGF and FGF are also considered to be closely related to the opening of the uterine implantation window and are used as one of the potential molecular markers (38). Xing et al. showed that acupuncture increased VEGF gene/protein expression in the endometrium of PCOS rat to improve endometrial receptivity (26). Our results show that the continuous dynamic expression of VEGF and FGF-2 protein were basically consistent with integrin β3 and LIF. The gene and protein level trends of FGF-2 and VEGF were consistent on D4 and D6, which is also consistent with our previous experiments (15). However, on D5, only VEGF mRNA expression was consistent with the protein expression, and there was no statistical difference in FGF-2 mRNA expression in each group. The reason for this may also be related to the unknown cascade effect in the process of gene-to-protein expression.

In a nutshell, the above mentioned continuous dynamic experimental observation results from the cellular level to the molecular level systematically confirm the first half of our hypothesis. The decrease in LIF, integrin β3, FGF, and VEGF indices of COH rats on D5 and D6 may be an artifact caused by withdrawal after the peak on D4. In addition, although the pregnancy rate of COH rats decreased on D6, there were still more implantation sites than the normal group, which also may suggest that the ability of the endometrium to accommodate embryo implantation after COH may not be much impaired. The advanced implantation window caused by COH may be an important factor associate with the reduction in pregnancy rate in COH rats, and the effective role of acupuncture may be to restore the implantation window to normal and improve the local angiogenesis of the endometrium during the peri-implantation period, thereby improving the endometrial receptivity and finally improving pregnancy outcomes. In addition, to explain the further mechanism of acupuncture, we tested serum estrogen and progesterone levels and endometrium ER and PR.

Estrogen and progesterone are upstream targets of many links in the implantation mechanism, which can activate multiple downstream links to guide the structural and functional remodeling in the process of implantation (17). The peak of estrogen can initiate embryo implantation, and progesterone could down-regulate ER and PR receptors in the endometrium (39) and maintain stromal decidualization of the endometrium (40). Down-regulation of ER were proved to be implicated in abnormal expression of other endometrial biomarkers, such as Integrin β3 (19). Hence, the endometrial receptivity is closely associated with the level of peripheral blood steroid hormones levels and ER and PR (41). Therefore, some scholars have proposed that the body produces too much progesterone after COH, resulting in the imbalance of the progesterone to estrogen ratio, which further leads to the unsynchronized opening of the implantation window and endometrial development, leading to implantation failure (4, 5). Our results showed that although there was no significant difference in the serum estrogen levels in rats in each group, the progesterone level in the model group rats increased significantly during the three-day peri-implantation period, and acupuncture reduced the progesterone levels. The changing trend of estrogen and progesterone levels in our COH rats is consistent with the results in the COH mouse model reported by Song et al. (42). On D5 and D6, the expression of ERα and PR protein and gene levels in the model group significantly decreased; however, acupuncture could increase the expressions of ERα and PR. We speculate that the super-physiological dose of progesterone disrupts the balance of estrogen and progesterone, and at the same time, affects the expressions of ERα and PR, which in turn leads to the advancement of the embryo implantation window and unsynchronized development of the endometrium. Acupuncture may play a certain role in improving all of the above links.

However, our research also has certain limitations. At present, acupuncture is generally regarded as a mechanism that exerts benefits through multi-target and multi-step overall regulation. Our research shows that acupuncture could reduce the

progesterone to estrogen ratio, and make the implantation window return to normal. However, our results may still be a phenomenon in which acupuncture takes effect. The specific efficacy mechanisms of acupuncture may involve many complexities including angiogenesis, immune tolerance regulation, and endocrine regulation, among others. The specific mechanisms of acupuncture for improving the pregnancy rate in IVF-ET need to be confirmed by future research.

5 Conclusions

In this study, we continuously and dynamically observed markers related to endometrial receptivity at three time points: before, during, and after implantation (D4, D5, and D6), and found that the embryo implantation window moves forward after COH and asynchrony with the development of the endometrium may be important factors associated with a reduced pregnancy rate in COH rats. The effective targets of acupuncture could not only improve the local angiogenesis of the endometrium, progesterone to estrogen ratio, and the levels of ERα and PR, improving the ability of the endometrium to withstand embryo implantation, but also make the implantation window tend to return to normal.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by Animal Experiment Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China (prove number: TJH-202009008).

Author contributions

HD contributed to the conception of this article. RH wrote the manuscript. RH and YH completed the study, RH, HD, MZ, GH and YS revised the manuscript. RH, KS and XW designed and illustrated the figures. RH, HD, MZ, GH and YS performed the literature search and interpretation. RH, YH, YS, XW, KS, GH, MZ and HD reviewed 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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1110266/full#supplementary-material

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Analysis of sperm chromatin packaging and reproductive biomarker to evaluate the consequence of advanced male age

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In this study, the semen parameters, sperm chromatin integrity, antioxidant enzyme levels, and reproductive hormone levels of subfertile male subjects from Pakistan were assessed in relation to their age. Data on the demographic characteristics of the 750 study participants, including their general health, body mass index (BMI), and reproductive status, were collected from subfertile men from Pakistan. Semen and blood were collected to determine standard semen parameters, sperm chromatin dispersion (Halosperm-SCD), sperm chromatin integrity using toluidine blue (TB) staining, sperm chromatin maturity using chromomycin A3 (CMA3+) staining, and reproductive hormone (FSH, LH, prolactin and testosterone levels). The patients were divided into three groups according to their age: Group 1 included male subjects aged 30 years or less (n = 90), Group 2 included male subjects between the ages of 31 and 40 years (n = 330), and Group 3 included male subjects over 40 years of age (n = 330). Conventional semen parameters, reactive oxygen species (ROS), superoxide dismutase (SOD), guaiacol peroxidase (GPX), catalase (CAT), and lipid peroxidation (MDA) did not statistically (p > 0.05) differ with increasing male age or between different age groups. When compared to younger men (<30 years), sperm SCD (23.2 $\pm$ 0.88%) was significantly (p = 0.01) lower as compared to male patients aged >40 years (26.6 $\pm$ 0.6%). The concentration of LH, FSH, and testosterone levels were comparable between the groups (p > 0.05), while a significant (p = 0.04) increase in sperm chromatin immaturity CMA3+ (30 $\pm$ 0.71%) was observed in the old age group (>40 years) compared to the <30-year group (26.6 $\pm$ 1.03%). A positive association was observed between advanced male age and sperm chromatin dispersion (SCD) (r = 0.124, p = 0.001) and decondensation (CMA3+) (r = 0.1, p = 0.009). Despite potential limitations, this study has been carried out with extensive information on the potential risk of male age on sperm integrity. The present study demonstrated

the impact of male age on male reproductive health, as these patients had a higher percentage of sperm chromatin damage (SCD) in their semen. Sperm DNA damage assessment will help in the evaluation and diagnosis of the underlying cause of poor fertility and can help clinicians in selecting the right treatment options. Male age is one of the factors that have an impact on the decline in male fertility. As a result, it is preferable for patients receiving assisted reproductive technology to be younger.

KEYWORDS

sperm chromatin integrity, assisted reproductive procedures, sperm deoxyribose nucleic acid fragmentation index, reproductive marker, male age

1 Introduction

The risk of infertility and poor child health increases with delayed family planning and older parents. While the effects of aging on oogenesis have been extensively studied, spermatogenesis has received less attention (1). It is estimated that the prevalence of male subfertility between the ages of 15 and 50 years is up to 6%. Approximately 25% of couples experience male factor subfertility (2, 3). It has been reported that in male partners opting for semen analysis, over 50% of men presented with abnormal semen parameters. In recent years, advancing age becomes a key factor contributing to debility in reproductive health indices in both sexes. Old male patients have augmented estrogen levels, due to the amplification of aromatase; through a negative feedback loop, men display indications of hypogonadotropic hypogonadism. These hormonal fluctuations, besides augmented oxidative stress, lipotoxicity, and instabilities in the absorptions of adipokines, directly distress the gonads, peripheral reproductive organs, and the embryo (4). It is generally well accepted that reproductive function highly correlates with the degree of adiposity, nutrition, or metabolic condition related to food intake in human medicine (5, 6). Male age >40 years is associated with reduced semen quality. Furthermore, infection, immunological factors, trauma, or surgical insult to the male reproductive organs, and exposure to toxic chemicals or other materials are all known acquired factors that contribute to male subfertility (2, 7, 8). Similarly, a direct association was found between men's age and semen quality even after adjustment for reproductive hormones (9).

Abbreviations: BMI, Body mass index; DFI, DNA fragmentation index; MFI, Male factor infertility; SCD, Sperm chromatin dispersion; TB, Toluidine Blue; CMA3+, chromomycin A3; IVF, Invitro-fertilization; ICSI, Intra cytoplasmic injection; ART, Assisted reproduction techniques; ROS, Reactive oxygen species; WBC, white blood cell; TMS, total motile sperm; HOS, hypo-osmotic swelling; SOD, superoxide dismutase; GPX, guaiacol peroxidase; CAT, catalase; MDA, lipid peroxidation; FSH, follicular stimulating hormone; LH, luteinizing hormone; TUNEL assay, Terminal transferase dUTP nick-end labeling; HCG, human chorionic gonadotropin; HOST, hypo-osmotic swelling test; SCSA, Sperm chromatin structure assay.

Semen analysis is a routine and simple method for assessing male fertility status. However, alone, it is not sufficient to predict assisted reproductive outcomes (10, 11). With the development of new predictive tools to identify male fertility potential, the sperm deoxyribonucleic acid fragmentation index is a commonly used technique involving different methods (10, 12). For identification of the DNA fragmentation index, we used SCD assay (13–15). Chromomycin A3 (CMA3) has been used for the evaluation of sperm chromatin condensation, which is indirectly associated with its integrity since this fluorochrome binds to the guanine–cytosine dinucleotide region of DNA competitively with protamines that bind to the same region (16). CMA3 has been used as an indirect measure of the protamination state of nuclear chromatin. On the other hand, several authors affirm that the presence of protamine-deficient spermatozoa CMA3+ is associated with DNA integrity (17, 18). They base it on the fact that protamines are nuclear proteins that play a key role in the integrity of sperm DNA since they are responsible for the integrity stability and packaging of sperm DNA until the paternal genome is introduced into the oocyte during fertilization.

The relationships between age, semen characteristics, male reproductive hormones, sperm DNA fragmentation, chromatin structure, and ART outcome have been inconsistently correlated, according to numerous studies and meta-analyses (1, 19, 20). Giving birth at an appropriate male age can reduce the risk of disease in future generations. Regarding IVF and/or ICSI, despite the fact that numerous clinical studies have been carried out to evaluate the negative effects of human sperm DNA damage on reproductive outcomes, the findings from these studies are still debatable. Some researchers claim that sperm DNA damage has no negative effects on the rate of fertilization and pregnancy rate (21–23), while others claim that there is a link between DNA fragmentation and decreased fertility and pregnancy outcome (2, 24, 25). Moreover, other factors such as age would be the leading cause of lower pregnancy rates and failure of reproductive outcomes. Therefore, the overall health and normal age of parents should be considered in couples as an important concern in attaining successful reproductive outcomes. We aimed to investigate the correlation of male age on semen parameters

(concentration, motility, morphology, and vitality), oxidative stress, hormonal levels, SCD, and chromatin compaction markers.

2 Materials and methods

2.1 Study design and ethical clearance

The research was conducted at the Faculty of Biological Sciences, Reproductive Physiology Laboratory, Department of Zoology, and Quaid-i-Azam University-Islamabad Pakistan. All study participants provided consent and signed informed consent forms. The criteria for participation in the study were that the couples give their informed written consent. The ethical approval to conduct this study was obtained from the Ethics Committee of Salma Kafeel Medical Centre Islamabad Pakistan No, SKMC&FGS-010-2016. and the Bio-Ethic committee of the Department of Zoology, Quaid-i-Azam University, and Islamabad # BEC-FBS-QAU2016-77.

2.2 Participants

Inclusion and exclusion criteria were as follows: couples undergoing their first ovarian stimulation (who remained unsuccessful in achieving pregnancy after trying for 12 or more months, with male partner age range between 20 and 49 years from January 2016 to October 2021); patients with recent fever, abnormalities of the external genitalia, abnormal karyotyping, cryptorchidism, varicoceles, presence of anti-sperm antibodies, azoospermia, or severe oligoasthenoteratozoospermia; those taking treatment that can alter spermatogenesis; patients with chronic diseases (e.g., liver/renal disease, patients with hypertension, diabetes, and andrological disorders); and those with an identified subfertility factor in the female partner were not included. All patients were properly advised of the associated risks of IVF therapy and completed an informed permission form to allow researchers to utilize their clinical data. The patients were divided into three groups according to their age: Group 1 included male patients aged 30 years or less (n = 90) (the data obtained from male patients aged less than 30 years compared to other groups were lesser in record and fewer responders were available), Group 2 included male subjects between the ages of 31 and 40 years (n = 330), and Group 3 included male subjects over 40 years of age (n = 330). The study protocol was developed following the Declaration of Helsinki (26). The sample size was calculated using the formula used before (27, 28).

2.3 Sampling technique and data collection

Data collection was done through face-to-face interviews and electronically and the following characteristics of the couple were documented and evaluated: age (full years), duration of subfertility (years), history of hypertension or diabetes mellitus, family history, obesity, subfertility, and genetic disease during the first visit by an informal interview with the couple. The research committee of the Quaid I Azam University in Islamabad's Department of Reproductive Physiology examined and approved the study protocol and questionnaire. The survey responses were kept private. The data collector and skilled medical personnel entered the information into a database. The data collector made sure that the interviews and data were kept private. The lead investigator was the only person with access to the complete collection of data. Before data and sample collection, couples were assured that their identity would be kept anonymous.

2.4 Body mass index

All couples' height and weight were measured by a skilled nurse at the initial visit. Weight divided by height squared was used to compute BMI according to the classification standards of the global organization.

2.5 Outcomes

Semen sample volume, concentration, motility, and morphology were evaluated. SCD assay, chromatin integrity using toluidine blue (TB) staining, and CMA3 staining have been used as an indirect measure of the protamination state of nuclear chromatin and chromatin integrity.

2.6 Semen standard parameter analysis

After masturbation, the semen sample was collected, after 2–5 days of abstinence, and the semen sample was analyzed after 30 min of liquefaction at 37°C. Each sample was subjected to analysis for seminal characteristics. Semen parameters were assessed according to WHO 2010 standards; to summarize, sperm number was determined, the sperm motility was determined using a Leica microscope DM300 scoring at least 100 spermatozoa/slide, and morphology was determined using Diff-Quik staining. Sperm deformity index (SDI) and Teratozoospermic index (TZI) are calculated as described by Cooper et al. (29).

According to Jeyendran et al., the hypo-osmotic swelling test (HOS-test) was used for the assessment of membrane integrity of spermatozoa. A 100-µl sample of sperm suspension was added to 1 ml of hypoosmotic solution (equal parts of 150 mOsmol fructose and 150 mOsmol sodium citrate solutions), followed by 60 min of incubation at 37°C. After incubation, a minimum of 200 spermatozoa were examined per slide under a light microscope and the percentage of spermatozoa that showed typical tail abnormalities (curly tail) indicative of swelling were calculated (30).

2.7 Biochemical studies

While oxidant concentration of the ROS assessment method was previously published in detail, semen samples were examined to

test antioxidant enzyme levels including superoxide dismutase (SOD) (units/mg of protein) (31), guaiacol peroxidase (GPX), catalase (CAT) (32), and lipid peroxidation *via* malondialdehyde (MDA) (33) on a UV spectrophotometer (Agilent 8453). ROS were estimated using the protocol of Novotný et al. (34); briefly, the liquefied semen was centrifuged at 300*g* for 7 min, seminal plasma was removed, and the pellet of cells was washed in PBS (isotonic solution, pH = 7.4) and spun again and decanted. Washed cells were suspended in PBS to adjust sperm concentration to 1.25×10^6/ml. ROS production was measured after the addition of 10 µl of 5 mM freshly prepared solution of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma Chemical Co., St. Louis, MO, USA) in dimethyl sulfoxide (DMSO, Sigma Chemical Co.) to 400 µl of spermatozoa suspension. A tube containing 400 µl of PBS and 10 µl of luminol solution served as a blank. Chemiluminescence was measured integrally for 15 min using the Digene DCR-1 single detector luminometer (Digene Diagnostics, Inc., Gaithersburg, MD, USA). Results were expressed in relative light units (RLU) per minute and 20×10^6 spermatozoa.

The other semen fraction was tested for sperm DNA fragmentation (SCD), and chromatin maturity (CMA3+, TB+) was evaluated.

2.8 Sperm chromatin dispersion assay

As previously reported, the SCD test was conducted using a Sperm Nucleus DNA Integrity Kit (SCD) from Shenzhen Huakang Biomed Co., Ltd., Shenzhen, China (35). The technique that was carried out was as follows: A tube containing fluidized agarose received 60 µl of semen sample before being dropped onto a glass slide and covered with a glass coverslip. After 4 min at 4°C, the coverslip was removed. Following acid denaturation for 7 min, lysis for 20 min was performed. The slide was then thoroughly cleaned for 3 min with plenty of distilled water before being dehydrated for 2 min in successive ethanol washes of 70%, 90%, and 100%. Wright's staining was followed by the manual counting of 500 spermatozoa per slide to assess the integrity of the sperm DNA under bright-field microscopy. To assess the level of sperm DNA integrity, the dispersion of sperm DNA was calculated. If the value of SCD was found to be less than 30%, it was considered to be normal (36).

2.9 Toluidine blue staining

TB was used to measure chromatin integrity (37). Spermatozoa's two smears were fixed with freshly prepared 96% ethanol and acetone (1:1), and the slides were treated with 0.1 M HCl at 4°C for 5 min and then rinsed three times with distilled water for 2 min each. After 5–10 min, the slides were rinsed with distilled water and coated with TB solution (0.05% TB in 50% McIlvain citrate phosphate buffer, pH 3.5–4). The slides were dehydrated in ethanol baths one after the other (70%, 96%, and 100%). Finally, per sample, 200 spermatozoa were counted under an optical microscope after the slides were coated and mounted with xylene at room temperature (2–3 min). A cationic dye is TB. It can attach to DNA with damaged or loosely packed phosphate residues that are negatively charged. The cells were divided into two groups: light blue cells (TB− cells; normal chromatin structure) and dark violet cells (TB+ cells; aberrant chromatin structure).

2.10 Chromomycin A3 staining

Semen smear slides were settled in a 3:1 solution of methanol and glacial acetic acid at 4°C for 20 min before actually air-drying at room temperature for 20 min. A 100-L CMA3 solution was added to the slides for 20 min (38). The CMA3 solution was composed of 0.25 mg/ml CMA3 in McIlvain's buffer (pH 7.0) with 10 mmol/L $MgCl_2$. The films were washed in a buffer before getting mounted in a 1:1 v/v PBS-glycerol solution. After that, these same slides were kept at 4°C for 24 h. A fluorescent microscope was used to assess luminescence. On every slide, 200 sperm cells are assessed at probability sampling. CMA3 immunofluorescence was tested by separating sperm cells that stain bright yellow (CMA3+) versus those that light-color a dull yellow (CMA3−).

2.11 Statistical analysis

Data were methodically imported to Microsoft Excel 2010 from the medical record and the interviewer. The Statistical Package for Social Sciences (SPSS) 20 IBM program was used for all statistical studies (Armonk, NY). Data were presented as mean ± SD. To compare the percentage, the ANOVA with Tukey's test was chosen for the statistical analysis. Age-based groupings of the male subjects recruited for the current study were created. Age was the independent variable, while sperm DNA damage, chromatin maturity parameter, ROS, and semen parameter were considered dependent variables and values were compared to male BMI. Pearson correlation analysis was performed between the various parameters. Simple linear regression analysis was conducted to identify the relationship between male age as an independent variable with dependent variables including CMA3+, SCD, ROS, and TMS. The Hosmer–Lemeshow goodness of fit test was used to determine the model's dependability. A p-value of <0.05 was considered to be statistically significant.

3 Results

3.1 Demographic parameters

The mean demographic parameters, including age (years), BMI (kg/m^2), and fertility duration (years) evaluated in 750 couples enrolled in this study, are reported in Table 1.

TABLE 1 Demographic characteristics of couples included in the study.

	<30 years (n = 90)	30–40 years (n = 330)	>40 years (n = 330)	Total (n = 750)
Male age (years)	28.06 ± 0.30	36.24 ± 0.18	45.40 ± 0.32	38.80 ± 0.35
Male BMI (kg/m^2)	22.79 ± 0.23	23.03 ± 0.11	22.71 ± 0.15	22.89 ± 0.08
Female age (years)	27.46 ± 0.66	32.35 ± 0.35	35.62 ± 0.45	32.79 ± 0.28
Female BMI (kg/m^2)	26.89 ± 0.40	27.16 ± 0.19	26.75 ± 0.26	26.99 ± 0.14
Infertility duration (years)	5.03 ± 0.36	8.40 ± 0.31*	12.50 ± 0.55**	9.29 ± 0.28

Values represent mean ± SEM; BMI, body mass index, n = number of patients.
*p < 0.05, **p < 0.01.

3.2 Semen standard parameters, and biochemical and hormonal analysis

The mean conventional semen parameters, including concentration, normal morphology, total motile sperms (TMS %), HOS %, ROS (U/min), GPX (nmol), SOD (U/min), MDA (nmol/ml), and hormonal levels [FSH (mIU/ml), LH (mIU/ml), prolactin (mIU/ml), and testosterone (ng/ml) levels], were comparable in all age groups (Table 2).

3.3 Sperm chromatin integrity parameters

We found that aged men (>40 years) had a higher percentage of sperm with DNA damage (26.6 ± 0.6, p = 0.001) compared to younger aged men (¾30 years age, SCD% = 23.2 ± 0.88) (Table 3, Figure 1A). Percentage of mature spermatozoa with intact chromatin (CMA3) significantly (p = 0.04) decreased with the age of men (Table 3, Figure 1B). A significant positive correlation was found between the age of men and percentage of spermatozoa with

TABLE 2 The effects of male age on semen parameters, biochemical profile, and reproductive hormone concentration in studied groups.

	Below 30 years (n = 90)	30 to 40 years (n = 330)	Above 40 years (n = 330)	Total (n = 750)
Semen parameters				
Semen volume (ml)	4.03 ± 0.18	3.74 ± 0.09	3.93 ± 0.14	3.85 ± 0.07
pH	8 ± 0.00	8.00 ± 0.01	8.15 ± 0.15	8.05 ± 0.05
Liquefaction time (min)	31.85 ± 0.93	33.36 ± 0.97	31.07 ± 0.39	32.43 ± 0.56
WBC/HPF	3.32 ± 0.26	3.01 ± 0.13	3.02 ± 0.18	3.06 ± 0.10
Concentration ×10^6/ml	62.02 ± 8.13	56.97 ± 3.29	53.32 ± 4.05	56.47 ± 2.47
Normal morphology %	4.00 ± 0.20	3.63 ± 0.12	3.47 ± 0.16	3.63 ± 0.09
TMS %	50.42 ± 3.20	44.82 ± 1.64	44.45 ± 2.08	45.46 ± 1.19
Viability (HOS) %	73.32 ± 2.47	70.13 ± 1.28	70.49 ± 1.57	70.70 ± 0.92
Oxidant/antioxidant concentrations				
ROS (U/min)	1.60 ± 0.12	1.71 ± 0.07	1.72 ± 0.08	1.70 ± 0.05
SOD (U/min)	13.73 ± 0.35	13.42 ± 0.17	13.47 ± 0.20	13.48 ± 0.12
GPX (nmol)	10.84 ± 0.08	10.63 ± 0.05	10.66 ± 0.05	10.67 ± 0.03
CAT (g/dl)	9.71 ± 0.14	9.62 ± 0.06	9.62 ± 0.09	9.63 ± 0.05
MDA (nmol/ml)	28.56 ± 0.24	28.82 ± 0.11	28.86 ± 0.18	28.80 ± 0.09
Reproductive hormone levels				
FSH (mIU/ml)	6.08 ± 0.40	5.50 ± 0.19	5.91 ± 0.35	5.71 ± 0.16
LH (mIU/ml)	8.10 ± 1.35	6.75 ± 0.52	8.67 ± 1.00	7.54 ± 0.46
Prolactin (mIU/ml)	10.84 ± 1.12	11.34 ± 0.52	13.13 ± 0.72	11.84 ± 0.40
Testosterone (ng/ml)	382.25 ± 31.82	365.55 ± 15.63	394.80 ± 19.55	377.18 ± 11.40

Values represent mean ± SEM; n = number of patients; WBC, white blood cell; TMS, total motile sperm; HOS, hypo-osmotic swelling, ROS, reactive oxygen species; SOD, superoxide dismutase; GPX, guaiacol peroxidase; CAT, catalase; MDA, lipid peroxidation; FSH, follicular stimulating hormone; LH, luteinizing hormone.

TABLE 3 Male age influence sperm chromatin dispersion (SCD), chromatin integrity (TB+), and chromatin compaction (CMA3+) in studied groups.

	Below 30 years (n = 90)	30 to 40 years (n = 330)	Above 40 years (n = 330)	Total (n = 750)
Sperm chromatin dispersion—SCD %	23.2 ± 0.88	25.1 ± 0.4	26.6 ± 0.6**	25.4 ± 0.34
Chromatin integrity—TB+ %	26.71 ± 1.83	26.47 ± 0.86	28.65 ± 1.14	27.23 ± 0.65
Chromatin compaction—CMA3+ %	26.6 ± 1.03	29.04 ± 0.50	30 ± 0.71*	28.9 ± 0.30

Values represent mean ± SEM; n = number of patients; SCD, sperm chromatin dispersion; TB+, toluidine blue staining; CMA3+, chromomycin A3 staining.
*p < 0.05, **p < 0.01.

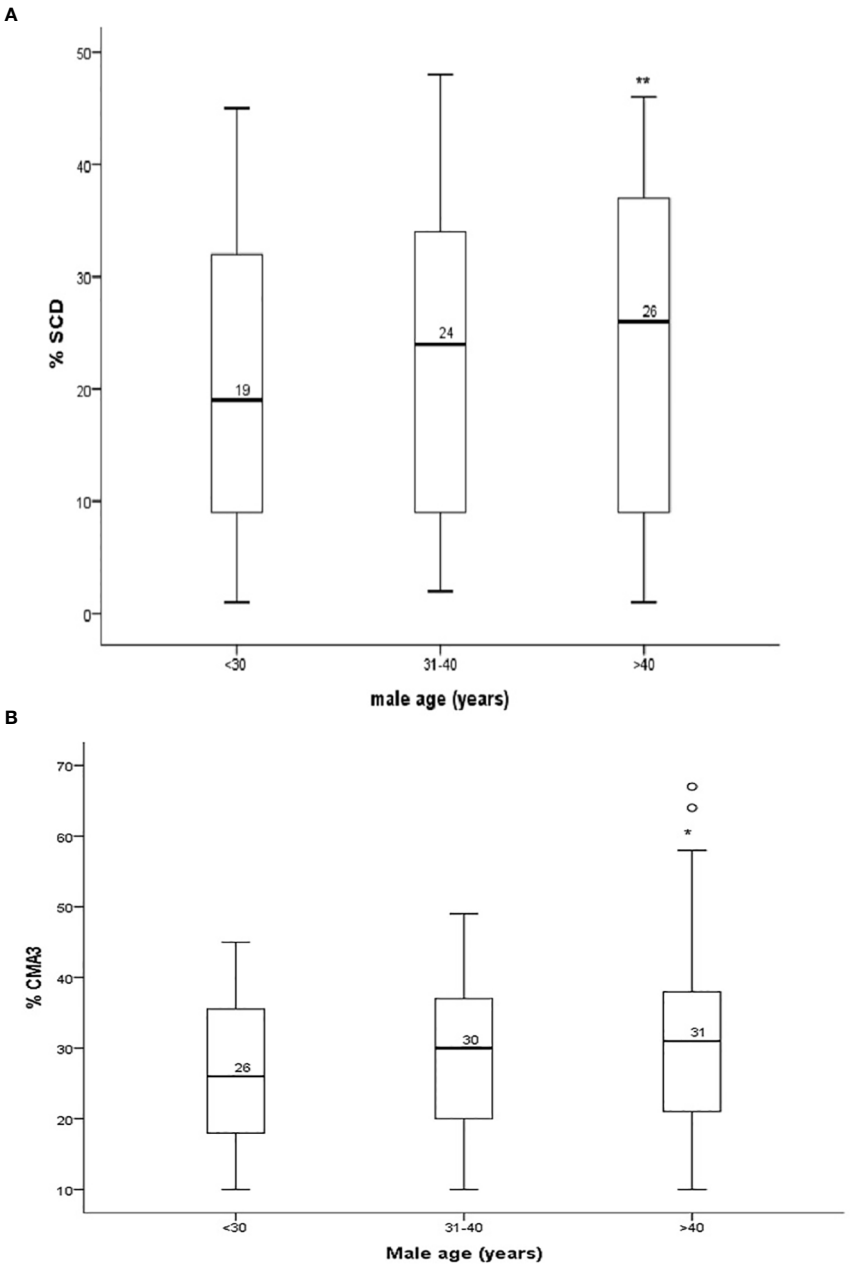

FIGURE 1
(A) Percentage of sperm chromatin dispersion (SCD) and **(B)** sperm protamine (CMA3+) content in sperm of males in different age groups. *P < 0.05, **P < 0.01.

DNA damage (SCD) (r = 0.124, p = 0.001) and percentage of immature spermatozoa with abnormal chromatin compaction (CMA3) (r = 0.1, p = 0.009) (Figures 2A, B). A significant positive linear association was found between male age and spermatozoa abnormal chromatin compaction (CMA3%) [β = 0.169, t = 2.63, 95% CI (0.042–0.295); p = 0.009] and spermatozoa with higher percentage of fragmented DNA (SCD %) [β = 0.195, t = 3.42, 95% CI (0.08–0.307); p = 0.001].

4 Discussion

The results of this study confirmed that advancing male age is associated with impaired sperm quality and sperm chromatin integrity. In the current investigation, we found an association between sperm DNA damage and rising male age. The alteration of sperm compactness (CMA3) in early stages of spermatogenesis leads to sperm DNA damage. Higher sperm DNA damage percentage was directly linked to increased male age. Male age harmed the integrity of sperm chromatin and its condensation, which represents a higher percentage of immature sperm with less compact chromatin (CMA3) (39, 40). It has been reported that, with age, ejaculated spermatozoa do exhibit changes, consistent with apoptosis in somatic cells, such as external of phosphatidylserine (PS), disrupted mitochondrial membrane potential, and/or DNA fragmentation (41). Recently, apoptosis has received much attention because of its vital role in reproduction, and early apoptosis indicated as the percentage of

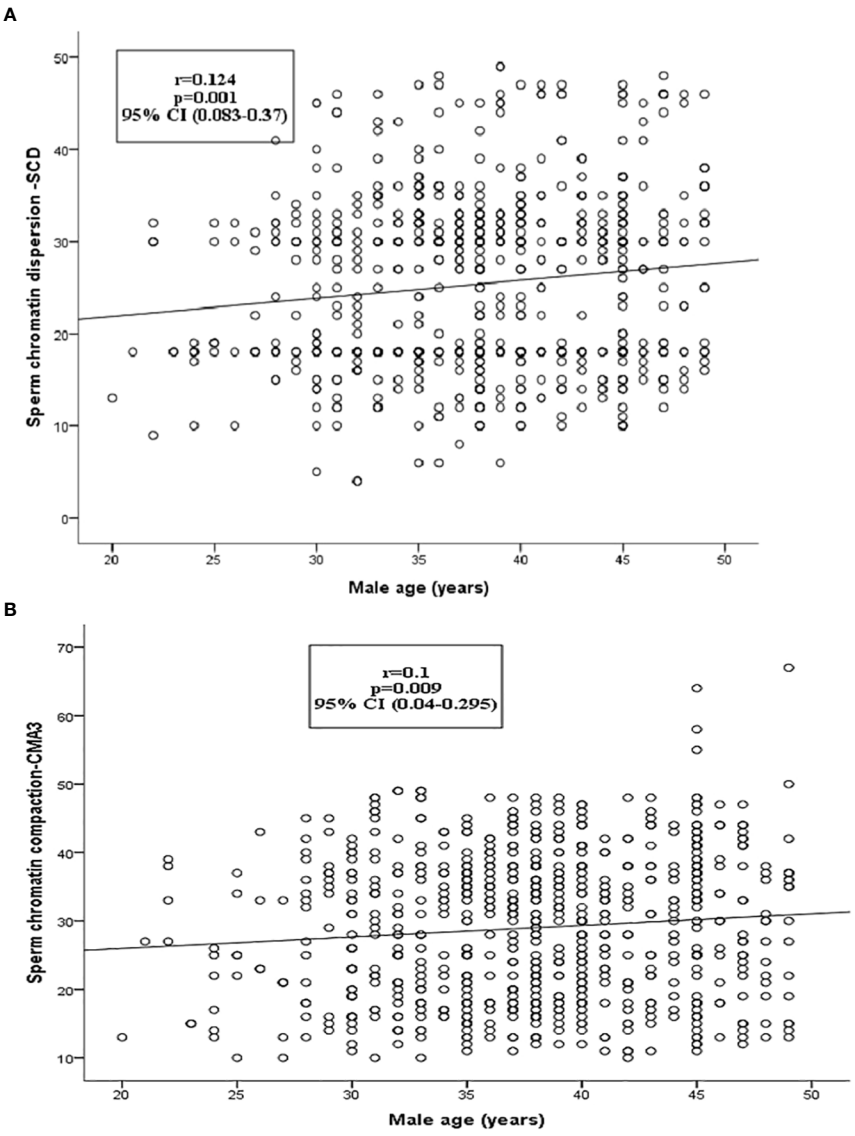

FIGURE 2
The relationship between sperm chromatin dispersion and sperm chromatin compaction (CMA3+) to male age. **(A)** The left charts show scatterplot correlation lines depicting the association between sperm chromatin dispersion (SCD) and male age. **(B)** Sperm chromatin compaction/protamine (CMA3+) and male age.

spermatozoa with PS, which is normally sequestered in the plasma membrane inner leaflet and appears in the outer leaflet, triggers non-inflammatory phagocytic reaction. Despite the effectiveness of DNA repair mechanisms, some DNA damage goes unrepaired, resulting in a gradual accumulation of DNA lesions in cells with mature age. As a result, the gradual but steady accumulation of damaged cells within tissues occurs with human aging (42). In the current investigation, we found an association between sperm DNA damage and rising male age (SCD); the present findings are consistent with other studies (43–45) in that higher sperm DNA damage percentage was directly associated with increased male age (46). A study showed a positive relationship between male age and sperm DNA damage in oligoasthenoteratozoospermia (OAT) but no difference in the control group (47), while some studies did not find any change in sperm DNA damage with an increase in male age (48–50). Male age harmed the integrity of sperm chromatin and its condensation, which represents a higher percentage of immature sperm CMA3.

In the current study, advanced men's age causes an increased risk of sperm chromatin de-condensation compared to younger men. The decrease in protamination, or possibly an issue with protamines caused by reduced thiol levels, would most likely explain the rise in CMA3+ staining. This would increase the histone-to-protamine ratio, which is what causes male subfertility (51). Alternate hypotheses for the etiology include immature spermatozoa shedding from the seminiferous tubes and abnormal protamine dephosphorylation (40, 52, 53). There was very limited literature on the influence of advanced male age on sperm chromatin packaging in humans and on data suggesting advanced human male age to be related to higher sperm chromatin damage (54).

Our analyses found no influence of male age on sperm morphology, motility, and concentration. Moreover, we looked into the relationship between male age and oxidative stress levels. A previous study showed a strong correlation between sperm DNA fragmentation and poor sperm quality, although no preferential effect on sperm concentration or morphology seemed to be present (55, 56). ROS production and levels of antioxidant enzyme imbalance result in impaired male fertility potential, and there are contradictory results on the relationship between levels of ROS production in semen with advanced male age (23, 49, 57–60). One study found a positive relationship (61), while another found no relationship between male age and ROS higher production. The present study found no link between male age with ROS production and no difference in ROS levels and antioxidative agents in all age groups (62). Given that the OS is a major factor affecting sperm function and that the balance between pro- and antioxidative agents is frequently shifted towards the pro-oxidizing condition in aging testis mitochondria, antioxidant interventions hold great promise as therapeutic strategies to lessen the negative effects of aging (and the resulting oxidative stress) on the male reproductive system (63, 64). The analysis of the present study revealed that there was no association between male age and reproductive hormone concentration. Androgen hormones are linked directly to sperm quality parameters and reproductive hormone imbalance leads to impaired spermatogenesis and poor male sexual health (65, 66). Male aging has previously been linked to a variety of factors, including decreased sperm quality, hormonal imbalances, and longer pregnancy times. Recent data, however, indicate that healthy aging does not impair spermatogenic output or hormone production from the testicles (1, 67).

As a result, we may also draw the additional conclusion that having older fathers has a deleterious effect on the molecular makeup of motile spermatozoa (23, 68). Given that it is established that sperm DNA is well protected because of chromatin condensation, which is essential at the time of sperm transit in the female reproductive system and additionally to manipulate epigenetic reprogramming at some point during the pre-implantation period, an increase in male age could result in impaired sperm chromatin integrity, making spermatozoa's genetic material vulnerable to the external environment insult (64, 69). Similar to this, poor fertility outcomes such as low fertilization rates, embryo morphokinetics, recurrent implantation failures, and miscarriages are associated with chromatin condensation and DNA integrity (67, 70–72). The process of sperm genome modification is believed to be due to highly hierarchical epigenetic changes occurring in the paternal genome after fertilization, including the dissolution of the sperm nuclear envelope, decondensation of the genetic material *via* the breakage of the disulfide bridges among protamines, substitution of maternal histones for male protamines, and genetic material rearrangement (64, 73). Understanding the body of available scientific evidence is the first step toward reducing or mitigating the negative effects of advanced male age. The present study sheds new light on the intricate associations between male age and concentrations of FSH and LH as well as DNA fragmentation and chromatin deficiency of spermatozoa among healthy men of reproductive age undergoing ICSI treatment. However, the results from this study should help provide critical information to assisted reproduction physicians and clinicians to understand the risks associated with male age and the resulting progenies after IVF/ICSI treatment. In the field of assisted reproduction, our study suggests that older men who are seeking fertility treatment may require more extensive testing and treatment than younger men. It also highlights the importance of seeking fertility treatment in younger age in male subjects; as the male age advances, fertility potential is reduced. Furthermore, the population in this study was homogeneous. Researchers, healthcare professionals, decision-makers, and patients, among others, should continue to discuss new data and their implications for individuals and society. Above all, it is critical that all parties work together to create a new agenda for reconsidering advanced male age management strategies in the context of protecting future parents' reproductive health.

The disadvantage of this study is that the sample size in Group 1 was smaller as compared to the rest, which is due to the recent social changes that enable men and women to choose to have a career first and delay childbearing and fatherhood to later age. The content and extent of fatherhood duties are filled in by traditional gender roles mainly set by society. The father provides protection and income for the mother and child. Financial and professional security and a greater motivation for parenthood usually characterized older couples. Moreover, the absence of an explicit condemnation of

the fatherhood age of men encourages a large number of men to delay fatherhood to advanced age. Secondly, it does not take into account other confounding factors, such as family histories and other diseases of old age. Subsequent cohort studies with older and younger men undergoing assisted reproductive treatment are recommended to investigate the effects of male advancing age on sperm chromatin packaging.

Male age identified by our investigation is an independent risk component for sperm DNA damage and chromatin condensation and influences reproductive health that could alter pre- and post-embryological developmental stages. This finding needs to be confirmed by future large prospective studies.

5 Conclusion

Old-aged men had a higher percentage of spermatozoa with sperm DNA damage (SCD %), significantly higher levels of immaturity (chromomycin staining, CMA3%), and a lower level of chromatin integrity. Male age is one of the factors contributing to the decline of male fertility. Therefore, younger age is advisable for patients who are undergoing assisted reproductive therapy.

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 ethical board of Quaid-i-Azam University and SKMC Islamabad Pakistan approved the experimental protocol # BEC-FBS-QAU2016-77 for the use of humans in this work. Participants were asked for their written consent. The patients/participants provided their written informed consent to participate in this study.

Author contributions

RB, SJ, SR, SQ, TA, AA, MQ, MH and HA significantly contributed to the design, experimental design, data collection, statistical evaluation, and article writing. RB, SJ, SQ, TA, SR, AA, MQ, MH and HA all contributed significantly to data interpretation and manuscript revision for intellectual content. 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.

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Traditional Chinese medicine Dingkun pill to increase fertility in women with a thin endometrium—a prospective randomized study

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Objective: The aim of this study is to optimize the treatment methods of infertility, which is suggested to be mainly caused by thin endometrium, using a special form of traditional Chinese medicine, the Dingkun pill (DKP), to increase the beneficial endometrial effect of conventional hormone/progestogen therapy.

Methods: A total of 307 patients visiting our specialized gynecological endocrinology department because of infertility, which we suggested to be caused by thin endometrium [endometrial thickness (EMT) < 7 mm], were randomly assigned to the experimental group and the control group. The experimental group was treated with estradiol + sequential dydrogesterone + DKP (every day); the control group received hormonal treatment without the Chinese medicine. All patients were monitored in terms of follicle diameter, EMT, and endometrial type every 2 days from the 8th to the 10th day of the menstrual cycle until ovulation day during three menstrual cycles. Serum progesterone levels on 7–8 days after ovulation were measured, and the cumulative pregnancy rate during three menstrual cycles between the two groups was compared.

Results: EMT on ovulation day in the experimental group was significantly higher than that in the control group (7.88 vs. 7.15 mm; $p < 0.001$). The proportion of type A and type B endometrium in total was significantly higher in the experimental group than that in the control group (83.2% vs. 77.7%; $p < 0.05$). Progesterone levels were significantly higher in the experimental group than those in the control group (10.874 vs. 10.074 ng/mL; $p < 0.001$). The cumulative pregnancy rate, the main outcome of the study, was significantly higher in the experimental group than that in the control group (29.2% vs. 15.7%; $p < 0.05$).

Conclusion: DKP added to conventional estrogen/progestogen therapy can significantly improve EMT and luteal function in patients attending due to infertility. Because this regimen increased the cumulative pregnancy rate in our study, we conclude that DKP can be used to increase the so-called "thin endometrium infertility".

KEYWORDS

infertility, thin endometrium, Dingkun pill, estrogen, traditional Chinese medicine

1 Introduction

In recent years, the incidence of female infertility has significantly increased due to various factors. It is reported that approximately 8%–12% of couples worldwide and 15% of couples in China are affected by infertility (1, 2). Effectively improving clinical pregnancy will benefit many patients, especially those who regard childbearing as a life mission.

The appropriate endometrium thickness (EMT) is an important single etiology of possible infertility (3). If the EMT is not high enough during ovulation, there is usually a poor reproductive outcome. Although there is no consensus regarding the cutoff value of EMT during ovulation, many studies have shown that the pregnancy rate will decrease significantly if the EMT measured by transvaginal ultrasound is less than 7 mm on the day of hCG or luteal support in assisted reproductive technology (ART) (4–6). Therefore, endometrium with an EMT of less than 7 mm during ovulation is considered thin, and the resulting infertility is regarded as "thin endometrium infertility".

Proliferation of the endometrium depends on the secretion of estrogen under normal physiological conditions. Hence, there is no doubt that estrogen supplementation is the first choice for thin endometrium infertility. In most cases, estrogen supplementation can actually promote endometrium proliferation, i.e., increase EMT and consequently improve the clinical pregnancy rate (7, 8). However, it is not effective for all patients, and some patients showed low response or no response to estrogen no matter whether it was used in the stimulating cycle, natural cycle, or high-dose hormone supplement cycle (9). The use of high-dose estrogen is limited due to the risk of hyperplasia, which could lead to endometrial cancer. Monitoring by assessing estradiol (E2) levels is difficult, because it has been shown that E2 levels were only correlated with endometrium thickness in patients with relatively low estrogen levels. Once E2 levels are >1,000 ng/mL, it has no further significant impact on endometrium thickness (3, 10). Therefore, it is necessary to explore another effective alternative method to improve EMT and pregnancy rates for those patients with less or even no response to estrogen supplement.

As the exclusive tribute medicine for the imperial palace of the Qing Dynasty, the effectiveness of Dingkun pill (DKP) on hypomenorrhea and female infertility has been repeatedly verified in China (11, 12). In this study, the main endpoint was EMT. The type of endometrium, progesterone (P), and cumulative pregnancy rate were assessed as secondary endpoints to verify the cooperation effect of DPK combined with estrogen in treating thin endometrium infertility.

2 Materials and methods

2.1 Ethical approval

This study was approved by the ethics committee of Beijing Obstetrics and Gynecology Hospital, Capital Medical University, China (Protocol number: 2018-KY-058-04). All patients provided their informed, signed consent.

2.2 Design and participants

This study was a prospective, randomized controlled study and the flowchart is shown in Figure 1. Patients attending the Department of Gynecological Endocrinology, Beijing Obstetrics and Gynecology Hospital, Capital Medical University between November 2018 and October 2022 were recruited. All patients who met inclusion criteria and exclusion criteria were randomly assigned to the control group or the experimental group in a 1:1 ratio according to the random numbers generated by the computer software and received different treatment schemes. The whole intervention process lasted three menstrual cycles. All patients were natural cycles and received treatments in the outpatient department. Since our research was not focused on evaluating the endometrial receptivity, we do not have sufficient evidence to consider if endometrial biopsies are essential or not. Thus, endometrial biopsy and/or hysteroscopy were only performed on patients who need further diagnostic procedures such as excluding polyps as reasons for bleeding problems.Inclusion criteria were as follows: (I) female infertility, (II) women aged between 20 and 40 years, and (III) EMT less than 7 mm on ovulation day in at least two natural menstrual cycles. Infertility was defined by failure to achieve a successful pregnancy after 12 months or more of appropriate, timed unprotected intercourse or therapeutic donor insemination (13).

Exclusion criteria were as follows: (I) no desire for pregnancy; (II) ovulation disorder, premature ovarian failure, hyperprolactinemia,

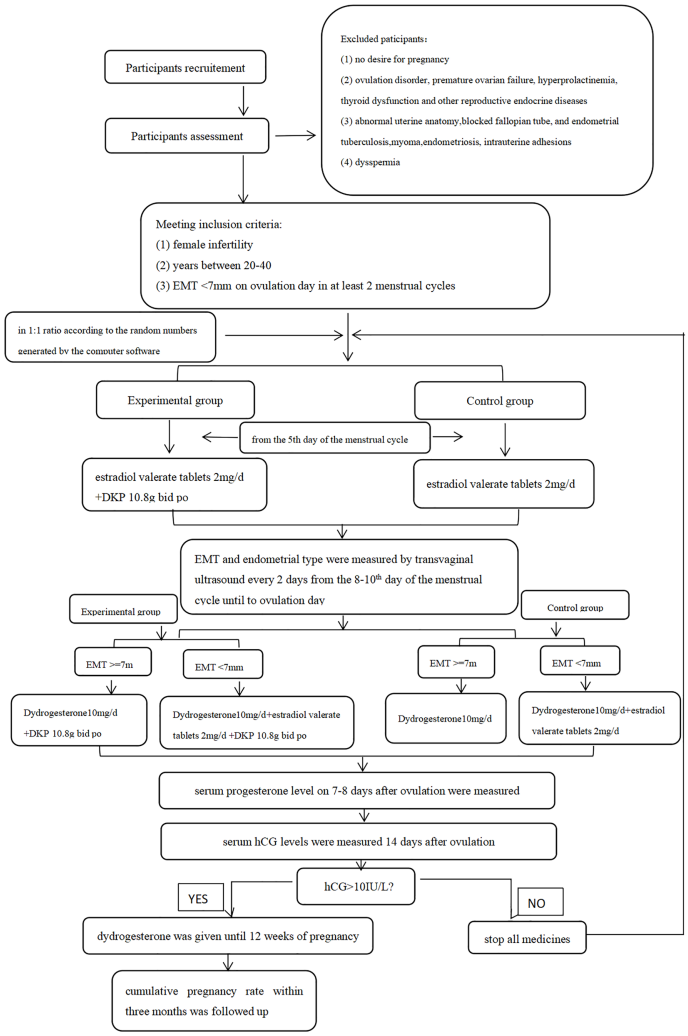

FIGURE 1
The flawchart.

thyroid dysfunction, and other reproductive endocrine diseases; (III) abnormal uterine anatomy, blocked fallopian tube, endometrial tuberculosis, myoma, endometriosis, and intrauterine adhesions; and (IV) dyspermia.

2.3 Measure parameters

2.3.1 Recruitment for the study

Serum endocrine hormones including follicle-stimulating hormone (FSH), luteinizing hormone (LH), and estradiol (E2) were measured on days 2–4 of the menstrual cycle. EMT and endometrium type on ovulation day were also measured by transvaginal ultrasound. The day when the dominant follicle (≥18 mm) disappeared was defined as ovulation day. Endometrium with an EMT of less than 7 mm on ovulation day was considered thin. Only these patients were recruited for the study, i.e., randomly selected for the experimental group or for the control group.

2.3.2 After randomized allocation to the two groups

All patients were monitored in terms of follicle diameters, EMT, and endometrial type every 2 days from the 8th day to the 10th day of the menstrual cycle until ovulation day during three menstrual cycles. Serum progesterone level on 7–8 days after ovulation were also measured. Cumulative pregnancy rate within 3 months was followed up.

2.3.3 EMT and type assay

After the uterus was scanned by the longitudinal section and finding out that the endometrial image was the clearest, the endometrium thickness was determined by measuring the maximum anteroposterior distance between the myometrial and the endometrium interface. The endometrial pattern was assessed according to the classification proposed by Gonen (14)as follows: Type A is a multilayered endometrium consisting of prominent outer and midline hyperechogenic lines and inner hypoechogenic regions. Type B is characterized by the same reflectivity of ultrasound as the

myometrium, with a non-prominent or absent central echogenic line. Type C is an entirely homogenous, hyperechogenic endometrium.

2.3.4 Cumulative pregnancy rate

We considered patients pregnant if serum hCG levels were more than 10 IU/L. Cumulative pregnancy rate: total number of pregnancies/total number of patients×100%.

2.4 Treatment in the two groups

2.4.1 Control group

The women were treated with estradiol valerate tablets (Bayer, Guangzhou, China) 2 mg/day orally starting on the fifth day of the menstrual cycle. If the EMT was measured by transvaginal ultrasound on ovulation day > 7 mm, the intake of estradiol valerate tablets was stopped, and only dydrogesterone 10 mg/day orally (Abbott Biologicals B.V., Netherlands) was used. If EMT on ovulation day remained< 7 mm, the treatment with estradiol valerate tablets was continued and, at the same time, dydrogesterone 10 mg/day orally was added (so-called "sequential hormone replacement therapy").

2.4.2 Experimental group

These women received DKP (Luwang, Jilin, China) 10.8 g twice a day orally from the fifth day of the menstrual cycle until 14 days after ovulation. The other drug regimen (hormonal treatment) was the same as that in the control group.

DKP is mainly composed of the following 30 medicinal herbs: Radix Ginseng, Cornu Cervi Pantotrichum, Radix Angelicae sinensis, Radix Rehmanniae Preparata, Stigma Croci, Caulis Spatholobi, Radix Notoginseng, Radix Paeoniae Alba, Rhizoma Atractylodis Macrocephalae, Fructus Lycii, Radix Scutellariae, Rhizoma Cyperi, Fructus Leonuri, Rhizoma Ligustici Chuanxiong, Cornu Cervi Degelatinatum, Colla Corii Asini, Rhizoma Corydalis, Flos Carthami, Herba Leonuri, Faeces Togopteri, Poria, Radix Bupleuri, Radix Linderae, Fructus Amomi Villosi, Cortex Eucommiae, Rhizoma Zingiberis, Herba Asari, Radix Cyathulae, Cortex Cinnamomi, and Radix Glycyrrhizae. The "Dingkun pill" standard is approved by the China Food and Drug Administration (CFDA).

Serum hCG levels were measured in both groups 14 days after ovulation. If hCG >10 IU/L, estradiol valerate tablets were stopped and only dydrogesterone was given until 12 weeks of pregnancy. If hCG<10 IU/L (i.e., no pregnancy), the study was continued accordingly; i.e., treatments were continued on the 5th day of the next menstrual cycle until patients became pregnant or until the end of the third cycle at the end of the study.

25 Statistical analysis

2.5.1 Sample size calculation and statistical analysis

We used the following formula for calculating the minimal sample size for this type of study:

$$N = \left[\frac{Z\alpha\sqrt{\pi_c(1-\pi_c)(1/Q_1+1/Q_2)} + Z_\beta\sqrt{\pi_1(1-\pi_1)/Q_1+\pi_2(1-\pi_2)/Q_2}}{\delta}\right]^2$$

Previous research estimated that the clinical pregnancy rate of infertile women with thin endometrium after estrogen treatment was approximately 10%, and the clinical pregnancy rate of other similar TCM was approximately 20% (15). The statistical significance level was set at 5% ($\alpha = 0.05$) using a one-sided test and 80% power ($1 - \beta$), Q1 = Q2 = 0.5, $\pi1 = 20\%$, $\pi2 = 10\%$, $\pi c = Q1\pi1 + Q2\pi2 = 0.5 * 0.20 + 0.5 * 0.1 = 0.15$, $\delta = \pi1 - \pi2 = 10\%$. We calculated that the minimum sample size should be about $n = 300$.

SPSS software 16.0 (SPSS Inc., Chicago, IL) was used to statistically describe and analyze the research results. The quantitative variables were described by mean ± standard deviation (SD) or median ± interquartile range (IQR). The qualitative variables were described by frequency and percentage. Two-sample *t*-tests were used to compare means. χ^2 tests were used to compare the frequency. A two-sided *p*-value of<0.05 was considered to indicate statistical significance.

3 Results

3.1 Clinical characteristics of the study population

We recruited 340 patients in total and 307 patients finished this study, including 154 patients in the experimental group and 153 patients in the control group. A total of 33 patients were lost to follow-up, namely, 17 patients in the experiment group and 16 patients in the control group. The baseline characteristics of all patients are shown in Table 1. There was no significant difference between the two groups regarding the baseline parameters including age, BMI, EMT/type on ovulation day, and the serum endocrine hormone levels measured on the 2nd to 4th day of menstruation.

3.2 Comparison of endometrium thickness/type and P levels between two groups after treatment

As shown in Table 2 and Figure 2, after three menstrual treatments, the average EMT of patients on the ovulation day in the control group and experimental group increased significantly, reaching 7.15 ± 1.40 mm and 7.88 ± 1.37 mm, respectively. The average EMT of the experimental group was significantly higher than that of the control group, with a statistically significant difference ($p< 0.001$). The proportion of type A and type B endometrium in total in the experimental group was significantly higher than that in the control group (83.2% vs. 77.7%; $p< 0.05$). In addition, the serum P level in the experimental group and control group was 10.874 ± 1.723 ng/mL and 10.074 ± 2.130 ng/mL, respectively. The serum P level in the experimental group was significantly higher than that in the control group, ($p< 0.001$).

TABLE 1 Basic statistical characteristics of the two groups of patients.

Variables	Experimental group (n = 154)	Control group (n = 153)	p-values
Age (years, $\bar{\chi}$± SD)	30.7 ± 4.09	30.9 ± 3.99	0.623
BMI	22.72 ± 2.482	22.45 ± 2.550	0.362
EMT (mm)	5.67 ± 0.83	5.71 ± 0.77	0.717
Count of type A and B Endometrium, n (%)	115 (74.7%)	109 (71.2%)	0.532
FSH (mIU/mL)	5.94 ± 1.41	5.80 ± 1.32	0.376
LH (mIU/mL)	6.29 ± 1.84	6.20 ± 1.73	0.665
E2 (pg/mL)	39.056 ± 11.016	38.167 ± 11.515	0.490

BMI, body mass index; EMT, endometrial thickness; FSH, follicle-stimulating hormone; LH, luteinizing hormone; E2, estradiol.

TABLE 2 Endometrium thickness/type and p level on ovulation for 7–8 days of the two groups.

Variable	Group		p-values
	Experimental	Control	
EMT (mm)	7.88 ± 1.37	7.15 ± 1.40	0.00***
P (ng/mL)	10.874 ± 1.723	10.074 ± 2.130	0.00***
Count of type A and B Endometrium, n (%)	347 (83.2%)	338 (77.7%)	0.047*

***p< 0.001, *p< 0.05; P, progesterone.

3.3 Cumulative pregnancy rate

As shown in Table 3, the cumulative pregnancy rate was 29.2% (45 of 154 patients) in the experimental group vs 15.7% (24 of 153 patients) in the control group. The cumulative pregnancy rate in the experimental group was significantly higher than that in the control group, with statistical significance.

3.4 Safety and tolerability

All participants rated the tolerability as good. No adverse events were associated with using DKP.

4 Discussion

In our study, we found that the average EMT of patients in the experimental group was significantly higher than that in the control group, exceeding the minimum threshold for pregnancy when the same dose of estrogen was supplemented. In contrast, the average EMT of patients in the control group on average just reached the pregnancy threshold, which means that, for some patients, the endometrium proliferating effect was perhaps not sufficient enough for an implantation during a possible pregnancy. Undoubtedly, proper EMT is crucial for embryo implantation. Once the EMT increased in those patients with thin endometrium infertility, the clinical pregnancy rate also increased significantly (16–18). The use of extended estrogen supplementation has solved the problem in some patients, but there were also risks that cannot be ignored. For example, the excessive use of estrogen during the early follicular phase will inhibit the production of follicle-stimulating hormone, affect the quality of oocytes, and increase the risk of endometrial hyperplasia and endometrial cancer. Short-term treatment can also increase the risk of venous thromboembolism. If the EMT can be effectively increased by using other treatment concepts without increasing the dose of estrogen, this will benefit many patients. In fact, DKP showed promising possibilities based on our research.

It was suggested that the mechanisms of DKP in treating infertility may be not only to increase EMT, but also to promote some changes that are beneficial for endometrial receptivity. Impaired endometrial receptivity may account for up to two-thirds of implantation failures in younger women (19). Our results also showed that the level of progesterone and the proportion of type A and B endometrium in the experimental group were significantly higher than those in the control group. These all can have a very favorable impact on endometrial receptivity and embryo implantation, which could be the reason that we achieved a significant increase in pregnancy rate in the experimental group in our study. Because our research was not focused on evaluating endometrial receptivity, we only performed endometrial biopsies for further diagnostics, for example, of unclear bleedings, not to assess endometrial receptivity. However, the improvement of EMT, corpus luteum function, and endometrial blood flow implies an increase in endometrial receptivity in many studies (19, 20). Furthermore, in relevant studies, DKP showed more complex potential mechanisms. DKP not only could increase E2 levels on the day of frozen–thawed embryo transfer and the

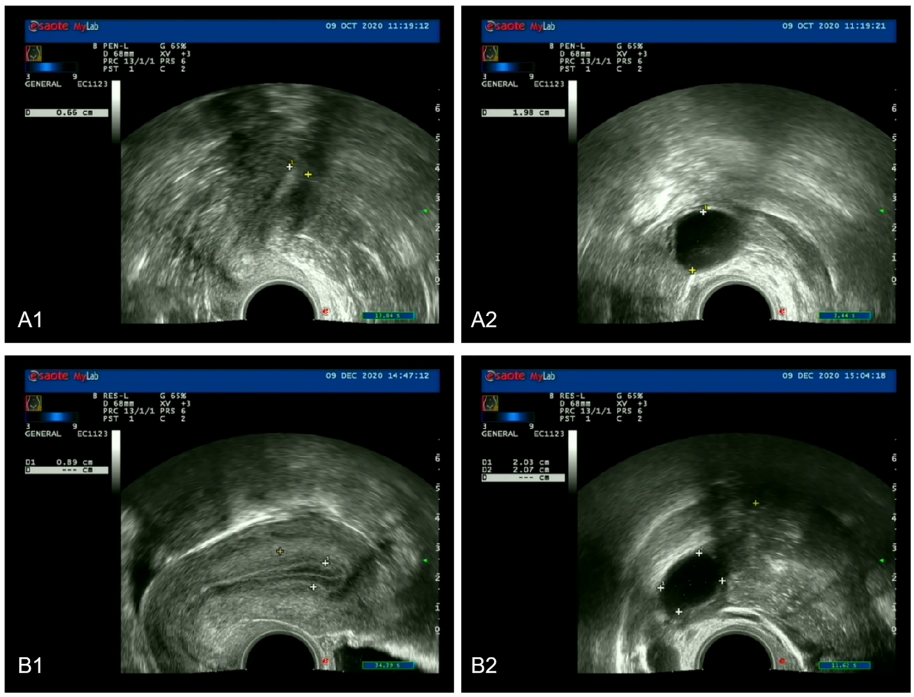

FIGURE 2
(A1, A2) Endometrium and follicle diameter during ovulation before treatments in experimental group. **(B1, B2)** Endometrium and follicle diameter during ovulation after treatments in the experimental group.

sensitivity to estrogen during the peri-implantation stage (21, 22), but also could increase numbers of oocytes retrieved, high-quality embryos, embryo implantation rate, and clinical pregnancy rate (23). Although the detailed molecular mechanisms of the effect of DKP on infertility are still unclear, traditional Chinese medicine theory provides a certain explanation. In traditional Chinese medicine theory, kidney function contributes to reproduction mechanisms, and infertility can be caused by kidney deficiency, liver depression, and blood stasis. In fact, modern pharmacology and clinical studies have confirmed that Chinese medicine with the effect of recuperating the kidney function and promoting blood circulation can indeed promote the proliferation of uterine glands and blood vessels in experimental animals, improve uterine artery perfusion, improve pelvic microcirculation, improve endometrial receptivity, and increase the clinical pregnancy rate in infertile patients (24–26). DKP is one of the most representative medicines; it contains Atractylodis Macrocephalae Rhizoma, Poria, Chuanxiong Rhizome, and Angelicae Sinensis Radix, which can recuperate the kidney function, and Leonuri Herba, Corydalis Rhizoma, and Angelicae Sinensis Radix, which can nourish blood and regulate menstruation. DKP alone has proved its effectiveness in treating hypomenorrhea and infertility in many traditional Chinese medicine studies (27). However, until now, there is a lack of studies that investigate the use of DKP combined with estrogen for “thin endometrium infertility”.

There are many factors that can cause infertility, and some are not very clear even. We focused on endometrial factors, but within our diagnostic and clinical procedures, we tried to exclude most of the other common potential causes, including ovulation disorder, premature ovarian failure, hyperprolactinemia, thyroid dysfunction and other reproductive endocrine diseases, abnormal uterine anatomy, blocked fallopian tube, endometrial tuberculosis, myoma, endometriosis, intrauterine adhesions, and dyspermia.

It can be stated that a strength of this study is that all EMT measurements, the main endpoint of this study, were performed by one experienced doctor. The sample size corresponds to the statistical calculation. The study design is prospective and clearly suitable to test the efficacy of DKP under routinely clinical

TABLE 3 Cumulative pregnancy rate between the two groups after 3 months.

Pregnancy	Group		*p*-values
	Experimental	Control	
Yes	45	24	
No	109	129	
Total	154	153	0.005**

**p< 0.01.

conditions. Only patients with thin endometrium have been included, as a precondition for randomization to the two groups.

The weakness of this trial is that there was no placebo–control due to the limited clinical conditions. Only one dose of estrogen supplement (2 mg per day) was studied. It remains unclear if the use of Dingkun alone, without addition of hormone therapy, would have similar beneficial results. Despite this question being of interest (to reduce hormone-dependent risks such as venous thrombosis), hormonal treatment is the "gold standard" for increasing endometrial thickness in women who wish to conceive. Therefore, our aim was to investigate whether the endometrium proliferating effect of hormones could be further increased by adding this form of traditional Chinese medicine, but without increasing the dosage of the estrogen. Further studies should investigate if the dose of hormones could be decreased, or if hormones could even be completely omitted.

5 Conclusion

DKP added to conventional estrogen/progestogen therapy can significantly improve EMT and luteal function in patients attending due to infertility with a thin endometrium. Because this regimen did increase the cumulative pregnancy rate in our study, we conclude that DKP can be used to increase the so-called "thin endometrium infertility".

Data availability statement

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

Ethics statement

The studies involving humans were approved by the ethics committee of Beijing Obstetrics and Gynecology Hospital, Capital Medical University, China (Protocol number: 2018-KY-058-04). 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

All authors qualify for authorship by contributing substantially to this article. FJ: article preparation and study implementation. XR: interpreted the results, provided critical comments, and revised the first draft. SQ, XX, YY, MG, YL, JC, JD, and XY: follow-up. AM: experimental supervision, interpretation of results, and article revision. 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.

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Comparison between the modified long gonadotropin-releasing hormone agonist protocol and the non-downregulation protocol in POSEIDON groups: a propensity score matching retrospective cohort study

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Background: *In vitro* fertilization (IVF) is the main technique to address the infertility issue in the patient-oriented strategy encompassing individualized oocyte number (POSEIDON) population. Adopting appropriate protocols for assisted reproduction technologies (ART) cycles in the POSEIDON group may attain more favorable pregnancy outcomes.

Objectives: This study aimed to compare the effectiveness of modified long gonadotropin-releasing hormone agonist protocol and non-downregulation protocol in POSEIDON patients undergoing ART, and to identify the factors affecting the pregnancy outcomes in this group.

Design: This study was designed as a propensity score-matched (PSM) retrospective analysis.

Participants: The study cohort consisted of 910 patients diagnosed with ovarian hyporesponsiveness and treated by IVF from January 2020 to June 2022. They were followed up until the transfer of the last embryo of the IVF cycle and/or pregnancy at 12 weeks. The study was conducted at the Center of Reproductive Medicine, Tongji Medical College, Wuhan Union Hospital, Huazhong University of Science and Technology.

Methods: The patients were divided into Group I and Group II. Group I was treated with modified long gonadotropin-releasing hormone agonist protocol while Group II was put on a non-downregulation protocol. Propensity score matching (PSM) was used to select patients for each group. The subjects were compared in terms of the baseline level, process of controlled ovarian hyperstimulation, and pregnancy outcomes. Binary logistic regression analysis

was performed to assess the difference in the cumulative pregnancy rate between the two groups.

Results: Of the 910 POSEIDON patients who underwent IVF, 213 received the modified long gonadotropin-releasing hormone agonist protocol and 697 were subjected to the non-downregulation protocol. From the original cohort, PSM matched 174 pairs of patients. No statistically significant difference was found in total gonadotropin (Gn) dose between the two PSM groups, but the average daily Gn dose was lower in Group I and the duration of Gn lasted longer. The number of retrieved oocytes, the number of metaphase II (MII) ooctyes retrieved, normal fertilization, and normal cleavage embryos was significantly higher in Group I than in Group II, but there existed no significant difference in the number of high-quality embryos between the two groups. The single-cycle CPR (cumulative pregnancy rate) was higher in Group I than in Group II (for Group I: before PSM, CPR = 52.6%; after PSM, CPR = 51.7%; for Group II: before PSM, CPR = 34.0%; after PSM, CPR = 34.5%), and the difference was statistically significant. A binary logistic regression analysis in the unmatched patients showed that the CPR of Group II was 0.486 times that of Group I (95% CI: 0.303 to 0.779).

Conclusions: The modified long gonadotropin-releasing hormone agonist protocol can be used as an optimal protocol for IVF or ICSI (Intracytoplasmic sperm injection) in POSEIDON patients.

Level of evidence: Level III

KEYWORDS

modified long gonadotropin-releasing hormone agonist protocol, nondown-regulation protocol, propensity score matching analysis, POSEIDON patients, ovarian hyporesponsiveness

1 Introduction

Controlled ovarian hyperstimulation represents a very important part of assisted reproduction technologies (ART). Through controlled ovulation stimulation, infertile patients can generate sufficient high-quality oocytes, which are the premise of subsequent embryo culture and clinical pregnancy. Patients with ovarian hyporesponsiveness respond poorly to gonadotropin (Gn) during ovulation hyperstimulation, resulting in low oocytes retrieval and high cycle cancellation rate. Due to policy and social factors, many women experience a poor ovarian response(POR) when they want to have babies. The proportion of this population in assistant reproductive technology is increasing, which poses great challenges for clinicians. How to provide assisted pregnancy counseling to patients with low ovarian response and develop individualized assisted pregnancy strategies have become issues that have to be addressed urgently.

To better stratify the low ovarian response population and facilitate assisted pregnancy counseling, researchers proposed the low-response patient-oriented strategy encompassing individualized oocyte number (POSEIDON) criteria in 2016 (1). The POSEIDON criteria categorizes patients into four groups in terms of age, anti-Müllerian hormone (AMH), antral follicle count (AFC), and other indicators. The assisted pregnancy strategies and clinical outcomes vary with different groups of POSEIDON patients. However, no definitive consensus has been reached regarding how to formulate individualized ovarian hyperstimulation protocols for each POSEIDON group to attain maximal benefit (2–4).

Some protocols do not require pituitary downregulation and these include the antagonist protocol, the non-downregulation protocol, and the progestin-primed ovarian stimulation protocol. The advantages of these protocols lie in that they do not require pre-treatment, have a short cycle preparation time, and are economical and convenient. They are the most popular ovulation stimulation protocols with low-response patients. However, some therapeutic drugs involved in these protocols may disturb the endometrial receptivity (5–7). Most patients require whole embryo freezing and frozen-thawed embryo transplantation, but the low response patients tend to have fewer oocytes, a high risk of blastocyst culture, and a high cancellation rate of the transfer cycle. On the other hand, the mid-luteal-phase short-acting long gonadotropin-releasing hormone agonist (GnRHa) long protocol and the modified long gonadotropin-releasing hormone agonist protocol entail the use of GnRHa (gonadotropin-releasing hormone agonist) for pituitary downregulation. The treatment cycle lasts longer, the Gn dosage is high, and the number of oocytes retrieved is low.

However, GnRHa can help improve endometrial receptivity (8, 9), and more patients can receive fresh embryo transfers. Until now, researchers have failed to agree on how to evaluate the efficacy of these two types of protocols. This study reviewed the *in vitro* fertilization cycles of patients with low ovarian response from January 2021 to June 2022 in our Center for Reproductive Medicine and comparatively examined the modified long gonadotropin-releasing hormone agonist protocol and the non-downregulation protocol with regards to their fertility-enhancing effect on the pregnancy result.

2 Materials and methods

2.1 Clinical data

Retrospective statistical analysis was performed on the data of patients who received IVF/ICSI (intracytoplasmic sperm injection) from January 2020 to June 2022 at the Center for Reproductive Medicine, Department of Obstetrics and Gynecology, Wuhan Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. The eligible subjects for inclusion in this study were those who (1) met the relevant criteria of POSEIDON groups I–IV and (2) had received one of the two clinical protocols (i.e., modified long gonadotropin-releasing hormone agonist protocol and non-downregulation protocol) in our center, and had been followed up until the last embryo of the oocyte retrieval cycle was transferred or the pregnancy was over 12 weeks. Exclusion criteria for this study included (1): patients who had concomitant uterine malformation, refractory intrauterine effusion, or intrauterine adhesion (2); patients who were undergoing preimplantation genetic testing (PGT); and (3) those who had other medical and surgical conditions, such as hypertension, diabetes, and tumors. In this study, 910 patients who satisfied the aforementioned inclusion criteria included 213 patients on the modified long gonadotropin-releasing hormone agonist protocol (Group I) and 697 patients receiving the non-downregulation protocol (Group II).

2.1.1 POSEIDON criteria

The POSEIDON criteria categorizes people with poor ovarian response into four groups.

POSEIDON group 1: Patients < 35 years with normal ovarian reserve parameters (AFC > 5, AMH > 1.2 ng/mL); however, they have an unexpected poor ovarian response.

POSEIDON group 2: Patients > 35 years with normal ovarian reserve parameters (AFC > 5, AMH > 1.2 ng/mL); they have an unexpected poor ovarian response.

POSEIDON group 3: Patients < 35 years with poor ovarian reserve parameters (AFC < 5, AMH < 1.2 ng/ml).

POSEIDON group 4: Patients > 35 years with poor ovarian reserve parameters (AFC < 5, AMH < 1.2 ng/mL).

2.2 Ovarian hyperstimulation protocol

This study was designed to compare the efficacy of two protocols, the modified long gonadotropin-releasing hormone agonist protocol and the non-downregulation protocol, in patients who met the POSEIDON criteria. Therefore, the ovarian hyperstimulation regimens in this study included the two above-mentioned protocols. The ovulation protocol employed the standard operating procedure (SOP) of the ovulation induction protocol of our center, and the procedure, detailed as follows:

2.2.1 Modified long gonadotropin-releasing hormone agonist protocol

Patients took 3.75 mg of gonadotropin-releasing hormone agonist (GnRHa) during day 2 to day 5 of the menstrual period and returned to the clinic for further consultation on day 28 after the downregulation. The starting time was determined according to the follicle size and the blood hormone levels. When the follicles reached the right size for ovulation, the ovulation was triggered with recombinant human chorionic gonadotropin/human chorionic gonadotropin (rHCG/HCG) injection and the oocytes were retrieved after 36-40 hours.

2.2.2 Non-downregulation protocol

The patients came to the hospital for ultrasound monitoring and hormone testing on day 2/day 3 of menstruation and the initiating dose of Gn was determined by the patient's age, body weight, AMH, and other factors. At the same time, the patient took oral clomiphene citrate at 100 mg per day. When the follicles reached the optimal size, the patient was administered GnRHa 0.2 mg/rHCG 250-500 μg/HCG 4,000-10,000 IU, alone or in combination with the trigger injection, and the oocytes were retrieved within 36-40 hours. Fresh embryo transfer was not conducted with this protocol, and all embryos were cultured into blastocysts and then frozen.

2.3 Transfer strategy

2.3.1 Fresh embryo transfer

Patients on a modified long gonadotropin-releasing hormone agonist protocol received luteal phase support (dydrogesterone tablets at 10 mg *po bid* and progesterone at 60 mg *im qd*) after oocyte retrieval. On the second day after egg retrieval, patients received type B ultrasound to measure endometrium thickness. If the endometrium was ≥ 7 mm, no uterine cavity effusion was found, and the diameter of both ovaries measured less than 70 mm, embryo transfer was performed on the third day post-oocyte retrieval. One or two embryos were transferred, depending on the embryo grade, and the remaining embryos were cultured to blastocysts and then cryopreserved.

2.3.2 Frozen embryo transfer

The patients who did not become pregnant after the whole cycle of embryo freezing or fresh embryo transfer were subjected to the hormone replacement therapy (HRT) protocol and underwent endometrial preparation on day 2 of menstruation. For HRT protocol, oral estradiol valerate tablets were given at 4-6 mg/d, starting on day 2 of menstruation. After 10 to 15 days, when the

endometrial thickness was more than 7 mm, blood samples were taken for estradiol and progesterone determination. When serum estradiol (E2) ≥ 200 pg/ml and serum progesterone (P) <1.5 ng/ml, progesterone was given to transform the endometrium. On the sixth day of endometrial transformation, the uterus was rechecked sonographically. When the endometrium reached a thickness greater than 7 mm, the embryos were thawed and transferred.

2.4 Outcome measures

This study compared the outcomes of the modified long gonadotropin-releasing hormone agonist protocol and the non-downregulation protocol in POSEIDON group patients by utilizing retrospective analysis plus propensity score matching (PSM). We matched the baseline conditions of the two groups of patients using PSM and then compared the ovulation and pregnancy outcomes of the two groups. The main outcome indicators covered by this study included:

2.4.1 Major outcome measures

The major outcome was the cumulative pregnancy rate of a single cycle upon use of the two different ovarian hyperstimulation protocols in the POSEIDON group patients. Single-cycle cumulative pregnancy rate = the number of pregnancy cycles after fresh or thawed transplantation of the same ovulation cycle/ total number of ovarian hyperstimulation cycles × 100%.

2.4.2 Secondary outcome measures

The secondary measures included total amount of Gn, Gn days, the number of oocytes retrieved, the number of MII ooctyes retrieved, the number of high-quality embryos, and the number of frozen embryos.

2.5 Statistical analysis

The SPSS 25.0 software package was employed for statistical analysis. The basic indicators, such as the age of the male and female patients, number of stimulation cycles, fertilization methods, infertility factors, years of infertility, body mass index (BMI), AFC, and AMH, were used as covariates for matching. The tolerance was set at 0.05. A new dataset was created for the matched cases. To understand whether there existed statistically significant differences among the above basic indicators, a multifactorial binary logistic regression was performed on the indicators with significant differences, to understand whether the ovulation stimulation protocol impacted the pregnancy outcome in patients with ovarian hyporesponsiveness.

The normality test was conducted on the measurement data, and the data that conformed to the normal distribution were expressed as mean and standard deviation (SD), and the independent sample t-test was used for statistical analysis. The data not following the normal distribution pattern were presented as median (the 25th percentile/the 75th percentile), and the comparison between groups was made using the Mann–Whitney U test. The enumeration data were reported as percentages (%), and the ratios of constituents between groups were comparatively analyzed by utilizing the Chi-square test. A P less than 0.05 indicated that the difference was statistically significant.

3 Results

3.1 Baseline data before and after matching in the two groups

The main objective of this study was to compare the pregnancy outcomes of two different ovulation stimulation protocols, i.e., the modified long gonadotropin-releasing hormone agonist protocol and the non-downregulation protocol in patients with low ovarian response against the POSEIDON criteria. The two groups were compared in terms of basic indicators including: the age of both the male and female patients, ovulation cycle(s), infertility years, BMI, AMH, AFC, POSEIDON grouping, infertility type, infertility causes, and fertilization technique used. The results are shown in Table 1. There were statistical differences in multiple baseline features between the two groups, suggesting that there existed a selection bias when clinicians chose protocols for patients with low prognosis.

To compare the effect of two different ovulation stimulation protocols on the clinical outcomes, we used the propensity scoring to screen the data of two groups. With the aforementioned basic indicators as covariates and the tolerance set at 0.05, the subjects were matched at 1:1 and the unmatched cases were excluded. As a result, a total of 174 patients remained in each group. Post-matching comparison of the basic data between the two matched groups revealed that there were no statistically significant differences between the two groups in age, number of stimulation cycles, POSEIDON group, type of infertility, and the causes of infertility. Nonetheless, upon propensity score matching, there still existed a statistically significant differences between the two groups in terms of AMH, AFC, and fertilization techniques.

3.2 Comparison of ovulation stimulation protocols and clinical outcomes between the two groups before and after matching

The two ovulation stimulation protocols and their pregnancy outcomes before and after matching were comparatively analyzed, and the results are given in Table 2. The post-matching data showed that the total amount of Gn used by patients on the non-downregulation protocol was higher, the number of Gn days was lower, and the average daily dosage of Gn and Gn initiation doses were significantly increased. Compared with the modified long gonadotropin-releasing hormone agonist protocol, the number of oocytes obtained, the number of mature oocytes, normal fertilization, and normal cleavage were significantly lower in the non-downregulation protocol. However, the high-quality embryo rate of the non-downregulation protocol was comparable to that of the modified long gonadotropin-releasing hormone agonist

TABLE 1 Comparison of baseline data before and after the matching of two protocols.

	Original data			P-value	Data after matching			P-value
	Group I (n=213)	Group II (n=697)	*T or* $\chi 2$		Group I(n=174)	Group II (n=174)	*T or* $\chi 2$	
Female age	33.11 ± 4.50	34.62 ± 5.18	-3.83	0.000[a]	33.762 ± 4.55	33.132 ± 5.35	1.20	0.232
Male age	34.092 ± 5.26	35.872 ± 6.20	-4.15	0.000[a]	34.612 ± 5.42	33.662 ± 5.59	1.62	0.107
Stimulation cycle(s)	1 [1/2]	1 [1/2]	2.20	0.028[a]	1 [1/2]	1 [1/2]	0.54	0.587
Infertility years	3 [1/4]	3 [1.5/4]	0.22	0.825	3 [1/4]	2 [1/4]	0.78	0.438
BMI	23.082 ± 3.61	23.162 ± 3.55	-0.28	0.778	23.032 ± 3.71	23.702 ± 4.06	-1.61	0.109
AMH	2.10 [1.12/3.56]	1.06 [0.61/1.58]	10.40	0.000[a]	1.69 [1.0/2.68]	0.92 [0.23/2.27]	5.24	0.000[a]
AFC	10 [8/13]	8 [6/10]	7.11	0.000[a]	9 [7/11]	9 [6/11]	2.71	0.007[a]
POSEIDON grouping			72.73	0.000[a]			1.15	0.765
Group 1	112	171			76	68		
Group 2	43	123			40	42		
Group 3	23	191			23	22		
Group 4	35	212			35	42		
Infertility type			5.11	0.024[a]			2.27	0.132
Primary infertility	107	289			86	101		
Secondary infertility	106	408			88	73		
Causes of infertility			10.37	0.016[a]			7.12	0.068
Female factor	170	596			141	158		
Male factor	14	28			12	5		
Couple factors	6	33			6	3		
Unknown reasons	23	40			15	8		
Fertilization techniques			23.84	0.000[a]			14.91	0.000[a]
IVF	137	338			106	130		
ICSI	68	318			61	44		
RICSI	8	10			7	0		

a. P<0.05.
BMI, body mass index; AMH, anti-Müllerian hormone; AFC, antral follicle count; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; RICSI, rescue intracytoplasmic sperm injection.

protocol, and the number of frozen blastocysts was higher than that of the modified long gonadotropin-releasing hormone agonist protocol. These results might be ascribed to the fact that over 80% of patients on the modified long gonadotropin-releasing hormone agonist protocol underwent fresh embryo transfer. With the non-downregulation protocol, the whole embryo culture and blastocyst freezing were used after oocyte retrieval, so the number of frozen blastocysts was more than that with the modified long gonadotropin-releasing hormone agonist protocol. Patients with ovarian hyporesponsiveness had fewer eggs and substantially fewer embryos and it was very likely that no blastocyst developed in the process of blastocyst culture. This resulted in a 33.7% cancellation rate, which was significantly higher than the 11.7% cancellation rate observed with the modified long gonadotropin-releasing hormone agonist protocol.

Post-matching data exhibited that there was no significant difference in the total amount of Gn used between the two protocols. The duration of Gn in the modified long gonadotropin-releasing hormone agonist protocol lasted longer, and the daily average Gn dosage and Gn initiation dosage were significantly lower than those of the non-downregulation protocol. After matching of the basic data, the number of oocytes retrieved and mature oocytes, the normal fertilization and normal cleavage with the modified long gonadotropin-releasing hormone agonist protocol were still significantly higher than those with the non-downregulation protocol, but there was no significant difference

TABLE 2 Comparison of ovulation process and clinical outcome before and after the matching of two protocols.

	Original/Pre-matching data			P-value	Data after matching			P-value
	Group I (n=213)	Group II (n=697)	*T or* $\chi 2$		Group I (n=174)	Group II (n=174)	*T or* $\chi 2$	
Total Gn (IU)	27,892 ± 1,014	31,652 ± 896	-3.66	0.000[a]	29,922 ± 1,055	31,932 ± 1,026	-1.48	0.139
Gn days	11 [9/12]	10 [8/11]	5.97	0.000[a]	11 [10/12]	10 [8/11]	5.25	0.000[a]
Average daily Gn (IU)	242 [187/279]	300 [292/355]	-14.93	0.000[a]	254 [206/286]	300 [291/347]	-9.88	0.000[a]
Gn activation doses (IU)	200 [150/225]	300 [300/375]	-21.31	0.000[a]	200 [150/225]	300 [300/375]	-14.29	0.000[a]
Trigger day LH (IU/L)	0.78 [0.57/1.35]	6.93 [4.69/10.27]	-18.52	0.000[a]	0.87 [0.54/1.30]	7.83 [5.47/11.69]	-7.221	<0.001[a]
Trigger day E2 (pg/ml)	881 [433/1219]	1,682 [1,057/2,397]	-11.11	0.000[a]	872 [383/1,192]	1,821 [855/2,505]	-13.53	0.000[a]
Trigger day P (ng/ml)	0.57 [0.40/0.85]	0.77 [0.50/1.15]	-4.99	<0.001[a]	0.55 [0.39/0.85]	0.86 [0.50/1.20]	-4.25	<0.001[a]
Add GnRH-A ratio		54/697 (7.75%)				9/174 (5.17%)		
GnRH-A dose		0.25 [0.25/0.50]				0.25 [0.25/1.0]		
Premature ovulation ratio		7/697 (1.00%)				2/174 (1.15%)		
Retrieved oocytes	6 [3/8]	4 [2/6]	5.42	0.000[a]	5 [3/8]	4 [2/7]	3.02	0.003[a]
Mature oocytes	5 [2/6]	3 [2/6]	3.96	0.000[a]	4 [2/6]	3 [1/6]	2.05	0.041[a]
Normal fertilization	3 [2/5]	2 [1/4]	3.80	0.000[a]	3 [1/6]	2 [1/4]	2.05	0.041[a]
Normal cleavage	3 [1/4]	2 [1/4]	3.83	0.000[a]	3 [1/4]	2 [1/4]	2.11	0.035[a]
High- quality embryo rate of D3	1 [0/2]	1 [0/1]	1.69	0.000[a]	1 [0/2]	1 [0/1]	0.90	0.368
Frozen blastocyst	0 [1/2]	1 [0/2]	-4.73	0.000[a]	0 [0/1]	1 [0/2]	-4.04	0.000[a]
Cumulative pregnancy rate	112/213 (52.6%)	237/697 (34.0%)	23.82	0.000[a]	90/174 (51.7%)	60/174 (34.5%)	10.55	0.001[a]
Transplant cancellation Rate	25/213 (11.7%)	235/697 (33.7%)	38.62	0.000[a]	23/173 (13.2%)	61/174 (35.1%)	22.66	0.000[a]
Fresh embryo transfer ratio	185/213 (86.9%)				148/174 (85.1%)			
Average transfer embryos	265/185(1.42)				212/148(1.43)			
Single embryo	107/185 (57.8%)				84/148 (56.8%)			
Double embryos	78/185 (42.2%)				64/148 (43.3%)			
	93/185 (50.3%)				76/148 (51.4%)			

a. P<0.05.

between the two groups in the number of high-quality embryos on day 3. After matching, the results still showed that the cumulative pregnancy rate of the modified long gonadotropin-releasing hormone agonist protocol was significantly higher than that of its non-downregulation counterpart. In the matched data, the transfer cancellation rate of the non-downregulation protocol, due to lack of embryo freezing, was also significantly higher than that of the modified long gonadotropin-releasing hormone agonist protocol.

3.3 Determination of the independent factors influencing the cumulative pregnancy rate

The baseline data of the two groups of POSEIDON group patients on different clinical protocols still exhibited significant differences after PSM matching. Statistical tests still showed significant differences between the two matched groups in AMH,

AFC, and fertilization technique. We subjected the three factors plus the group factors to the logistic regression to see whether the clinical ovulation stimulation protocol was an independent factor influencing the cumulative pregnancy rate of POSEIDON group patients. As shown in Table 3, the ovulation stimulation protocol group was an independent factor influencing the cumulative pregnancy rate in POSEIDON group patients. The cumulative pregnancy rate of the non-downregulation protocol was 0.486 times that of the modified long gonadotropin-releasing hormone agonist protocol, with a 95% confidence interval of 0.303-0.779, and the difference was statistically significant. In addition to the clinical ovulation stimulation protocol, the number of basal antral follicles and the group of fertilization methods were also independent influencing factors. As the number of basal antral follicles increased, the cumulative pregnancy rate also increased, with the OR value being 1.128 and its 95% confidence interval 1.046-1.218, and the difference was statistically significant. With respect to the group of different fertilization methods, the cumulative pregnancy rate of ICSI (intracytoplasmic sperm injection) was lower than that of IVF, while the difference between RICSI (rescue ICSI) and IVF was not statistically significant. In this study, the proportion of ICSI and RICSI was relatively small. These results did not rule out the possibility of bias. In future, larger-sized studies are warranted for further verification.

4 Discussion

Controlled ovarian stimulation (COS) is a pivotal part of ART. Controlled ovarian hyperstimulation allows infertile women to yield a sufficient number of oocytes, which is important for attaining high pregnancy and live birth rates. Ovarian hyporesponsiveness is principally characterized by a poor response to Gn. With low responders, the dose of Gn used in the ovulatory cycle is high but the quality and quantity of oocytes are poor, resulting in low pregnancy rates and high cycle cancellation rates. In fact, POR poses a major challenge for ART. For the POR patients, it is particularly important to work out an ovulation stimulation protocol to each individual.

In 1984, Porter et al. (10), for the first time, successfully used GnRHa in combination with gonadotropin for ovulation induction. Since then, pituitary downregulation was extensively employed as ovarian hyperstimulation treatment in in vitro fertilization and embryotransfer (IVF-ET). GnRHa binds stably to the pituitary gonadotropin-releasing hormone receptor. After a short flare-up period, pituitary is functionally suppressed, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels are reduced, and eventually pituitary function is effectively inhibited. Functional downregulation of the pituitary synchronizes the recruitment of follicles. Upon the downregulation, exogenous Gn dosing can achieve the synchronous and uniform the growth of follicles, thereby improving the outcome of ART. However, patients with low ovarian function already respond poorly to Gn medication. After downregulation and inhibition of endogenous Gn secretion, even with additional higher doses of exogenous Gn, follicular dysplasia or even aplasia may still occur. Therefore, our early practice was to treat most POR patients with a non-downregulation protocol. In this protocol, patients were given sufficient doses of Gn to promote ovulation during the menstrual period, with oral clomiphene citrate (CC) serving as adjuvant therapy. CC is a selective estrogen receptor modulator chemical. In general, it exerts predominantly an estrogenic antagonist or anti-estrogenic effect. As an anti-estrogenic agent, CC can act directly on hypothalamic gonadotropin-releasing hormone (GnRH) neurons and indirectly promote the release of GnRH by inhibiting the negative feedback of endogenous estrogens in hypothalamus. GnRH secreted enters the pituitary portal system, stimulating the secretion of pituitary FSH and LH, which stimulate ovarian activity, and promote the growth, development, and maturation of follicles and ovulation (11). A prospective, randomized, controlled trial by Al-Inany Hesham et al. demonstrated that the addition of CC to hMG could effectively reduce premature LH surges without compromising the pregnancy rate (12). However, this protocol may be associated with early onset of LH peak and early ovulation due to lack of downregulation. In order to prevent early ovulation, a GnRH antagonist is occasionally used if serum LH exceeds 10 mIU/ml during superovulation monitoring in the

TABLE 3 Regression analysis of factors influencing cumulative pregnancy rate in patient-oriented strategy encompassing individualized oocyte number (POSEIDON) group patients.

	B values	SD	P-value	OR	OR (95% CI)	
					5%	95%
Protocol grouping	-0.722	0.241	0.003[a]	0.486	0.303	0.779
AMH	0.164	2.697	0.101	1.178	0.969	1.432
AFC	0.121	0.039	0.002[a]	1.128	1.046	1.218
Fertilization methods			0.004[a]			
ICSI/IVF	-0.835	0.273	0.002[a]	0.434	0.254	0.741
RICSI/IVF	-1.521	0.889	0.087	0.218	0.038	1.248
Constant	-1.036	0.388	0.008[a]	0.355		

a. P<0.05.
AMH, anti-Müllerian hormone; AFC, antral follicle count; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; RICSI, rescue intracytoplasmic sperm injection.

non-downregulated protocol. With the non-downregulation protocol, CC plus high-dose Gn entails no pretreatment, does not affect follicular development, and involves only a short stimulation time. Its use is more economical and efficient in patients with POR. However, due to the anti-estrogen effect of CC, which affects the growth and development of the endometrium, the endometrium of most patients on the ovulation stimulation cycle cannot satisfy the requirements for transplantation (13). Therefore, in using the non-downregulation protocol, we employ the "freeze all" strategy. All the embryos obtained by this protocol are cultured to blastocysts and frozen. In general, the ovarian response of POR patients is poor and fewer oocytes are retrieved. In particular, a significant number of patients are unable to have blastocysts available for transfer under the strategy. For the past two years, our center has been trying to use the modified long gonadotropin-releasing hormone agonist protocol for ovarian hyperstimulation in poor responders. The long-acting GnRHa in the modified long gonadotropin-releasing hormone agonist protocol can inhibit immune and inflammatory factors, upregulate the expression of the endometrial cell adhesion molecule integrin, enhance endometrial pinocytosis in the implantation window, and thereby improve endometrial receptivity (5, 6, 8, 9, 14). Patients with ovarian hyporesponsiveness have poor responses to Gn, the number of oocytes retrieved is low, and the number of embryos available is few. The long-acting GnRHa used in the modified long gonadotropin-releasing hormone agonist protocol can improve endometrial receptivity and the POR patients with this protocol potentially have more opportunity for fresh embryo transfer. This study showed that the modified long gonadotropin-releasing hormone agonist protocol could accomplish a fresh embryo transfer rate of more than 85%.

Due to the presence of selection bias, it is meaningless to directly compare the two protocols in terms of pregnancy outcomes. Therefore, our study performed a propensity score matching on the retrospective data and comparatively analyzed the two protocols in terms of outcomes. We found that although there were more frozen blastocysts with the non-downregulation protocol, its cumulative single cycle pregnancy rate was still significantly lower than that with the modified long gonadotropin-releasing hormone agonist protocol. This study suggests that the modified long gonadotropin-releasing hormone agonist protocol can be used as an option for individualized ovarian hyperstimulation in POSEIDON group patients. Nevertheless, although the single-cycle cumulative pregnancy rate of the modified long gonadotropin-releasing hormone agonist protocol is not an optimal alternative, it is still worth future investigation and exploration as a pregnancy strategy for low responders.

Other studies also examined the use of the modified long gonadotropin-releasing hormone agonist protocol in patients with low response. A retrospective study by Huang MC et al. (3) also suggested that the modified long gonadotropin-releasing hormone agonist protocol might have more advantages over the GnRH-antagonist protocol when used in young POR populations. The modified long gonadotropin-releasing hormone agonist protocol in a young POR population yielded a lower transplant cancellation rate and attained a higher implantation rate, which might be attributed to the improved embryo quality and endometrial receptivity. Another study by Guo Y et al. (2) suggested that low-response patients with normal AFC and low AMH levels might benefit from a modified long gonadotropin-releasing hormone agonist protocol. However, women with normal AMH but low AFC appeared to have more favorable clinical outcomes with the mid-luteal-phase short-acting GnRH-agonist long protocol. Li w et al. (15) compared the pregnancy promoting outcomes of 451 IVF/ICSI patients in the POSEIDON 3 group between June 2017 and June 2020 under three different ovarian stimulation protocols, and the results suggested that the cumulative pregnancy rate and cumulative live birth rate were significantly higher in patients using the modified ultra-long protocol than those using the antagonist protocol and the mild stimulation protocol (50.88% vs 32.02% and 31.88%, respectively, for cumulative pregnancy rate, and 48.25% vs 26.97% and 28.99%, respectively, for cumulative live birth rate). Women in the POSEIDON 3 group who underwent IVF-ET with the modified ultra-long protocol had higher stimulation duration and total Gn dose and thicker endometrial thickness. The findings suggest that the modified ultra-long protocol increases the cumulative pregnancy and cumulative live birth rates in women with a poor ovarian response in the POSEIDON 3 group. Currently, the selection of an individualized ovulation induction protocol for patients with a low ovarian response remains controversial. Bias can make the study results less consistent. The aforementioned studies attempted to reduce the impact of bias by stratifying them in terms of age or by subgrouping. In contrast, our study aimed to minimize the effect of bias between the two groups of patients by using PSM. This ensured the reliability of our results.

While our study provides data for the further exploration of the individualized ovulation induction protocol for treating patients with low response, it is subject to some limitations. Firstly, the sample size of the modified long gonadotropin-releasing hormone agonist protocol was small, making it difficult to make comparison between groups in terms of the POSEIDON groups. The exclusion of some cases for matching might lead to selection bias. Secondly, this study was a retrospective study. Despite the use of statistical methods to minimize bias, it was impossible to completely prevent bias. In future, larger-sized studies or prospective research are needed to further verify or modify the above conclusions.

5 Conclusion

In summary, the selection of ovarian stimulation strategies for patients with poor ovarian response has been a challenge. The modified long gonadotropin-releasing hormone agonist protocol improves the endometrial receptivity, increases the fresh embryo

transfer rate, and reduces the cycle cancellation rate, giving patients more opportunities to receive an embryo transfer. The protocol accomplishes better clinical outcomes compared to its non-downregulation counterpart. Therefore, the modified long gonadotropin-releasing hormone agonist protocol can be an alternative individualized ovulation induction protocol for patients with ovarian hyporesponsiveness.

Data availability statement

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

Author contributions

LL and YG contributed to the paper design and writing, CC and XZ contributed to the data processing and paper writing, All authors contributed to data collection. All authors contributed to the article and approved the submitted version.

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.2023.1189357/full#supplementary-material

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Sequential 2.5 mg letrozole/FSH therapy is more effective for promoting pregnancy in infertile women with PCOS: a pragmatic randomized controlled trial

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Study question: In infertile women with polycystic ovary syndrome (PCOS), is the sequential use of letrozole 2.5 mg/follicle stimulating hormone(FSH) more effective than letrozole 5 mg/FSH in stimulating ovulation and promoting pregnancy?

Research design and methods: The study was designed as a prospective, single-center, randomized, controlled pragmatic clinical trial. 220 infertile women between the ages of 20 and 40, who matched the Rotterdam criteria for PCOS and had no other identified reasons for infertility were enrolled from April 2023 to July 2023.The participants were randomly assigned to two groups in a 1:1 ratio. One group received 2.5 mg of letrozole on cycle days 3-7 with a sequential injection of 75 IU FSH on cycle days 8-10 (n = 110), while the other group received 5 mg of letrozole on cycle days 3-7 with a sequential injection of 75 IU FSH on cycle days 8-10 (n = 110). The duration of FSH treatment varied depending on the follicular development stage. Each participant underwent one to three treatment cycles until achieving pregnancy.The primary outcome was the cumulative pregnancy rate of all the participants. Secondary outcomes included characteristics and clinical pregnancy rates of all the intervention cycles.

Results: For all 220 participants, the sequential letrozole 2.5 mg/FSH treatment group had a significantly higher cumulative pregnancy rate compared to the letrozole 5 mg/FSH treatment group (72.7% versus 59.1%, RR (95%CI) = 1.23 (1.02, 1.49), P-value = 0.033). For all 468 intervention cycles, letrozole 2.5 mg/FSH group had a significantly higher clinical pregnancy rate than the letrozole 5 mg/FSH group (36.2% versus 26.3%, P-value = 0.021), no statistically significant differences were observed in ovulation rates or adverse effects.

Conclusions: The data indicate that the sequential letrozole 2.5mg/FSH protocol may be more effective than the sequential letrozole 5mg/FSH protocol for promoting pregnancy in infertile women with PCOS.

Clinical trial registration: www.chictr.org.cn, identifier ChiCTR2300069638.

KEYWORDS

polycystic ovary syndrome, infertility, ovulation induction, letrozole, sequential therapy

1 Introduction

Polycystic ovary syndrome (PCOS) is an endocrine condition that is commonly characterized by persistent anovulation, hyperandrogenism, and insulin resistance in women of gestational age (1, 2). PCOS is the most prevalent cause of anovulatory infertility in women, accounting for approximately 75% of anovulatory infertility, with a prevalence rate between 5% and 10% (3). Ovulation abnormalities are responsible for roughly one quarter of all cases of infertility in couples (4). PCOS is the most prevalent reason for anovulatory infertility, accounting for about 70 percent of the overall cases (5). For PCOS patients, the standard method of treatment for anovulatory infertility is restoring mono-ovulation (6). Consequently, ovulation induction is the most crucial treatment option for infertile patients with PCOS, despite various therapeutic medications have varying ovulation induction effects (7).

There are currently primarily three types of medications for PCOS patients to induce ovulation (8–10): (i) Clomiphene citrate (CC), which has been used as a first-line ovulation induction drug for PCOS patients for decades, approximately 20% to 25% of patients with PCOS still have CC resistance in clinical practice. Besides, it has some drawbacks, including a long half-life and the occupation of estrogen receptors, which can have negative effects on the endometrium and cervical mucus and result in unsatisfactory pregnancy rates. Additionally, Clomiphene citrate is associated with a 10% increased risk of multiple pregnancies, while hyperstimulation syndrome is uncommon, which makes its clinical application subject to some restrictions (11). (ii) Letrozole (LE), which as a third-generation steroidal aromatase inhibitor, reversibly binds to aromatase enzyme and inhibits estrogen production (12). By reducing the process of converting androgens to estrogens, it lowers the body's estrogen levels, relieves the negative feedback inhibition on the hypothalamus-pituitary gland, and increases the secretion of gonadotropin-releasing hormone (GnRH), which contributes to the development of follicles, and has the advantages of a shorter half-life and no effect on estrogen receptors compared with CC. It has gotten a lot of interest since Mohamed et al. used LE for the first time to promote ovulation and achieved good clinical efficacy (13). (iii) Follicle stimulating hormone (FSH), which activates aromatization of androgens to estrogen by granulosa cells and follicle maturation, acts on the ovary to help grow and mature small follicles (14). Although FSH can be used to treat CC-resistant PCOS patients, its tendency to induce the development of multiple follicles at the same time, which increases the incidence of multiple pregnancies and the incidence of ovarian hyperstimulation syndrome (OHSS), and its price is relatively expensive, which limits its use in clinical practice (15).

Since the highest pregnancy rate with the fewest problems is considered to be the optimal outcome of ovulation induction, the procedure was accomplished by administering consecutive injections of FSH after receiving letrozole or other medications (16). This procedure has been referred to as minimal stimulation, and Kistner RW initially proposed the concept of minimal stimulation (17). It has been demonstrated to raise overall pregnancy rates while reducing adverse consequences; notably, ovarian hyperstimulation syndrome rarely occurs (18).

Studies have continued over the last few decades employing a variety of drugs together with varying gonadotrophin dosages and types (19). Despite the diversity of prior research, which ranged from retrospective evaluations to prospective randomized controlled trials, combination procedures have yielded clinical pregnancy rates equivalent to or greater than gonadotrophin-only therapies with less gonadotrophin usage and fewer multi-ovulation (20–23).

Even though numerous studies on the optimal dose of letrozole have been conducted in recent years, the optimal dose of letrozole for inducing ovulation remains debatable (24). Moreover, the most common doses of letrozole are 2.5 mg, 5 mg, and 7.5 mg, with a few reports of doses as high as 20 mg (25, 26). We analyzed the data of previously reported clinical trials on letrozole for inducing ovulation. Some studies concluded that 5 mg was more effective than 2.5 mg inducing ovulation, whereas others determined that 2.5 mg was more efficient than 5 mg and 7.5 mg in promoting ovulation (27–29).

Our recent clinical trial using sequential letrozole/gonadotrophin to induce ovulation in infertile women with PCOS identified that the sequential protocol's ovulation rate and live birth rate are higher than the letrozole protocol (20). In the present pragmatic clinical trial study, we aim to identify the optimal dose of letrozole by comparing the ovulation rate, pregnancy rate, and adverse effects of two doses of letrozole in sequential letrozole/FSH therapy.

2 Materials and methods

2.1 Trial design

This pragmatic clinical trial was performed at Union Hospital of Tongji Medical College, Huazhong University of Science and Technology, to compare treatment outcomes of different letrozole doses for ovulation induction in infertile women with PCOS, using sequential letrozole/FSH. Each individual paid for their own medication and examination, and no extra compensation was offered. The study was approved by the Medical Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology (approval number 0002-02; approval date March 5, 2023), and it was registered on the Chinese Clinical Trial Registration website (www.chictr.org.cn; identifier ChiCTR2300069638; registration date March 22, 2023). Before participation, signed informed consent with self-signature was obtained from each participant. Full details of the trial protocol can be found in the Chinese Clinical Trial Registry, available at https://www.chictr.org.cn/showprojEN.html?proj=193178.

2.2 Participants

The first participant became involved on 3 April 2023, and the final participant accomplished the study on 31 July 2023. Outpatients with PCOS and infertility who desired to conceive were evaluated using inclusion and exclusion criteria to identify those who were suitable and had no other definite causes of infertility. Inclusion criteria involved: (i) PCOS patients who met the Rotterdam criteria in 2003 (diagnosis can be created if two of three are present): oligo-ovulation or anovulation, clinical or biochemical hyperandrogenemia, polycystic ovary/ies changes under ultrasound (Rotterdam ESHRE/ASRM Sponsored PCOS Consensus Workshop Group, 2004) (30); (ii) 20 - 40 years old, normal intercourse without protection, duration of infertility longer than 1 year; (iii) hysterosalpingography indicates that normal uterine morphology and at least one Fallopian tube is unobstructed; (iv) the sperm analysis of the spouse shows no abnormality; (v) normal organ function without any endocrine disease such as hypopituitarism, hyperthyroidism, hyperprolactinemia, congenital adrenal hyperplasia, Cushing's syndrome, tumor of ovaries or adrenal gland, etc. Exclusion criteria involved: allergy to relevant drugs, failure to cooperate, severe cardiovascular disease, severe defects of liver or kidney function, pregnancy or lactation, previous history of abnormal uterine or uterine cavity disease, and previous contact with mutagenic toxins and radiation.

2.3 Intervention

Outpatients who met the inclusion and exclusion criteria for this research were selected as potential candidates for this study. Prior to their participation, the researcher provided a description of the study's purpose, methodology, potential benefits, and risks. Participants were, only after they signed the informed consent form, recruited for the study. A computer-generated random number table was used to allocate eligible participants into two groups (Group A and Group B) in a 1:1 ratio. Group A participants (letrozole 2.5 mg/FSH group) received a daily dosage of 2.5 mg letrozole (Furui, Jiangsu Hengrui Medicine Co., Ltd., Lianyungang, China) from cycle days 3 to 7, followed by a sequential therapy of 75 IU FSH (urofollitropin, Lizhu Pharmaceutical Factory, Zhuhai, China) daily from cycle days 8 to 10. Participants in Group B (letrozole 5 mg/FSH group) were given 5 mg of letrozole daily from cycle days 3 to 7, followed by sequential therapy with 75 IU of FSH daily on cycle days 8 to 10. The duration of FSH administration varies according to follicular development. Vaginal B-ultrasound was utilized to monitor follicular development beginning on day 11 of the cycle. During scanning, the number/size of follicles and endometrial thickness were measured. Blood was drawn to detect the sex hormone concentration when at least one dominant follicle reached ≥ 18 mm, and then 10,000 IU human chorionic gonadotrophin (HCG) was injected intramuscularly to trigger ovulation. Participants were advised to engage in intercourse 24 to 36 hours later. In order to monitor ovulation, a vaginal B-ultrasound was conducted 48 hours after HCG injection. Two weeks of Dydrogesterone were consumed orally. Quantitative HCG testing was carried out two weeks later to diagnose conception, and B-ultrasound was conducted four weeks later to diagnose clinical pregnancy. Each participant underwent one to three cycles of treatment until pregnancy was achieved.

2.4 Outcome measures

The primary outcome was the cumulative pregnancy rate for all participants. Secondary outcomes included the characteristics and clinical pregnancy rates of all intervention cycles. These characteristics were listed as follows: (i) ovulation characterized by a decrease in ovarian follicles and fluid in the pouch of Douglas, detected through B-mode ultrasonography 2 days after HCG administration; (ii) cancellation cycle referred to a cycle that has been discontinued due to premature ovulation, follicular dysplasia, or ovarian hyperstimulation syndrome (OHSS); (iii) biochemical pregnancy defined as a positive β-HCG pregnancy test 2 weeks after ovulation, clinical pregnancy defined as the observation of a gestational sac with fetal echoes and primitive cardiac tube pulsation inside the uterine cavity via B-ultrasound 2 weeks after a positive pregnancy test, singleton pregnancy (the sample for singleton pregnancies was derived from ongoing pregnancies), multiple pregnancy, early abortion(gestational age ≤ 14 weeks), ectopic pregnancy; (iv) duration of FSH treatment; (v) rate of one mature follicle (diameter ≥ 18 mm) and ovulation with one follicle; (vi) days required for follicles to mature and ovulate; (vii) sex hormone concentration on HCG injection day; (viii) endometrial thickness on HCG injection day and ovulation day.

2.5 Sample size

The number of samples estimated by G*Power 3.1 software was 91 subjects per group with a two-sided probability value of 0.05 and 0.9 statistical power using Pearson's chi-squared test, based on the

findings of the previous studies (31, 32). In consideration of non-compliance and loss to follow-up, the ultimate sample size per group was 110 after correction, allowing for a dropout rate of 17%.

2.6 Randomization and masking

Using SPSS software (Version 26, IBM, Armonk, NY), a statistician generated a randomization scheme that allocated the enrolled participants in a 1:1 ratio to two groups. The randomization scheme consisted of an enrollment order combined with treatment codes (A and B). Participants were assigned by a clinician who was aware of the order of the randomization scheme but unaware of the meaning behind each code. The assignment was made based on the enrollment order specified in the randomization scheme. Sonographers, statisticians, and outcome evaluators remained blinded to the allocation, while physicians and patients were informed.

2.7 Statistical analysis

The intervention cycle (IC) analysis comprised all intervention cycles, while the complete data (CD) analysis comprised only those intervention cycles for which complete data were collected. Statistical analysis was conducted using SPSS for Windows (Version 26). For qualitative (categorical) data, the outcome was expressed as number of cases and percentage, and differences between the two groups were analyzed using chi-squared test at a two-sided significance value of 0.05. Fisher's exact test was used if the total number of cases was less than 40, or if a theoretical frequency was equal to 0, or if two theoretical frequencies were greater than or equal to 1 but less than 5.If the total number of cases was greater than 40, a theoretical frequency was greater than or equal to 1 but less than 5, P-value required continuity correction. For quantitative (numerical) variables, the Shapiro-Wilke test for a single sample was used to evaluate the normality of distribution. In the case of normally distributed data, Student's t-test was performed to two independent samples, and the mean ± standard deviation was presented. The Mann-Whitney U-test was utilized to asymmetrical data presented as a median and interquartile range. A stratification analysis was performed when evaluating cumulative pregnancy rate and clinical pregnancy rate. A degree of significance of less than or equal to 0.05 was considered statistically significant. Figures were generated using GraphPad Prism 9 software.

3 Results

3.1 Participation flow chart

The complete procedure for study participants is depicted in Figure 1. Two hundred twenty participants were randomly assigned from 230 invited women who were initially considered suitable; 110

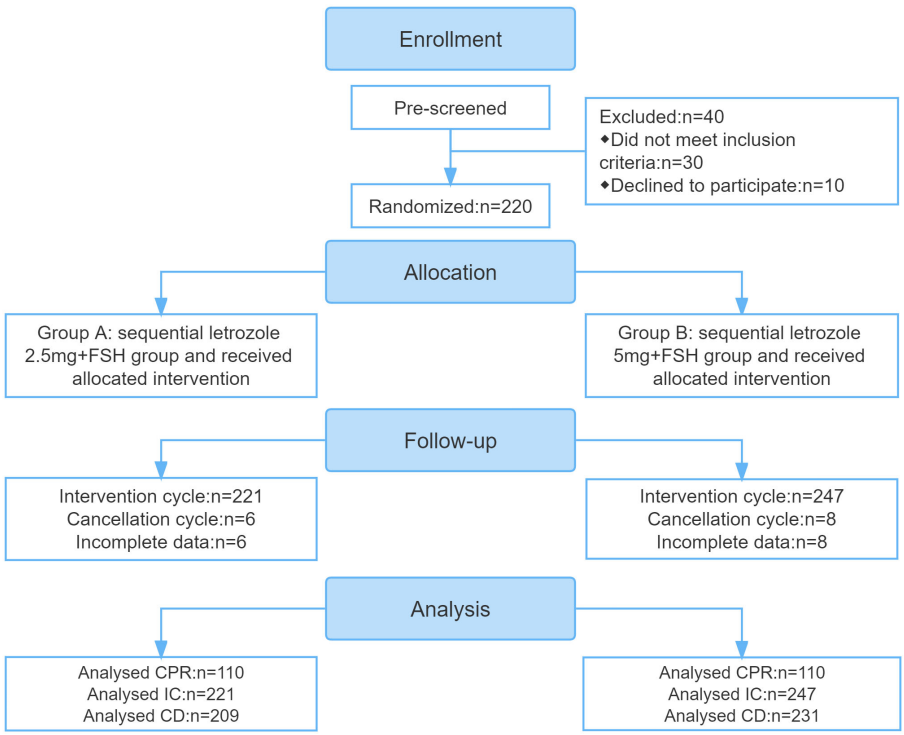

FIGURE 1
CONSORT flow diagram revealing the participation process for each stage of the study. CONSORT (Consolidated Standards of Reporting Trials); FSH, follicle stimulating hormone; CPR, cumulative pregnancy rate; IC, intervention cycle; CD, complete data.

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were assigned to the letrozole 2.5 mg/FSH group, and 110 were assigned to the letrozole 5 mg/FSH group. All 220 participants completed the process from corresponding treatment to pregnancy evaluation; they became included in the CPR analysis. Fourteen out of 468 intervention cycles were canceled, 14 cycles had incomplete data recordings, thus 440 cycles were included in the complete data analysis.

3.2 Baseline data

Table 1 depict baseline demographic, clinical and endocrine characteristics. No significant differences existed between the two groups.

TABLE 1 Baseline characteristics of participants.

Characteristic	Group A (n = 110) Letrozole 2.5 mg + FSH	Group B (n = 110) Letrozole 5 mg + FSH	*P*-value
Age, years	28(26~30)	28(26~29)	0.554
BMI, kg/m^2	23.24(21.56~25.69)	23.4(21.45~25.7)	0.96
HOMA-IR	2.65(1.86~3.62)	2.66(1.89~3.59)	0.654
Sex hormone			
LH, IU/L	8.56(5.89~10.66)	8.13(4.75~11.64)	0.523
FSH, IU/L	6.78(5.91~7.74)	6.53(5.58~7.51)	0.362
LH/FSH	1.2(0.93~1.57)	1.24(0.72~1.8)	0.841
PRL, ng/ml	18.65(13.49~24.15)	16.11(12.2~22.37)	0.132
AMH, ng/ml	8.25(5.9~10.07)	8.4(5.85~12.29)	0.158
Stratification			
Age≤ 28, years	64(58.2%)	69(62.7%)	0.491
BMI<24, kg/m^2	70(63.6%)	64(58.2%)	0.407
No insulin resistance	82(74.5%)	83(75.5%)	0.876
Primary infertility	94(85.5%)	91(82.7%)	0.58
LH ≤ 12, IU/L	93(84.5%)	84(76.4%)	0.126
FSH ≤ 8, IU/L	88(80%)	92(83.6%)	0.484
LH/FSH<2	97(88.2%)	92(83.6%)	0.333
PRL ≤ 30, ng/ml	96(87.3%)	97(88.2%)	0.837
T ≤ 2, nmol/L	104(94.5%)	101(91.8%)	0.422
AMH>4, ng/ml	100(90.9%)	104(94.5%)	0.299

Values are presented as n (%) or median (interquartile range). P-values were evaluated using chi-squared test for qualitative data and Mann-Whitney U-test for quantitative data. FSH, follicle stimulating hormone; BMI, body mass index; HOMA-IR, homeostasis model assessment of insulin resistance; LH, luteinizing hormone; PRL, prolactin; AMH, anti-Müllerian hormone; T, testosterone.

3.3 Primary outcome

Table 2 depicts cumulative pregnancy rate among participants. The cumulative pregnancy rate was substantially higher in Group A than in Group B (72.7% versus 59.1%, RR (95%CI) = 1.23 (1.02, 1.49), P-value = 0.033). Age>28 years, BMI ≥24 kg/m^2, LH ≤ 12IU/L, FSH ≤ 8IU/L, PRL ≤ 30ng/ml, T ≤ 2nmol/L and AMH>4ng/ml were subgroups where the positive effect of Group A on cumulative pregnancy rate was evident (P-value<0.05).

3.4 Secondary outcomes

Fourteen out of 468 intervention cycles were canceled, while 14 cycles had incomplete data recordings; thus, a total of 440 cycles were included in the complete data analysis. Table 3 illustrate the characteristics of these cycles. For all the intervention cycles, there were no significant differences observed between the two groups in terms of ovulation rate, cancellation rate, incidence of early ovulation, follicular dysplasia, and OHSS. However, Group A exhibited significantly higher rates of biochemical (40.7% versus 28.3%, P-value = 0.005) and clinical pregnancy (36.2% versus 26.3%, P-value = 0.021).

Moreover, Group A showed a significantly higher rate of obtaining only one mature follicle compared to Group B (80.9% versus 64.5%, P-value <0.01), whereas there was no significant difference in the ovulation rate with one follicle (72.7% versus 62.7%, P-value >0.05). The duration of FSH treatment (4.47 ± 0.15 versus 4.51 ± 0.14, P-value >0.05) and days required for follicles to mature (13.3 ± 0.16 versus 13.12 ± 0.14, P-value >0.05) or ovulate (15.51 ± 0.16 versus 15.27 ± 0.15, P-value >0.05) were similar between both groups. The hormone concentration on HCG injection day significantly differed between the two groups. The endometrial thickness on ovulation day was significantly less in Group A (P-value<0.05), whereas there was no substantial difference on the day of HCG administration.

Table 4 displays the clinical pregnancy rate for the intervention cycle with complete data recorded. The clinical pregnancy rate in Group A was significantly greater than in Group B (38.3% versus 27.7%, RR (95%CI) = 1.38(1.05,1.81), P-value = 0.018). The beneficial effect of Group A on clinical pregnancy rate was apparent (P-value<0.05) in subgroups with ages >28 years old, cycles with only one mature follicle and endometrial thickness on ovulation day>10 mm. There was no significant difference between groups for participants who received treatment once; however, for participants who received treatment for 2 or more cycles, Group A had a significantly higher clinical pregnancy rate (P-value<0.05).

4 Discussion

Our present study in infertile women with PCOS revealed that letrozole 2.5 mg/FSH sequential therapy was superior at ovulation induction and pregnancy rates than letrozole 5 mg/FSH sequential therapy. Specifically, there were no notable disparities in adverse drug reactions.

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TABLE 2 Cumulative pregnancy rate of participants.

Item		Group A, n(%) Letrozole 2.5 mg + FSH	Group B, n(%) Letrozole 5 mg + FSH	RR (95%CI)	*P*-value
CPR		80/110(72.7%)	65/110(59.1%)	1.23(1.02,1.49)	0.033
Age, years	≤ 28	45/64(70.3%)	42/69(60.9%)	1.16(0.9,1.48)	0.253
	> 28	35/46(76.1%)	23/41(56.1%)	1.36(0.99,1.86)	0.048
BMI, kg/m^2	< 24	53/70(75.7%)	44/64(68.8%)	1.1(0.89,1.36)	0.368
	≥ 24	27/40(67.5%)	21/46(45.7%)	1.48(1.01,2.17)	0.042
Insulin resistance	No	61/82(74.4%)	52/83(62.7%)	1.19(0.96,1.46)	0.105
	Yes	19/28(67.9%)	13/27(48.1%)	1.41(0.88,2.25)	0.139
Infertility	Primary	69/94(73.4%)	56/91(61.5%)	1.19(0.97,1.46)	0.085
	Secondary	11/16(68.8%)	9/19(47.4%)	1.45(0.81,2.59)	0.306[a]
LH, IU/L	≤ 12	68/93(73.1%)	49/84(58.3%)	1.25(1.01,1.56)	0.038
	> 12	12/17(70.6%)	16/26(61.5%)	1.15(0.75,1.77)	0.543
FSH, IU/L	≤ 8	66/88(75%)	54/92(58.7%)	1.28(1.04,1.58)	0.02
	> 8	14/22(63.6%)	11/18(61.1%)	1.04(0.64,1.69)	0.87
LH/FSH	< 2	72/97(74.2%)	56/92(60.9%)	1.22(1,1.49)	0.05
	≥ 2	8/13(61.5%)	9/18(50%)	1.23(0.66,2.31)	0.711[a]
PRL, ng/ml	≤ 30	70/96(72.9%)	56/97(57.7%)	1.26(1.02,1.56)	0.027
	> 30	10/14(71.4%)	9/13(69.2%)	1.03(0.63,1.69)	> 0.1[a]
T, nmol/L	≤ 2	76/104(73.1%)	59/101(58.4%)	1.25(1.02,1.53)	0.027
	> 2	4/6(66.7%)	6/9(66.7%)	1(0.48,2.08)	> 0.1[a]
AMH, ng/ml	≤ 4	6/10(60%)	3/6(50%)	1.2(0.47,3.09)	> 0.1[a]
	> 4	74/100(74%)	62/104(59.6%)	1.24(1.02,1.51)	0.029

[a]P-values were calculated using Fisher's exact test. RR, relative risk; CI, confidence interval; CPR, cumulative pregnancy rate.

Currently, the majority of frequently used to stimulate ovulation are clomiphene, letrozole, and FSH, with FSH being the most effective and clinically valuable (33). Nevertheless, FSH is associated with an increased risk of multiple pregnancies and OHSS (34). Letrozole is an aromatase inhibitor of the third generation, and its effects on the human body are primarily reflected in two aspects (35). Firstly, aromatase inhibition diminishes estrogen production, which relieves estrogen's inhibiting effect on the hypothalamus and pituitary gland, resulting in the release of FSH and LH and an increase in follicular recruitment (36). Secondly, the increase of androgens in the ovary elevates the sensitivity of insulin-like growth factor, which together with FSH, promotes follicular development (37).

Letrozole is associated with comparable ovulation rates and reduced rates of complications such as multiple births and OHSS than clomiphene. The 2018 European Society of Human Reproduction and Embryology (ESHRE) "International Evidence-Based Guidelines for the Evaluation and Management of Polycystic Ovarian Syndrome" recommends letrozole as a first-line ovulation induction agent for infertile patients with PCOS (38). Letrozole is the initial treatment option for infertile patients with PCOS (39). Obstetricians and gynecologists tend to use urinary follicle-stimulating hormone (uFSH) in combination with letrozole for ovulation induction in infertile women in order to reduce the risk of the aforementioned adverse events and reduce the financial burden (40).

Our recent report suggested that sequential letrozole/gonadotrophin is superior to letrozole alone for inducing ovulation and facilitating pregnancy in infertile women with PCOS (20). Sequential letrozole/gonadotrophin is recommended as a high-reward, low-risk ovulation promotion regimen. There is disagreement regarding the optimal dose of letrozole in the clinical treatment of PCOS. Previous investigations have demonstrated that 5 mg/day of letrozole is more efficacious than 2.5 mg/day but that 7.5 mg/day has no advantage over 5 mg/day (41, 42). Other studies have suggested that the efficacy of the 2.5 mg dose is more confident and promotes ovulation more effectively than the 5 mg dose and the higher 7.5 mg dosage (31, 43). The present study found that letrozole at 2.5 mg/day was more effective than letrozole at 5 mg/day. The current investigation revealed that the utilization of sequential therapy involving letrozole 2.5 mg/FSH did not necessitate an escalation in the dosage of FSH in order to attain an equivalent therapeutic outcome. Moreover, the synergistic effect of FSH made it possible to achieve a better therapeutic effect with

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TABLE 3 Cycle characteristics.

Characteristic	Group A Letrozole 2.5 mg + FSH	Group B Letrozole 5 mg + FSH	*P*-value
Intervention cycle analysis	n=221	n=247	
Ovulation rate	217/221(98.2%)	239/247(96.8%)	0.329
Cancellation rate	6/221(2.7%)	8/247(3.2%)	0.740
Ovulate prematurely	2/221(0.9%)	0/247(0%)	0.222[a]
Follicular dysplasia	2/221(0.9%)	3/247(1.2%)	>0.1[a]
OHSS	0/221(0%)	1/247(0.4%)	>0.1[a]
Reproductive outcomes overall			
Biochemical pregnancy	90/221(40.7%)	70/247(28.3%)	0.005
Clinical pregnancy	80/221(36.2%)	65/247(26.3%)	0.021
Singleton pregnancy	60/80(75%)	46/65(70.8%)	0.568
Multiple pregnancy	10/80(12.5%)	14/65(21.5%)	0.145
Early abortion	9/80(11.3%)	4/65(6.2%)	0.285
Ectopic pregnancy	1/80(1.3%)	1/65(1.5%)	>0.1[a]
Complete data analysis	n=209	n=231	
Days of uFSH treatment	4.47 ± 0.15	4.51 ± 0.14	0.852
One mature follicle	169/209(80.9%)	149/231(64.5%)	<0.01
Ovulation with one follicle	152/209(72.7%)	154/231(66.7%)	0.168
Days required for follicles to mature	13.3 ± 0.16	13.12 ± 0.14	0.373
Days required for follicles to ovulation	15.51 ± 0.16	15.27 ± 0.15	0.202
Hormone concentration on HCG injection day			
LH, IU/L	10.14 (7.54~14.35)	8.86(6.35~13.1)	0.014
Oestradiol, pg/ml	228(156.35~355)	188.5 (120.55~302.5)	0.001
Progesterone, ng/ml	0.23(0.1~0.38)	0.26(0.16~0.49)	0.011
Endometrial thickness, mm			
HCG injection day	9.16 ± 0.14	9.36 ± 0.15	0.367
Ovulation day	10.16 ± 0.14	10.67 ± 0.15	0.015

[a]P-values were calculated using Fisher's exact test. Values are showed as n (%) or median (interquartile range). When the medians are equal, values are showed as mean ± SD.

2.5 mg of letrozole without the need to increase the letrozole dosage additionally.

In this study, there was no statistically significant difference in fundamental characteristics between the letrozole 2.5mg/FSH group and the letrozole 5mg/FSH group. However, the incidence of single follicular development was statistically significantly higher in the former group, with a 16.4% absolute difference. Statistically significant improvements were also observed in the biochemical and clinical pregnancy rates in the letrozole 2.5 mg/FSH group. Furthermore, this analysis was stratified by age and body mass index (BMI) (44). The group receiving letrozole 2.5 mg/FSH had a statistically significant higher cumulative pregnancy rate among patients older than 28. The cumulative pregnancy rate in the letrozole 5 mg/FSH group was considerably lower in patients over 28 years of age compared to patients under 28. This indicates letrozole 2.5 mg/FSH is more suitable for patients over 28.

A large BMI is disadvantageous to ovulation in infertile patients with PCOS, and treatment with ovulation-promoting medications is less effective in obese patients than in patients with a normal BMI (45, 46). In the current study, the cumulative pregnancy rate decreased in both groups of patients with BMI of 24; however, the decrease was more pronounced in the BMI-stratified letrozole 5 mg/FSH group. The cumulative pregnancy rate was statistically higher in the letrozole 2.5 mg/FSH group for patients with a BMI below 24. Therefore, the letrozole 2.5 mg/FSH protocol is suggested for ovulation in patients with a BMI less than 24. It has also demonstrated that the letrozole 2.5 mg/FSH regimen is superior concerning pregnancy rate.

We investigate further why the letrozole 2.5 mg/FSH group in this study performed better regarding pregnancy rate. The single mature follicle development rate was significantly higher in the letrozole 2.5 mg/FSH group, suggesting that multiple follicle development is more likely to occur in the letrozole 5 mg/FSH group. It may be because a higher dose of letrozole inhibits the conversion of androgens to estrogens in the body to a greater extent (47, 48). This decrease in estrogen contributes to the discharge of the negative feedback inhibition of the hypothalamic-pituitary gland, increasing gonadotropin-releasing hormone (GnRH) secretion and, ultimately, the development of multiple follicles (49, 50). Group B, which had a high incidence of considerable follicle development, had statistically lower estrogen levels than group A on the day of HCG injection. We hypothesized that this could be due to the elevated rate of multiple follicle development in group B. We further speculate that the inferior egg quality in group B, compared to group A, ultimately leads to a low pregnancy rate and poor embryo quality. This possible explanation is consistent with the earlier observations that specifically examined egg quality and pregnancy outcome (51, 52).

The benefit of the present research was that, with the exception of the ovulation therapy, there was no disparity between the two groups regarding the other treatments, ranging from pretreatment to dydrogesterone application or the baseline characteristics. Consequently, the two treatment groups are identical at this point. The diagnostic criteria depend on the standard PCOS diagnostic criteria (53), which can be employed as a guide for treating the same group of patients in various clinical environments.

The present study has limitations that should be acknowledged. This study was open-label, so neither participants nor physicians were uninformed of the medication regimen. However, the patient assignment was determined by a computer-generated randomization algorithm implemented by a statistician who had no responsibility for patient enrolment (54). All selected outcomes were objective indicators, with the exception of B-ultrasound, which

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TABLE 4 Clinical pregnancy rate of complete data cycles.

Item		Group A, n(%) Letrozole 2.5 mg + FSH	Group B, n(%) Letrozole 5 mg + FSH	RR (95%CI)	*P*-value
Complete data analysis		n=209	n=231		
Clinical pregnancy rate		80/209(38.3%)	64/231(27.7%)	1.38(1.05,1.81)	0.018
Age,years	≤ 28	45/119(37.8%)	41/139(29.5%)	1.28(0.91,1.81)	0.158
	> 28	35/90(38.9%)	23/92(25%)	1.56(1,2.41)	0.044
BMI, kg/m^2	< 24	53/137(38.7%)	44/134(32.8%)	1.18(0.86,1.62)	0.315
	≥ 24	27/72(37.5%)	20/97(20.6%)	1.82(1.11,2.97)	0.015
One mature follicle	No	15/40(37.5%)	23/82(28%)	1.34(0.79,2.27)	0.29
	Yes	65/169(38.5%)	41/149(27.5%)	1.4(1.01,1.93)	0.039
Hormone concentration on HCG injection day					
LH, IU/L	≤ 10	40/104(38.5%)	37/136(27.2%)	1.41(0.98,2.04)	0.064
	> 10	40/105(38.1%)	27/95(28.4%)	1.34(0.9,2)	0.148
Oestradiol, pg/ml	≤ 200	25/82(30.5%)	24/122(19.7%)	1.55(0.95,2.52)	0.076
	> 200	55/127(43.3%)	40/109(36.7%)	1.18(0.86,1.62)	0.302
Progesterone, ng/ml	≤ 0.25	47/119(39.5%)	34/113(30.1%)	1.31(0.92,1.88)	0.133
	> 0.25	33/90(36.7%)	30/118(25.4%)	1.44(0.96,2.18)	0.08
Endometrial thickness on ovulation day, mm	≤ 10	40/130(30.8%)	30/119(25.2%)	1.22(0.82,1.83)	0.33
	> 10	40/79(50.6%)	34/112(30.4%)	1.67(1.17,2.38)	0.005
Cycle	1	40/106(37.7%)	31/105(29.5%)	1.28(0.87,1.88)	0.207
	2~3	40/103(38.8%)	33/126(26.2%)	1.48(1.01,2.17)	0.041

was administered by a physician who was oblivious to the treatment. There was no selection bias because the investigators did not know which group the next subject would be assigned to (55, 56). The preceding should reduce any bias induced by the open-label design of the study. When patients were aware of their own regimen and drug side effects, a bias toward side effects may have been introduced.

Nonetheless, this bias should have a tendency to cause more adverse events in sequential groups, which is contrary to the majority of adverse events observed here. Considering the existence of bias, the actual adverse events of the sequential group should be lower than the current information, which is more advantageous to the present conclusion (57, 58). Consequently, it could be stated that the subjective perception bias for adverse effects had little impact on the conclusion of the present investigation.

5 Conclusion

In conclusion, the current clinical trial demonstrated that letrozole 2.5mg/FSH was preferable to letrozole 5 mg/FSH for inducing ovulation and promoting pregnancy in infertile women with PCOS. Therefore, letrozole 2.5 mg/FSH is recommended as a superior protocol for ovulation induction. Consequently, in clinical practice, the starting dose of letrozole for ovulation induction is suggested to be 2.5 mg. If this dosage proves ineffective, alternative therapeutic regimens should be considered rather than increasing the dose of letrozole since it does not enhance the patient's cycle pregnancy rate.

Data availability statement

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

Ethics statement

The studies involving humans were approved by The Medical Ethics Committee of Tongji Medical College of Huazhong University of Science and Technology. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

L-JC: Conceptualization, Data curation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. YL: Conceptualization, Data curation, Methodology, Project administration, Supervision, Writing – review & editing. LZ: Data curation, Writing – review & editing. J-YL: Data curation, Investigation, Writing – review & editing. W-QX: Data curation, Investigation, Writing – review & editing. TL: Data curation, Writing – review & editing. HD: Data curation, Writing – review & editing. B-JL: Data curation, Formal analysis, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

Publisher's note

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Anastrozole monotherapy further improves near-adult height after the initial combined treatment with leuprorelin and anastrozole in early-maturing girls with compromised growth prediction: results from the second phase of the GAIL study

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Background: The first phase of the GAIL study ("Girls treated with an Aromatase Inhibitor and Leuprorelin," ISRCTN11469487) has shown that the combination of anastrozole and leuprorelin for 24 months is safe and effective in improving the predicted adult height (PAH) in girls with early puberty and compromised growth prediction by +1.21 standard deviation score (SDS; +7.51 cm) compared to inhibition of puberty alone, +0.31 SDS (+1.92 cm).

Objectives and hypotheses: In the second phase of the GAIL study, we assessed the adult height (AH)/near-adult height (NAH) at the end of the first phase and, in addition, the efficacy of anastrozole monotherapy thereafter in further improving NAH.

Methods: We measured the AH (age 16.5 years)/NAH [bone age (BA), 15 years] of the 40 girls included, divided into two matched groups: group A (20 girls on anastrozole + leuprorelin) and group B (20 girls on leuprorelin alone). Group A was further randomized into two subgroups: A1 and A2. Group A1 (n = 10), after completion of the combined therapy, received anastrozole 1 mg/day as monotherapy until BA 14 years, with a 6-month follow-up. Group A2 (n = 10) and group B (n = 20), who received only the combined treatment and leuprorelin alone, respectively, were recalled for evaluation of AH/NAH.

Results: AH or NAH exceeded the PAH at the completion of the 2-year initial phase of the GAIL study in all groups, but the results were statistically significant only in group A1: NAH−PAH group A1, +3.85 cm (+0.62 SDS, p = 0.01); group A2, +1.6 cm (+0.26 SDS, p = 0.26); and group B, +1.7 cm (+0.3 SDS, p = 0.08). The gain in group

A1 was significantly greater than that in group A2 (p = 0.04) and in group B (p = 0.03). Anastrozole was determined to be safe even as monotherapy in Group A1.

Conclusions: In early-maturing girls with compromised growth potential, the combined treatment with leuprorelin and anastrozole for 2 years or until the age of 11 years resulted in a total gain in height of +9.7 cm when continuing anastrozole monotherapy until the attainment of NAH, as opposed to +7.4 cm if they do not continue with the anastrozole monotherapy and +3.6 cm when treated with leuprorelin alone. Thus, the combined intervention ends at the shortest distance from the target height if continued with anastrozole monotherapy until BA 14 years.

KEYWORDS

aromatase inhibitors, anastrozole, early puberty, precocious puberty, adult height, LHRH analogue, girls, GAIL study

1 Introduction

The GAIL study ("Girls treated with an Aromatase Inhibitor and Leuprorelin," ISRCTN11469487) was a prospective phase IIa study assessing 40 girls consecutively referred for early puberty (onset, 7.5–9 years) with a predicted adult height (PAH) less than −2 or >1.5 SD lower than their target height (TH) (1). All these girls had initiated central puberty, but very few of them might have had precocious puberty, according to the age limit of 7.5 years, as defined by Greek data to distinguish precocious from early puberty (2). In the first phase of the GAIL study, 20 girls were treated with leuprorelin 11.25 mg depot injection (3) plus anastrozole, and 20 girls were treated with leuprorelin alone for 2 years or until the age of 11 years, as further continuation of pubertal inhibition after the age of 11 years might have resulted in a loss rather than a further gain as far as PAH is concerned, which is in accordance with Carel et al. (4). The two groups did not differ in age, height, body mass index (BMI), bone age advancement (BAA), TH, or distance of PAH from TH. Their bone age (BA) was inappropriately advanced compared to their TH percentile, which was higher than the percentile on which they were growing, as these girls did not follow the pattern of constitutional advancement of growth and puberty (CAGP) (5), which is the major determinant of precocious or early puberty. The first phase of the study clearly showed that the combination of anastrozole and leuprorelin for up to 24 months and until the age of 11 years was safe and effective in ameliorating PAH in girls with early puberty and compromised growth by +1.21 standard deviation score (SDS; +7.51 cm) compared to inhibition of puberty alone, +0.31 SDS (+1.92 cm). Although these results were straightforward, they dealt only with PAH, and the real impact of this strategy on adult height (AH) per se or at least near-adult height (NAH) remains open.

Thus, in the second phase of the GAIL study, we studied whether the gain attained in PAH was indeed preserved after cessation of the combined treatment and translated into a real increase in AH/NAH and whether the continuation of anastrozole monotherapy until BA 14 years resulted in a further improvement in AH, or at least NAH. We also evaluated the safety of anastrozole as monotherapy in these girls.

2 Materials and methods

At the end of the combined treatment with leuprorelin and anastrozole, the 20 girls in Group A of the GAIL study (1) were further randomized into two subgroups using their electronic health record numbers only (6). A total of 10 girls forming subgroup A1 continued anastrozole as monotherapy until BA 14 years, while the 10 girls forming subgroup A2 did not receive any therapy. Assignment to either group was presented as our medical decision as this was an open-label trial. The two subgroups did not differ in median age, BMI, TH, and PAH ($p < 0.05$), as shown in Table 1 (1).

Treatment with anastrozole tablets was at the dose of 1 mg (p.o.) once daily (Arimidex®). Patients in subgroup A1 were followed at 6-month intervals. The patients and their parents were advised to report any sign of hyperandrogenism (e.g., acne, hirsutism, or hair loss) and incidents of peculiar feelings or behavior. Medication was electronically prescribed as an off-label treatment, with the costs covered by the patients' social security at 75%, which ensured treatment compliance.

In the follow-up visits, a complete physical examination with accurate height measurements, pubertal Tanner staging, a BA X-ray, a pelvic ultrasound by a pediatric radiologist, and biochemical testing (at 0800 hours and after an overnight fast) were obtained. Dual-energy X-ray absorptiometry (DEXA) and anterior–posterior/lateral X-rays of the lumbar spine were performed annually. All the

TABLE 1 Characteristics of the girls in subgroup A1 (who continued anastrozole as monotherapy until bone age 14 years) and subgroup A2 (who did not receive any therapy after completion of the first phase of the GAIL study) at the inclusion of the second phase of the GAIL study.

	Age (years)	BMI (SDS)	TH (cm)	PAH (cm)
Group A1 (n = 10)	11.0	1.18	160.98	152.36
Group A2 (n = 10)	10.7	1.16	161.33	153.92
p-value	*0.23*	*0.12*	*0.31*	*0.31*

For age, the values shown are medians.
BMI, body mass index; SDS, standard deviation score; TH, target height; PAH, predicted adult height.

methodology and statistical analyses of the GAIL study were followed as previously presented and published in detail (1).

NAH was defined as the height at BA 15 years (7) and adult (final) height as the age or BA at 16.5 years (8).

All procedures were in accordance with the ethical standards and with the approval of the institutional research committees as described in the BMC ISRCTN registry (ISRCTN11469487, https://doi.org/10.1186/ISRCTN11469487), as the second phase of the GAIL study with anastrozole monotherapy was included in the original GAIL study design. Additional scientific and ethical approval (No. 6762) was also obtained from the relevant committee of the University General Hospital of Larisa, Greece, for the second phase of the GAIL study. Informed consent was obtained from the parents of all individual participants.

3 Results

The results on the median AH/NAH (in centimeters) and the distance from TH (NAH–TH, in centimeters) at the end of the GAIL study compared to the first phase inclusion and its end are shown in Table 2. In early-maturing girls with compromised growth, initial treatment for 2 years or until the age of 11 years with leuprorelin 11.25 mg/12 weeks + anastrozole 1 mg/day resulted in a gain of +9.7 cm in total when treated with anastrozole monotherapy until BA 14 years, which was +2.3 cm more than the gain of +7.4 cm if they did not continue with anastrozole monotherapy and +6.1 cm more than those treated with a luteinizing hormone–releasing hormone analog (LHRHa) alone, who gained only +3.6 cm. The combined therapy continued with anastrozole monotherapy, which ended in the shortest distance of NAH from TH, i.e., −4.7 cm (from −14.48 at inclusion) compared to −5.7 cm (from −13.48 at inclusion) in those with LHRHa + anastrozole alone and −8.7 cm (from −12.82 at inclusion) in girls treated with LHRHa alone. AH or NAH exceeded that of the PAH at the completion of the first phase of the GAIL study in all three groups, but the results were statistically significant only for group A1: NAH–PAH group A1, +3.85 cm (+0.62 SDS, p = 0.01); group A2, +1.6 cm (+0.26 SDS, p = 0.26); group B, +1.7 cm (+0.3 SDS, p = 0.08). The extra gain in height of group A1 was significantly higher than that of group A2 (p = 0.04) and of group B (p = 0.03). It has to be noted that while there was a significant difference between the A1 and A2 groups at the end of the first phase when their PAH had a greater distance from the NAH, these groups finally had better

TABLE 2 Results on the median adult height/near-adult height (NAH) and the distance from target height (NAH–TH) at the end of the GAIL study compared to the initial (first phase inclusion and its end).

Group	PAH at initial (first phase inclusion)	PAH (end of the first phase)	NAH (cm)	NAH–PAH ((end of the first phase)	NAH–PAH at initial (first phase inclusion)	TH–NAH (cm)
A1	146.5	152.36	156.21	3.85	9.7	4.7
p-value		*0.01*		*0.001*		
A2	148.1	153.92	155.58	1.66	7.4	5.7
p-value		*0.26*		*0.006*		
p-value (A1 *vs.* A2)	*0.11*	*0.12*		*0.04*	*0.03*	
B	151.08	153.0	154.7	1.7	3.6	8.7
p-value		*0.08*		*0.004*		
p-value (A1 *vs.* B)				*0.03*	*0.002*	*0.01*
p-value (A2 *vs.* B)				*0.47*	*0.020*	*0.02*

Group A1: 10 girls that continued anastrozole as monotherapy until BA 14 years.
Group A2: 10 girls that did not receive any therapy after completion of the first phase of the GAIL study.
Group B: 20 girls on leuprorelin alone at the first phase of the GAIL study.

growth and reached a shorter distance from their TH compared to the A2 group (3.85 *vs.* 1.66 cm, $p = 0.04$), indicating that anastrozole monotherapy could have been even more effective than it appears when comparing only the AH/NAH.

The evolution of the PAH, BAA, BMI, and height velocity (HV) of the subjects in subgroup A1 is presented in Table 3. The PAH was significantly higher in the girls in subgroup A1 at 24 months (155.9 cm, $p = 0.04$) and 30 months (156.34 cm, $p = 0.03$) of treatment compared to the PAH of 152.36 cm at the beginning of this second phase of the GAIL study. This was achieved due to the reduction in the advancement rate of BA, practically extending the growth period in combination with the increase in the girls' height velocity SDS (statistically significant at 12, 18, 24, and 30 months). Anastrozole monotherapy until BA 14 years further improved the AH/NAH by +3.85 cm (+0.62 SDS, $p = 0.001$). The gain in BAA that was achieved with the combined therapy in the initial phase of the GAIL study was preserved until completion of growth, with the BA even lagging behind the chronological age in the first 6 months with anastrozole monotherapy (Figure 1). Thus, the greatest effect on BA advancement appears to be that of the combined LHRHa + anastrozole treatment. The HV presented a gradual increase in each 6-month follow-up visit, becoming statistically significant at the third visit at 12 months (Table 3). This was due to the initiation of a pubertal growth spurt after the cessation of pubertal inhibition. The BMI SDS remained unchanged (Table 3).

None of the girls presented clinical signs of hyperandrogenism (e.g., acne, hirsutism, or hair loss). The testosterone concentrations are shown in Table 4. Testosterone rose slightly above 0.5 ng/ml in three girls, but none developed clinical hyperandrogenism. One girl presented with ovarian stromal hyperplasia, with an ovarian volume slightly above 10 ml in pelvic ultrasound compatible with a polycystic ovary syndrome image, but without any cycle disturbances or any biochemical or clinical signs of hyperandrogenism. Overall, the hematocrit, lipid, and biochemical profiles did not change significantly during treatment. None reported any adverse events, nor were there any signs of emotional instability reported. The DEXA scans showed normal median (range) bone mineral density (BMD) *z*-scores for BA without significant inter-patient changes: −0.4 (−0.5 to 1.0) at inclusion, −0.4 (−0.5 to 1) at 1 year, and −0.3 (−0.4 to 1.1) at 2 years on anastrozole monotherapy. The time to menarche data after discontinuation of leuprorelin were compared between the three subgroups and were found to be practically identical [(median (range), years]: group A1 at 12.3 (11.65–12.8), group A2 at 12.3 (11.2–12.75), and group B at 11.9 (11.3–13).

4 Discussion

In early-maturing girls with compromised growth, continuation of anastrozole monotherapy until BA 14 years after an initial combined treatment with leuprorelin and anastrozole for 2 years or until the age of 11 years not only preserved the initial gain in PAH but also further improved it, with a statistically significant further gain of +3.85 cm, corresponding to +2.3 cm more in NAH than in those who completed only the initial combined treatment. Thus, the aromatase inhibitor (AI) anastrozole appears to be an effective and safe treatment even as monotherapy in ameliorating NAH in girls with accelerated BA and a compromised growth prediction. The fact that the initial combined treatment with leuprorelin and anastrozole resulted in a total height gain of +9.7 cm when continuing anastrozole monotherapy until attainment of NAH, compared to +7.4 cm if the combined treatment is stopped and only +3.6 cm when treated with leuprorelin alone, clearly shows that the combined intervention continued with anastrozole monotherapy ends at the shortest distance from TH. These girls reached and probably exceeded (as NAH leaves a margin for an additional 2% until AH is attained) the total pubertal gain of around 27 cm expected in normal Greek girls (9). Thus, the addition of anastrozole to an LHRHa, apart from being safe, is effective in substantially ameliorating the NAH of girls with early puberty and compromised growth potential, making the intervention meaningful.

The GAIL study has several limitations, which have been thoroughly discussed (1), the most significant being the study design, as this is not a randomized double-blind placebo-controlled trial. Another extremely important one is the limited number of patients included, especially in the second phase. However, the study design simulated randomization in its first phase as close as possible to the real-world setting in practicing clinical pediatric endocrinology, and a control group was included. In its second phase with anastrozole monotherapy, randomization was absolute, but based on electronic health records only. To our

TABLE 3 Evolution of the median predicted adult height (PAH), bone age advancement (BAA; delta bone age/chronological age), body mass index [BMI, standard deviation score (SDS)], and height velocity (HV, SDS) of subgroup A1 on anastrozole monotherapy.

	Inclusion	6 months	12 months	18 months	24 months	30 months
PAH (cm)	152.36	154.00	155.17	155.4	155.9	156.34
p-value		*0.18*	*0.059*	*0.06*	*0.04*	*0.03*
BAA (years)	0.14	−0.24	−0.21	−0.22	−0.23	−0.29
p-value		*0.12*	*0.16*	*0.12*	*0.16*	*0.11*
BMI	1.14	1.03	0.90	0.96	0.91	0.97
p-value		*0.35*	*0.20*	*0.29*	*0.23*	*0.31*
HV	−3.44	−3.39	−0.73	−0.42	0.94	2.58
p-value		*0.47*	*0.002*	*<0.001*	*<0.001*	*<0.001*

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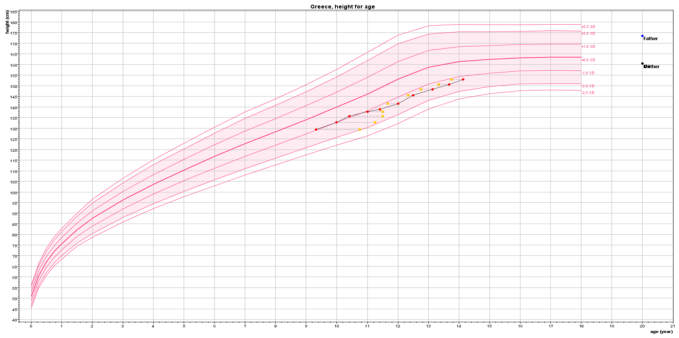

FIGURE 1
Virtual growth curve of the girls included in the GAIL study based on the median age, height, and bone age of the girls treated with leuprorelin + anastrozole until the age of 11 years and anastrozole monotherapy thereafter until bone age 14 years.

knowledge, this is the first study to show data on AH or NAH in early-maturing girls with a compromised growth potential who were treated with an initial combination of an LHRHa and an AI for 2 years or until the age of 11 years, and then with an AI alone as monotherapy or with no further therapy until practical completion of growth, i.e., BA 14 years, compared to a control group treated alone with an LHRHa for 2 years or up to the age of 11 years.

The frequency of visits to pediatric endocrinology outpatient clinics for precocious puberty is rapidly increasing (10), particularly for girls (2). The recognition of the pattern of CAGP by general physicians following children and pediatricians apart from specialists in pediatric endocrinology is therefore of fundamental importance (11). This pattern, which is the mirror image of the constitutional delay of growth and puberty (CDGP) (12, 13), is the major determinant of borderline precocious or early puberty in girls (14) and is associated with early onset of adiposity rebound and obesity (11, 15), which can lead to premature adrenarche (16), which is linked to early thelarche and menarche (17). The improvement in socioeconomic conditions that took place in the second half of the 20th century resulted in an earlier onset of puberty in children (18), with a decrease in the age at menarche, leveling off, however, at least in developed countries (9).

Thus, a significant proportion of children presenting with concerns about early pubertal development represent physiological variations that do not require treatment (19). The major concern, as in the girls treated in the GAIL study, is the compromised AH prediction due to advanced skeletal maturation. This is why therapeutic interventions must be timely and individualized, and ideally, gonadotropin-releasing hormone analogs (GnRHa) must be started before the initiation of the growth spurt (20). Most studies agree that inhibition of puberty is useful and effective in progressive precocious puberty (21). However, the subset of precocious, slowly progressive puberty probably corresponds to the pattern of CAGP, which is a normal variation of growth and pubertal maturation and does not require pubertal inhibition (22–24). Similarly, early puberty starting at 7.5–8.5 years with an initial normal height prediction does not require pubertal inhibition (25). Girls with advanced progressive puberty starting between 8 and 9 years of age (26) and advanced-normal puberty with onset between 8.5 and 10 years (27) with an initial height prediction 3–5 cm lower than their TH (practically up to −1 SD) have been found to have no benefit in final height from pubertal inhibition, practically reaching their TH even without treatment, showing that gonadotropin-suppressive therapy in the above groups affects the pace of puberty but not the total pubertal growth or final height. However, in selected girls with rapidly progressive borderline early puberty starting between 7 and 10 years, treatment with GnRHa may be considered (28). If untreated, these girls were found to lose 3.6 cm compared to normal controls, which was exactly the real gain found in group B in the GAIL study, i.e., in the girls treated with leuprorelin alone.

However, a subset of girls—and boys probably so—with constitutional–idiopathic short stature and normal early pubertal development (29, 30) growing with a normal HV at or even below their projected TH curve, initially implying a possible pattern of CDGP, ultimately begin their pubertal maturation either at a particularly low height and/or with a markedly advanced or at least not delayed BA without presenting an early-onset growth spurt. These children, if untreated, reach AHs considerably lower than their THs and at the lower end or below normal for the population. This is exactly the subset of children included in the GAIL study.

Third-generation AIs have been used to increase the PAH in boys (31–34), in girls in the context of McCune–Albright syndrome (35), and, recently, even in girls with congenital adrenal hyperplasia (CAH) (36). The concept of using AIs in girls lies in the fact that

TABLE 4 Evolution (average ± SD) of the testosterone concentrations in Group A1.

	Inclusion	6 months	12 months	18 months	24 months	30 months
Testosterone (ng/ml)	0.23 ± 0.14	0.3 ± 0.17	0.38 ± 0.20	0.33 ± 0.14	0.37 ± 0.19	0.32 ± 0.04
p-value		*0.18*	*0.06*	*0.08*	*0.06*	*0.15*

peripheral aromatization of mainly the adrenal but also ovarian androgens is the main mechanism of BAA (37), with extragonadal estrogen biosynthesis, particularly in the bone, deploying a "paracrine" or "intracrine" action (38). An increase in AH can be attained in growing adolescents by inhibiting estrogen action, providing a rationale for studies aimed at delaying the maturation of growth plates and increasing AH when the growth potential is compromised (39). The use of selective inhibitors of the aromatase enzyme with AIs (also in combination with pubertal inhibition and growth hormone) represent therapeutic choices that have been studied as strategies to maximize pubertal growth in children with compromised growth (33). Anastrozole appears to be more effective in slowing epiphyseal maturation and in increasing PAH, even if it is less potent than letrozole (40). Furthermore, previous concerns about the skeletal safety of AIs are gradually subsiding (41), and they have been increasingly used for functional (42) or obesity-induced hypogonadism (43, 44) in men, with anastrozole being the drug of choice for the treatment of infertility in men when using AIs (45), as well as for improving fertility by inducing ovulation in women with polycystic ovary syndrome (PCOS) (46).

Back in 2011, it was stated that "the use of aromatase inhibitors to promote growth in girls should be pursued only in the context of a clinical trial" (34). In 2016, the first phase of the GAIL study showed positive results on the PAH (1); now, after a preliminary report of the second phase in 2020 (47), the results on AH/NAH are finally available. The use of off-label medications in children, however, remains a common practice for pediatric providers (48), and according to the American Academy of Pediatrics policy statement on the use of off-label medications in children (49), "Off-label is the use of a drug that is not included in the package insert (FDA-approved labelling) [and] does not imply improper, illegal contraindicated or investigational use. Off-label use does not necessarily require prescribers to obtain informed consent if the decision to use the medication is supported by scientific or even anecdotal evidence and is not investigational in nature. The purpose of off-label use is to benefit the individual patient and practitioners use their professional judgment to determine these uses. Therapeutic decision-making must always rely on the best available evidence and the importance of the benefit for the individual patient." While AI therapy may be associated with a positive height outcome, clinicians need to be cautious when counseling families about the potential height outcome with and without intervention, as the difference might be completely unpredictable (50). However, it is important that the use of growth-promoting therapies, including AIs, be found psychosocially beneficial in adolescents with idiopathic short stature (51). The results of the second phase of the GAIL study also imply the possible use of AIs in the treatment of short stature in girls, which is an established off-label treatment in the clinical setting for boys (52).

5 Conclusion

While most of the early-maturing girls present a normal variation of CAGP, which does not require treatment, there is a subset who, nonetheless, are not taller or—even worse—are shorter than their projected TH curve with a particularly advanced BA and a severely compromised growth prediction. In these girls, with treatment for 2 years or until the age of 11 years with leuprorelin 11.25 mg/12 weeks combined with anastrozole 1 mg/day (p.o.), the gain in NAH is +9.7 cm in total if treatment is continued with anastrozole monotherapy until BA 14 years, ending in the shortest distance and within the normal range of TH −4.7 cm, compared to +7.4 cm if they do not continue with anastrozole monotherapy (−5.7 cm from TH) and only +3.6 cm (−8.7 cm from TH) when treated with leuprorelin alone. The above findings indicate that while there is some gain with classical pubertal inhibition using the standard approach with LHRHa, an initial combination therapy until 11 years of age, with the AI anastrozole continued as monotherapy until BA 14 years, ends in the shortest distance and within the normal range from TH. This approach is not only safe but also appears particularly effective in substantially ameliorating AH/NAH, making the decision to intervene in the pubertal maturation of these girls incisively meaningful.

Author's note

This work is part of the PhD Thesis of E.D.

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 Athens Medical Center Ethics Committee and General University Hospital of Larisa Scientific Committee. The studies were conducted in accordance with local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

DP: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. ED: Data curation, Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing. PC: Investigation, Methodology, Resources, Writing – review & editing, Writing – original draft. SL: Formal analysis, Writing – review & editing, Writing – original draft. IG: Supervision, Validation, Visualization, Writing – review & editing, Writing – original draft. GM: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing – review & editing, Writing – original draft.

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Effect of medroxyprogesterone acetate dose in progestin-primed ovarian stimulation on pregnancy outcomes in poor ovarian response patients with different body mass index levels

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Background: For the poor ovarian response (POR) population, the relationship between medroxyprogesterone acetate (MPA) dose in progestin-primed ovarian stimulation (PPOS) and clinical outcome is still unclear. This study aims to explore the effect of MPA dose in PPOS on clinical outcomes in POSEIDON group 3 and 4 patients with different body mass index (BMI) levels, hoping to provide clinical doctors with better options for controlled ovarian hyperstimulation (COH) programs.

Methods: This is a retrospective analysis of 253 oocyte retrieval cycles of POSEIDON group 3 and 4 patients who underwent PPOS protocol in IVF/ICSI treatment at the Reproductive Medical Center of Renmin Hospital of Wuhan University from March 2019 to April 2022. The effects of different MPA doses (8 mg/d or 10 mg/d) on pregnancy outcomes were compared in normal BMI (18.5–24 kg/m^2) and high BMI (≥24 kg/m^2) patients, and multivariate logistic regression analysis was performed to analyze the factors affecting pregnancy outcomes.

Results: For normal BMI patients, the 8-mg/d MPA group had a higher embryo implantation rate (33.78% vs. 18.97%, P = 0.012). For high BMI patients, the 10-mg/d MPA group had a higher HCG positive rate (55.00% vs. 25.00%, P = 0.028), clinical pregnancy rate (50.00% vs. 20.00%, P = 0.025), and cumulative pregnancy rate (37.74% vs. 13.79%, P = 0.023) compared with the 8-mg/d MPA group. There was no significant difference in cumulative live birth rate between the 8-mg/d and 10-mg/d MPA groups in patients with normal or high BMI. The results of multivariate logistic regression showed a significant correlation between MPA dose and cumulative pregnancy in the high BMI population (OR = 0.199, 95% CI: 0.046~0.861, P = 0.031).

Conclusions: For POR patients with high BMI, 10 mg/d of MPA in the PPOS protocol had a higher cumulative pregnancy rate than 8 mg/d of MPA, but it had no significant effect on the cumulative live birth rate.

KEYWORDS

progestin-primed ovarian stimulation, medroxyprogesterone acetate, POSEIDON criteria, body mass index, pregnancy outcome

1 Introduction

With the increased number of late marriage and childbearing couples, poor ovarian response (POR) patients receiving assisted reproductive technology (ART) fertility treatment have also increased. POR refers to a pathological state in which the ovaries respond poorly to exogenous gonadotropin stimulation in patients receiving ART, which is characterized by an increased cycle cancellation rate and decreased number of oocytes and clinical pregnancy rate (1). It has been reported that more than 30% of women can be diagnosed with POR during controlled ovarian hyperstimulation (COH) (2). Therefore, the diagnosis and treatment of POR patients need to be given more attention.

Currently, many ovarian stimulation regimens have been proposed to improve the prognosis of POR patients. For example, progestin-primed ovarian stimulation (PPOS) proposed in recent years can effectively suppress the premature luteinizing hormone (LH) surge and increase the number of oocytes retrieved by using the exogenous progesterone medroxyprogesterone acetate (MPA) and has gradually been widely used in POR patients (3–6). The commonly used dose of MPA in the PPOS protocol is 10 mg/d, and the lowest dose that can suppress the premature LH surge is 4 mg/d (7). Previous studies have found that in a population with normal ovarian reserve, the dose of MPA is related to clinical outcome, and the use of higher doses of MPA in patients with high body mass index (BMI) is beneficial for achieving higher embryo implantation rates, clinical pregnancy rates, and live birth rates. However, a high dose of MPA may have deep pituitary suppression, thus increasing the total dose and duration of gonadotropin (8, 9). Whether MPA has a dose-dependent effect on IVF/ICSI outcomes and is influenced by BMI has attracted the attention of researchers. However, there is no literature on the relationship between MPA dose and IVF/ICSI pregnancy outcomes in POR patients with different BMI levels according to the POSEIDON criteria. Therefore, we conducted a retrospective study to investigate the effect of different MPA doses (8 mg/d and 10 mg/d) in the PPOS protocol on the clinical outcomes of POSEIDON group 3 and 4 patients with different BMI levels and whether using smaller doses of MPA (8 mg/d) is more beneficial for clinical outcomes.

2 Materials and methods

2.1 Study design and patients

This was a retrospective study of POR patients who underwent PPOS protocol in IVF/ICSI treatment at the Reproductive Medical Center of Renmin Hospital of Wuhan University from March 2019 to April 2022. This study was approved by the Ethics Committee of Renmin Hospital of Wuhan University (WDRY2018-K009) and accorded with the basic principles of the Helsinki Declaration.

In this study, we included POSEIDON group 3 and 4 patients. According to the POSEIDON criteria (10), POSEIDON group 3 is defined as female age <35 years, antral follicle count (AFC) <5, and/or anti-Müllerian hormone (AMH) <1.2 ng/ml, and POSEIDON group 4 is defined as female age ≥35 years, AFC <5, and/or AMH <1.2 ng/ml. The exclusion criteria included patients >45 years (11), uterine malformation, polycystic ovary syndrome, endometrial disease, severe endometriosis, chromosomal abnormality, recurrent miscarriage, and endocrine diseases (thyroid dysfunction, hyperprolactinemia, diabetes). The cycle of mixed transfer of embryos obtained from other cycles is also excluded.

A total of 253 oocyte retrieval cycles were included according to the above inclusion and exclusion criteria. According to the China BMI classification (12, 13), patients were divided into two categories: normal BMI (18.5–24 kg/m^2) and high BMI (≥24 kg/m^2). The two categories of patients were further divided into the 8-mg/d MPA group and the 10-mg/d MPA group.

2.2 Clinical setting

All patients received the PPOS protocol. Based on previously published articles (5), patients were orally administered 8 mg/d or 10 mg/d of MPA (Beijing ZhongXin Pharmaceutical, China) and injected with 150–300 IU/d of human menopausal gonadotrophin (hMG, Livzon, China) or recombinant human follitropin (Gonal-F, Merck, Switzerland) from menstrual cycle day 2 or 3 until the trigger day. Gonadotropin dose was adjusted every 2 to 3 days after assessing follicle development based on transvaginal ultrasound and blood hormone levels. Final follicle maturation was triggered with 10,000 IU of human chorionic gonadotropin (hCG, Livzon, China) when at least one follicle reached the diameter of 18 mm or two follicles reached the diameter of 17 mm. After 34 to 36 h, the oocytes with diameter ≥12 mm were retrieved under the guidance of transvaginal ultrasound. The oocytes were fertilized by conventional IVF/ICSI according to semen parameters.

Fertilization was assessed 16 h to 18 h after oocyte retrieval. Seventy-two hours after oocyte retrieval, the cleavage status and fragmentation ratio of embryos were observed. Cleavage embryos were scored and graded as follows: grade I (uniform blastomere size, no fragmentation), grade II (uniform blastomere size, fragmentation <20%), grade III (uneven blastomere size, fragmentation <50%), and grade III (uneven blastomere size, fragmentation >50%). High-quality embryos were defined as blastomeres with six to eight cells and fragmentation <20%; transferable embryos were defined as blastomeres with six or more cells and embryo grade III or higher. Blastocyst culture was performed according to embryo quality and the patient's wishes. The blastocyst was graded according to the Gardner scoring standard (14). Due to the effect of high progesterone levels on endometrial receptivity, all embryos obtained were frozen.

2.3 Frozen-thawed embryo transfer and follow-up

Frozen embryo transfer (FET) was performed 2 months after oocyte retrieval. Patients with regular menstrual cycles underwent natural cycles, and one to two cleavage embryos or blastocysts were transferred 3 or 5 days after ovulation. Patients with irregular menstruation underwent hormone replacement therapy (HRT);

3–6 mg/d of estradiol valerate tablets (Progynova, Bayer, Germany) were administered from day 3 of the menstrual cycle, and when the endometrial thickness ≥8 mm, 40 mg/d of progesterone and 30 mg/d of dydrogesterone were administered orally. For patients using gonadotropin-releasing hormone agonist (GnRH-a) downregulation combined with hormone replacement therapy (GnRH-a + HRT), 3.75 mg of GnRH-a (leuprorelin acetate, China) was given subcutaneously during menstruation, and the HRT was started 30 days later to prepare the endometrium. Luteal support was maintained until 10–12 days after transplantation. Serum β-HCG was detected 10–12 days after transplantation, and β-HCG >10 IU/L was defined as HCG positive. Clinical pregnancy was defined as gestational sac observed by vaginal ultrasonography 30 days after transplantation; pregnancy loss that occurred before 12 weeks was defined as early miscarriage; live birth after 28 weeks of gestation was defined as live birth. The deadline for follow-up of the included patients was August 2023.

2.4 Outcome measures and definition

The primary outcomes included cumulative pregnancy rate and cumulative live birth rate (CLBR) per oocyte retrieval cycle. The secondary outcomes included the number of oocytes retrieved, the number of mature oocytes, mature oocyte rate, 2PN fertilization rate and cleavage rate, high-quality embryo rate, the number of transferable embryos, cycle cancellation rate, HCG positive rate, embryo implantation rate, clinical pregnancy rate, and early miscarriage rate.

When there were no oocytes retrieved or no transferable embryos, the cycle was canceled. Cumulative pregnancy rate per oocyte retrieval cycle was defined as the ratio of the number of clinical pregnancy after all embryos have been transferred to the total number of oocyte retrieval cycles. CLBR was defined as the ratio of the number of live births after all embryos have been transferred to the total number of oocyte retrieval cycles (15).

2.5 Statistical analysis

SPSS 26.0 (IBM Corp., USA) was used for analysis. Measurement data conforming to a normal distribution were expressed as mean ± SD, and the independent sample *t*-test was used to compare variables between groups. Enumeration data were expressed as frequency (%). Categorical variables were compared using the chi-square test or Fisher's precision probability test. Multivariate logistic regression was used to analyze the relationship between various factors and pregnancy outcome, and the odds ratio (OR) with 95% confidence intervals (CI) was calculated. Statistical significance was defined as *P*-value <0.05.

3 Results

The research flowchart is shown in Figure 1. A total of 253 oocyte retrieval cycles were included in this study, with 171 oocyte retrieval cycles for normal BMI patients (18.5–24 kg/m^2) and 82 oocyte retrieval cycles for high BMI patients (BMI ≥24 kg/m^2). The baseline characteristics of the different MPA dose groups in normal BMI and high BMI patients are shown in Table 1 and indicate that there were no significant differences in these indicators.

The comparison of laboratory parameters and pregnancy outcomes of the different MPA dose groups in normal BMI and high BMI patients is shown in Table 2. For normal BMI patients, the embryo implantation (33.78% vs. 18.97%, $P = 0.012$) was higher in the 8-mg/d MPA group than in the 10-mg/d MPA group. For high BMI patients, the HCG positive rate (55.00% vs. 25.00%, $P = 0.028$), clinical pregnancy rate (50.00% vs. 20.00%, $P = 0.025$), and cumulative pregnancy rate (37.74% vs. 13.79%, $P = 0.023$) were higher in the 10-mg/d MPA group than in the 8-mg/d MPA group. There were no statistically significant differences in the other indexes between the 8-mg/d MPA group and the 10-mg/d MPA group in both normal BMI and high BMI patients.

Multivariate logistic regression was used to analyze the effects of MPA dose, age, BMI, AFC, and the number of oocytes retrieved and mature oocytes on cumulative pregnancy and cumulative live birth. As shown by the results in Table 3, AFC and the number of mature oocytes were positively correlated with cumulative pregnancy (OR = 1.166, 95% CI: 1.010~1.347, $P = 0.036$; OR = 1.565, 95% CI: 1.066~2.300, $P = 0.022$) and cumulative live birth (OR = 1.169, 95% CI: 1.006~1.359, $P = 0.041$; OR = 1.500, 95% CI: 1.014~2.219, $P = 0.042$) in normal BMI patients, but there was no significant correlation between MPA dose and pregnancy outcome. In patients with high BMI, the dose of MPA was significantly correlated with cumulative pregnancy (OR = 0.199, 95% CI: 0.046~0.861, $P = 0.031$) but had no significant effect on cumulative live birth.

4 Discussion

The growth and development of follicles is accompanied by an increase in estradiol concentration, and estradiol induces LH surge through positive feedback to promote follicle maturation and ovulation (16). Early LH surge is the main cause of unexpected ovulation and cycle cancellation during COH, which is usually inhibited by GnRH agonists and GnRH antagonists (17). The PPOS protocol was first proposed in 2015 and was proven more effective in inhibiting premature LH surge by using exogenous progesterone than the GnRH antagonist protocol (18, 19). The theoretical basis of the PPOS protocol is that exogenous progesterone inhibits the appearance of LH surge by blocking the positive feedback of estradiol (16). In our study, patients in the different MPA dose groups did not have an early LH surge, which also suggested the effectiveness of the PPOS protocol in suppressing LH surge. However, due to the effect of progestogen on endometrial receptivity, all the embryos obtained need to be frozen (20). With the development of embryo freezing and warming technology, FET has been widely utilized. Consequently, the clinical application of the PPOS protocol has been further improved. It has been confirmed in subsequent studies that the PPOS protocol can improve pregnancy outcomes of POR patients (21–23). In addition, the PPOS protocol has the advantages of low cost, oral

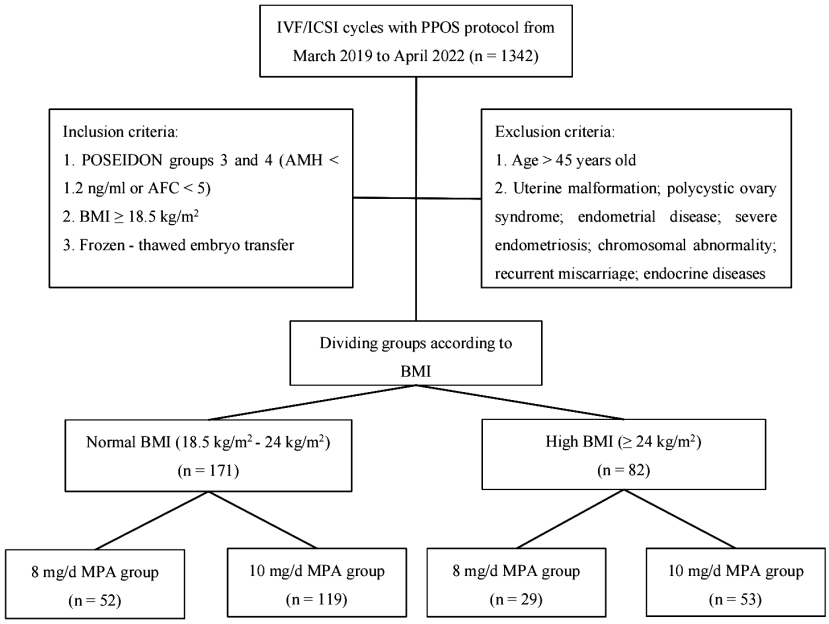

FIGURE 1
Flowchart of this study.

administration, and easy availability, making it a more economical and convenient option for POR patients.

At present, the pathogenesis of POR has not been fully elucidated, and it is difficult to formulate clinical management strategies. The 2011 Bologna criteria were the first criteria to standardize the definition of POR patients (1). However, the Bologna criteria lack age-stratified management, cannot sufficiently address the problem of population heterogeneity, and have limited predictive power for pregnancy outcomes (24). To better manage POR patients, the 2016 POSEIDON criteria classified POR patients into POSEIDON groups 1 and 2 due to abnormal ovarian response to exogenous gonadotropin and POSEIDON groups 3 and 4 due to decreased ovarian reserve based on age and the ovarian reserve parameters AFC and AMH, which improved the homogeneity and comparability of clinical studies and was conducive to providing more accurate assisted pregnancy strategies for POR patients (10, 25). Female age, BMI, AFC, and COH protocols were independent predictors of live birth in POR patients according to the POSEIDON criteria (26, 27). The previous retrospective analysis on the impact of BMI on the pregnancy outcome of FET showed that low BMI had no significant impact on the live birth rate, while obesity was closely associated with decreased clinical pregnancy rate and live birth rate (28, 29).

Our study first evaluated the effect of different MPA doses on clinical outcomes of POSEIDON group 3 and 4 patients with different BMI levels and found that the higher dose of MPA (10 mg/d) was associated with higher cumulative pregnancy rates in high BMI patients. Our results show that the 8-mg/d MPA group has a higher embryo implantation rate than the 10-mg/d MPA group in normal BMI patients, while the 10-mg/d MPA group has a higher HCG positive rate, clinical pregnancy rate, and cumulative pregnancy rate than the 8-mg/d MPA group in high BMI patients. The CLBR had a similar trend, but the difference was not statistically significant in normal BMI and high BMI patients, probably because of insufficient sample size. The results of multivariate logistic regression suggest a significant correlation between MPA dose and cumulative pregnancy in patients with high BMI. Our findings confirm that a sufficiently large dose of MPA (10 mg/d) is beneficial for cumulative pregnancy in patients with high BMI, although MPA (8 mg/d) is also enough to inhibit early LH peaks. Previous research showed that 10 mg/d of MPA and 4 mg/d of MPA had similar pregnancy and live birth outcomes in patients with normal ovarian reserve or normal BMI (18.5–25 kg/m^2) (7). However, using 10 mg/d of MPA in infertile women with high BMI (≥25 kg/m^2) resulted in higher embryo implantation rate, clinical pregnancy rate, and live birth rate rather than using 4 mg/d of MPA, possibly because the high MPA dose rescued physiological progesterone deficiency in patients with high BMI, which was partially consistent with our research results (8). Contrary to our expectations, there was no significant difference in the total dose and duration of gonadotropin between the 8-mg/d MPA group and the 10-mg/d MPA group, which was different from previous research results (7, 8). This difference may be due to the different populations included, different MPA doses, and BMI classification criteria in the studies.

Previous studies showed that obese patients often had poor IVF outcomes related to oocyte quality (8, 30). A single-center cohort study showed that embryo quality parameters in high BMI patients were not age-related but correlated with fatty acids in follicular fluid (31). A randomized controlled trial demonstrated that using MPA in the PPOS protocol can increase lipid levels in follicular fluid, promoting follicular development and oocyte maturation (32). This may be the reason why the clinical pregnancy rate of patients with high BMI in the 10-mg/d MPA group is better than that in the 8-mg/d MPA group. Interestingly, in our study, the mature oocyte rate and high-quality embryo rate in the 10-mg/d MPA group

TABLE 1 Baseline characteristics of the different MPA dose groups.

Variable	Normal BMI			High BMI		
	8-mg/d MPA group (*n* = 52 cycles)	10-mg/d MPA group (*n* = 119 cycles)	*P*-value	8-mg/d MPA group (*n* = 29 cycles)	10-mg/d MPA group (*n* = 53 cycles)	*P*-value
Age (years)	34.90 ± 5.23	34.62 ± 4.21	0.709	35.48 ± 4.72	34.32 ± 4.94	0.304
BMI (kg/m^2)	21.35 ± 1.64	21.27 ± 1.56	0.761	26.38 ± 1.30	25.95 ± 1.86	0.273
Duration of infertility (years)	4.27 ± 4.17	4.25 ± 3.12	0.976	5.60 ± 5.31	4.74 ± 4.13	0.419
Basal FSH (IU/L)	11.19 ± 3.99	10.78 ± 5.16	0.611	11.91 ± 5.74	10.10 ± 5.75	0.176
Basal LH (IU/L)	3.75 ± 1.43	3.41 ± 1.82	0.235	3.40 ± 1.96	3.24 ± 2.16	0.728
Basal estradiol (pg/ml)	53.03 ± 26.33	49.60 ± 24.80	0.417	40.42 ± 14.62	43.25 ± 22.89	0.550
AFC	6.17 ± 3.36	6.25 ± 2.74	0.872	5.86 ± 2.88	6.92 ± 3.38	0.156
AMH (ng/ml)	0.70 ± 0.35	0.67 ± 0.33	0.577	0.55 ± 0.29	0.60 ± 0.35	0.554
Gn total dose (IU)	2,489.33 ± 860.00	2,352.42 ± 738.00	0.291	2,535.86 ± 816.30	2,457.26 ± 979.05	0.714
Gn duration (days)	9.67 ± 2.33	9.32 ± 2.30	0.358	9.31 ± 2.29	9.47 ± 2.83	0.793
Trigger day						
Estradiol (pg/ml)	1,384.42 ± 811.90	1,410.55 ± 745.00	0.838	1,090.43 ± 736.75	1,020.75 ± 686.40	0.670
Progesterone (ng/ml)	0.64 ± 0.37	0.70 ± 0.60	0.490	0.56 ± 0.26	0.76 ± 1.09	0.327
LH (IU/L)	2.89 ± 3.67	2.29 ± 1.73	0.267	2.21 ± 1.56	1.84 ± 1.38	0.273

Dates were shown as mean ± SD or n (%).
FSH, follicle-stimulating hormone; LH, luteinizing hormone.

TABLE 2 Laboratory parameters and pregnancy outcomes of the different MPA dose groups.

Variable	Normal BMI			High BMI		
	8-mg/d MPA group (*n* = 52 cycles)	10-mg/d MPA group (*n* = 119 cycles)	*P*-value	8-mg/d MPA group (*n* = 29 cycles)	10-mg/d MPA group (*n* = 53 cycles)	*P*-value
No. of oocytes retrieved	4.31 ± 3.20	3.87 ± 2.44	0.334	2.93 ± 2.15	3.25 ± 2.79	0.601
No. of mature oocytes	3.73 ± 2.92	3.18 ± 2.25	0.185	2.59 ± 2.01	2.60 ± 2.26	0.972
Mature oocyte rate (%)	85.57 ± 23.65	80.41 ± 30.64	0.234	72.32 ± 37.17	81.20 ± 36.40	0.301
2PN fertilization rate (%)	58.84 ± 33.49	53.22 ± 37.30	0.352	50.82 ± 37.51	51.40 ± 39.33	0.949
2PN cleavage rate (%)	83.97 ± 36.45	75.99 ± 41.90	0.211	65.52 ± 48.37	69.18 ± 46.16	0.736
No. of high-quality embryos	1.50 ± 1.54	1.45 ± 1.53	0.857	1.07 ± 1.51	1.26 ± 1.33	0.547
High-quality embryo rate (%)	49.57 ± 44.34	51.08 ± 43.42	0.835	44.02 ± 46.04	50.89 ± 44.44	0.511

(Continued)

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TABLE 2 Continued

Variable	Normal BMI			High BMI		
	8-mg/d MPA group (*n* = 52 cycles)	10-mg/d MPA group (*n* = 119 cycles)	*P*-value	8-mg/d MPA group (*n* = 29 cycles)	10-mg/d MPA group (*n* = 53 cycles)	*P*-value
No. of transferable embryos	2.12 ± 1.84	1.90 ± 1.68	0.453	1.55 ± 1.48	1.58 ± 1.68	0.929
Cycle cancellation rate, n (%)	17 (32.69)	38 (31.93)	0.922	13 (46.43)	20 (38.46)	0.531
No. of FET cycles	41	97		20	40	
Type of embryos transferred, n (%)			0.226			0.677
Cleavage-stage embryos	53 (71.62)	137 (78.74)		24 (75.00)	59 (78.67)	
Blastocysts	21 (28.38)	37 (21.26)		8 (25.00)	16 (21.33)	
HCG positive rate, n (%)	20 (48.78)	38 (39.18)	0.296	5 (25.00)	22 (55.00)	0.028
Embryo implantation rate, n (%)	25 (33.78)	33 (18.97)	0.012	7 (21.88)	24 (32.00)	0.290
Clinical pregnancy rate, n (%)	19 (46.34)	29 (29.90)	0.064	4 (20.00)	20 (50.00)	0.025
Early miscarriage rate, n (%)	1 (5.26)	4 (13.79)	0.643	1 (25.00)	8 (40.00)	1.000
Cumulative pregnancy rate, n (%)	19 (36.54)	27 (22.69)	0.060	4 (13.79)	20 (37.74)	0.023
Cumulative live birth rate, n (%)	16 (30.19)	25 (21.01)	0.169	3 (10.34)	11 (20.75)	0.373

Dates were shown as mean ± SD or n (%).
FET, frozen embryo transfer.

TABLE 3 Multivariate logistic regression analysis of pregnancy outcomes.

Pregnancy outcome	Variable	Normal BMI		High BMI	
		OR [95% CI]	*P*	OR [95% CI]	*P*
Cumulative pregnancy	8 mg/d vs. 10 mg/d MPA	1.914 [0.852–4.300]	0.116	0.199 [0.046–0.861]	0.031
	Age	0.956 [0.879–1.040]	0.299	0.954 [0.842–1.081]	0.461
	BMI	0.919 [0.720–1.173]	0.499	1.071 [0.780–1.471]	0.672
	AFC	1.166 [1.010–1.347]	0.036	1.212 [0.975–1.507]	0.083
	No. of oocytes retrieved	0.655 [0.655–1.284]	0.614	0.928 [0.616–1.702]	0.928
	No. of mature oocytes	1.565 [1.066–2.300]	0.022	1.527 [0.804–2.899]	0.196
Cumulative live birth	8 mg/d vs. 10 mg/d MPA	1.642 [0.705–3.823]	0.250	0.569 [0.124–2.611]	0.468
	Age	0.950 [0.870–1.038]	0.254	0.889 [0.762–1.037]	0.135
	BMI	1.113 [0.860–1.441]	0.415	1.045 [0.733–1.488]	0.809
	AFC	1.169 [1.006–1.359]	0.041	1.114 [0.883–1.405]	0.364
	No. of oocytes retrieved	0.957 [0.673–1.361]	0.806	1.163 [0.681–1.988]	0.580
	No. of mature oocytes	1.500 [1.014–2.219]	0.042	1.151 [0.614–2.161]	0.661

showed an increasing trend compared with the 8-mg/d MPA group, but there was no significant statistical difference, possibly due to the small sample size. In addition, due to the fact that current embryo evaluations are based on morphological scores, there are subjective biases and individual differences. Although there is no difference in embryo grading between the groups, different doses of MPA may affect the developmental potential of embryos through potential epigenetic effects. The specific reasons and possible mechanisms still need further investigation. In the future, we will focus on the individualized treatment of POR patients to find the optimal dosage of MPA in the PPOS protocol, in order to increase the number of oocytes obtained and improve the quality of oocytes while effectively suppressing the early LH surge.

This study had some limitations. First, the retrospective study has selective bias. Second, our research sample size was small. Third, although the baseline data between the different MPA dose groups were comparable, many confounding factors existed during the ovulation induction cycle and FET cycle. Therefore, large-sample prospective trials or multicenter randomized controlled trials are needed for further studies in the future.

In conclusion, our study confirmed for the first time that in POR patients, high BMI patients who used 10 mg/d of MPA had better cumulative pregnancy rate than those who used 8 mg/d of MPA, although there is no significant difference in cumulative live birth rate. In normal BMI patients, the 8-mg/d MPA group had a higher embryo implantation rate than the 10-mg/d MPA group, while cumulative pregnancy and cumulative live birth rates were similar. The impact of MPA on patient pregnancy outcomes still needs further prospective trials with a large sample size to verify.

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 Renmin Hospital of Wuhan University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

QZ: Data curation, Writing – original draft, Writing – review & editing. SH: Data curation, Writing – original draft. YM: Writing – original draft. TY: Conceptualization, Writing – review & editing. LM: Methodology, Writing – review & editing. JY: Supervision, Writing – review & editing. SL: Conceptualization, Funding acquisition, Methodology, 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

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Pregnancy outcomes in women with primary ovarian insufficiency in assisted reproductive technology therapy: a retrospective study

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Purpose: This study aims to retrospectively estimate cumulative reproductive outcomes in women with primary ovarian insufficiency (POI) in assisted reproductive technology (ART) therapy.

Methods: A total of 139 patients diagnosed with POI were reviewed in this study. Firstly, they were divided into two groups according to oocyte origin: using their own oocytes (OG group) or accepting oocyte donations (OD I group). Secondly, the patients were split depending on the pregnancy outcome. In the OG group, nine patients decided to use others' oocytes after a failure of attempting to use their own, and this population was the oocyte donation II group (OD II group).

Results: There were 88 patients who used their own oocytes, while 51 patients accepted oocyte donations. In the OG group, there are only 10 (7.2%) patients who got pregnant, and patients in the OD group had worse hormone levels (FSH 71.37 ± 4.18 vs. 43.98 ± 2.53, AMH 0.06 ± 0.04 vs. 1.15 ± 0.15, and AFC 0.10 ± 0.06 vs. 1.15 ± 0.15) and more years of infertility (5.04 ± 0.48 vs. 3.82 ± 0.30), which explained why they choose oocyte donation. In all the three groups, baseline characteristics were comparable between pregnant women and non-pregnant women. Of the 10 pregnant patients in the OG group, four of them used luteal-phase short-acting long protocol and had pregnancies successfully in their first cycles.

Conclusion: Ovarian stimulation in POI women requires more cost and time. For those with a stronger desire to have genetic offspring, luteal-phase short-acting long protocol may help them obtain pregnancy rapidly.

KEYWORDS

primary ovarian insufficiency, pregnancy outcomes, ovarian stimulation, oocyte donation, ART

Introduction

Premature ovarian insufficiency (POI) is one of the most common and intractable causes of infertility in women of childbearing age. Due to the reduction of follicle pool or the abnormality of follicle function, patients under 40 years old lose normal ovarian function. The incidence rate of POI in women aged under 40 and 30 is 1% to 2% and 0.1%, respectively (1). The clinical manifestations are menopausal symptoms and long-term sequelae. The long-term sequelae include accelerated cognitive impairment, accelerated cardiovascular aging, infertility, and menopausal symptoms in advance (2). Infertility is the most important one in women during their childbearing years (3). The etiology of POI is diverse and complex, including genetic, autoimmune, metabolic, and iatrogenic, while most patients cannot find a clear cause (4). For women with POI, hormone replacement therapy (HRT) may help to improve the estrogen deficiency symptoms, such as hot flashes, sweating, and osteoporosis, and also prevent cardiovascular diseases. For women with POI with fertility requirements, oocyte donation, for POI patients, is an effective method to make their dream of becoming parents come true, and an increasing number of patients are beginning to receive this treatment (5). However, limiting the number of egg donation and medical ethics restrict this treatment. In recent years, stem cell therapy, *in vitro* activation (IVA), is the new treatment that could be applied (6, 7). Until now, there are several publications that reported a total of 51 patients with POI undergoing IVA therapy. In those studies, 29.4% (15/51) patients with POI had oocyte development, 7.8% (4/51) patients with POI achieved clinical pregnancy, and 5.9% (3/51) patients with POI achieved successful vaginal delivery (6, 8, 9). In clinical trials, there are 29 studies associated with POI but which lack information on pregnancy outcomes. Nevertheless, here are still some issues worth exploring. Since some patients with POI have a very strong wish to obtain a biological child, our study focuses on estimating cumulative pregnancy outcomes in patients with POI in ART therapy.

Materials and methods

Selection of patients

In this retrospective study, a total of 269 women were reviewed. A total of 139 women were included. The participants had undergone ART cycles (including fresh cycles and freeze–thaw cycles) and were diagnosed with POI according to the 2016 ESHRE guidelines, namely: (i) 4 months of persistent oligomenorrhea or amenorrhea and (ii) two tests with follicle-stimulating hormone (FSH) levels above 25 (at an interval of at least 4 weeks) (10). Participants who (1) had endometriosis, chromosomal abnormalities, and other endocrine diseases and (2) had ovarian surgery or gynecological tumors were excluded (Figure 1).

Grouping method

Firstly, the 139 female patients were split into two groups based on oocyte origin: using their own oocytes (OG group) or accepting oocyte donations (OD I group). In the OG group, nine patients decided to use others after a failure of attempting to use their own oocytes, and this population was the oocyte donation II group (OD II group). Each of the three groups was then divided into pregnant or non-pregnant group.

Ovarian stimulation protocols

Follicular-phase long-acting protocol

The patients were injected with 3.75 mg GnRH antagonist (GnRH-a, Tryptorelin, Ferring, Germany) on the 2nd day of menstruation if the ultrasound did not find cysts and follicles >10 mm. The patients will visit the hospital 28 days after the injection to be examined by ultrasound and to check the serum FSH, LH, E2, and progesterone (P) levels. A starting dose of 225–300 IU of human recombinant FSH (rFSH, Gonal F, Serono. Ltd., Switzerland) was administered, with subsequent adjustment of gonadotropin (Gn) use according to follicular growth. The starting dose is according to age, AFC, and basal hormone levels.

Luteal-phase short-acting long protocol

Subsequent to the administration of 0.1 mg GnRH antagonist (GnRH-a, Tryptorelin, Ferring, Germany) on day 21 of menstruation, it was used throughout the COH period. rFSH (Gonal F, Serono. Ltd., Switzerland) was given when the patients reached the downregulation criteria. The downregulation criteria were as follows: FSH <5 mIU/mL, LH <5 mIU/mL, E2 <30 pg/mL, and P <0.6 ng/mL; endometrial ≤5 mm; and antral follicle diameter 4–7 mm. Gn was used to induce follicle development. The administration of a starting dose of 225–300 IU of human recombinant FSH (rFSH, Gonal F, Serono. Ltd, Switzerland) lead to a subsequent adjustment of gonadotropin (Gn) use according to follicular growth. The starting dose is according to age, AFC, and basal hormone levels.

Mild stimulation protocol

From the 3rd day of menstruation, 2.5 mg letrozole (Furui, Ltd., Jiangsu, China) was used per day, and 300 IU human menopausal gonadotropin (HMG; 75 U/ampoule, Ferring GmbH, Germany) was added 2 days after. When the follicle diameter was >14 mm, monitoring the growth of the follicles was carried out by ultrasound every day.

Natural cycle

The follicle monitoring begins around the 6th–8th day of menstruation according to the menstruation cycles. Transvaginal sonography as well as serum sex hormone levels were monitored throughout the whole cycle based on the growth of the follicles.

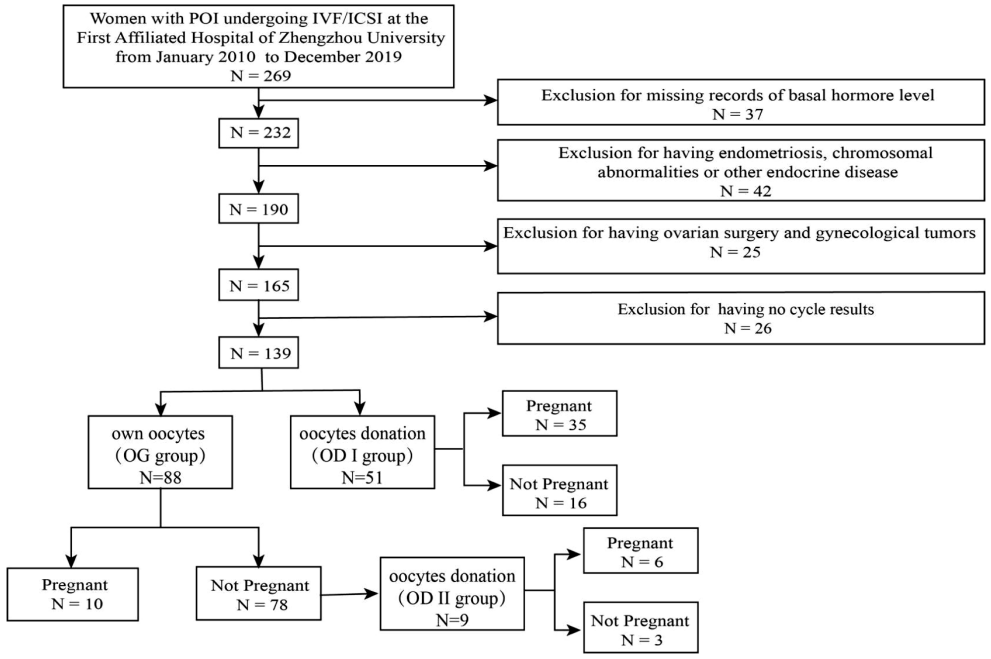

FIGURE 1
Flowchart of patient screening.

GnRH antagonist protocol

rFSH was administrated on the 2nd day of the cycle, and GnRH antagonist (Cetrorelix, Merck Serono, Europe) was given on the 6th day from the beginning of using rFSH. The follicles were monitored by ultrasound, and serum FSH, LH, E2, and P levels were examined according to the growth of the follicles.

Progestin-primed ovarian stimulation

On day 3 of the cycle, medroxyprogesterone acetate (MPA) (Xianju, Ltd., Zhengjiang, China) at 10 mg and HMG 150-225 IU were administered daily. The follicles were monitored after 5 days, and the HMG dose was adjusted depending on the growth of the follicles. The MPA dose was consistent with the trigger day.

In the above-mentioned protocol, ovulation is performed using human chorionic gonadotropin (HCG) (2,000 IU) (Lizhu. Ltd., Guangdong, China) and recombinant HCG (250 μg) (Serono. Ltd., Switzerland) for the follicular-phase long-acting protocol, GnRH antagonist protocol, and luteal-phase short-acting long protocol and using human chorionic gonadotropin (HCG) (10,000 IU) (Lizhu. Ltd., Guangdong, China) for mild stimulation protocol, natural cycle, and progestin-primed ovarian stimulation (PPOS). Egg retrieval was performed 34–36 h later. Depending on the quality of the male partner's sperm, the fertilization method will be decided.

Endometrial preparation

Endometrial preparation in all of the patients involved hormone replacement therapy. The detailed endometrial preparation protocol for freeze–thaw cycles has been described in a previous article, including the classification of endometrial types and thickness measurement methods (11). For estrogen–progesterone (EP) cycles, oral estradiol (Progynova, Bayer, Germany) administration began on days 2 to 3 of the target cycle and lasted for about 2 weeks. When the thickness of the endometrium reaches 8 mm and above, the patient is asked to add oil-based progesterone (60 mg). On the same day, the thickness of the endometrium was recorded using transvaginal ultrasound examination. To avoid cavity fluid and other unfavorable conditions, the patients were hospitalized and re-measurement of endometrial thickness was done in the morning of the transplantation day. Luteal supplement was altered to vaginal progesterone gel (90 mg, Crinone 8%; Merck Serono) and oral dydrogesterone (20 mg Duphaston; Abbott) after embryo implantation. Clinical pregnancy was confirmed by ultrasound observation after embryo transfer.

Embryo cryopreservation and thawing

The cleaved embryos were incubated in an incubator with 6% CO_2 at 37°C and individually in microdrops (50 μL) containing G1 (Vitrolife) + 5% HSA (Vitrolife). Embryo quality was assessed on day 3 based on the Peter cleavage stage embryo scoring system. Vitrification was used for embryo cryopreservation and thawing.

Statistical methods

IBM SPSS, 21.0 (IBM Corp., Armonk, NY, USA) was employed. Numerical data were shown as mean ± standard deviation (SD),

while categorical variables were shown as % (*n*/*N*). Mann–Whitney test and chi-square test were utilized for continuous and categorical variables, respectively. The threshold was set as two-tailed P <0.05.

Results

Baseline characteristics and hormone levels of patients with POI undergoing IVF/ICSI

There were 88 (63.3%) patients who used their own oocytes, while 51 (36.7%) patients accepted oocyte donations (Table 1). No significant difference was found between the two groups in age (32.73 ± 0.51 vs. 31.27 ± 0.61) and body mass index (BMI) (22.04 ± 0.28 vs. 22.73 ± 0.40). The women in the OG group had lower basal serum FSH (43.98 ± 2.53 vs. 71.37 ± 4.18) and LH (22.45 ± 1.76 vs. 34.53 ± 2.17). The women in the OG group had higher serum E2 (44.24 ± 7.49 vs. 9.97 ± 1.09) and AMH (0.08 ± 0.01 vs. 0.06 ± 0.04). In addition, the women in the OG group had more AFC (1.15 ± 0.15 vs. 0.10 ± 0.06), fewer years of infertility (3.82 ± 0.30 vs. 5.04 ± 0.48), and more numbers of previous pregnancies (0.85 ± 0.14 vs. 0.24 ± 0.06).

TABLE 1 Baseline characteristics and hormone levels of patients with POI undergoing IVF /ICSI.

	OG	OD	P value
Number	88	51	
Age	32.73±0.51	31.27±0.61	0.064
BMI	22.04±0.28	22.73±0.40	0.167
Baseline hormone levels			
FSH (IU/L)	43.98±2.53	71.37±4.18	<0.001
E2(pg/mL)	44.24±7.49	9.97±1.09	<0.001
LH (IU/L)	22.45±1.76	34.53±2.17	<0.001
AMH(ng/ml)	0.08±0.01	0.06±0.04	<0.001
AFC	1.15±0.15	0.10±0.06	<0.001
Type of infertility			0.227
Primary infertility	55	37	
Secondary infertility	33	14	
Years of infertility	3.82±0.30	5.04±0.48	0.031
Number of previous pregnancies	0.85±0.14	0.24±0.06	0.012

Numbers are mean ± standard deviation; BMI, body mass index; FSH, follicle-stimulating hormone; E2, estradiol; LH, luteinizing hormone; AMH, anti-mullerian hormone; AFC, antra follicular count.

Baseline characteristics and hormone levels of patients in different groups undergoing IVF/ICSI

There were nine patients who accepted oocyte donations after trying using their own oocytes (Table 2). In the OG group, the results were comparable between pregnant patients and non-pregnant patients in age (33.40 ± 1.30 vs. 32.64 ± 0.55), BMI (22.58 ± 0.59 vs. 21.98 ± 0.30), and AFC (1.70 ± 0.58 vs. 1.08 ± 0.15), FSH(52.23 ± 11.91 vs. 42.93 ± 2.43, E2 (49.16 ± 26.43 vs. 43.61 ± 7.81), LH (20.00 ± 7.01 vs. 22.76 ± 1.78), and AMH (0.14 ± 0.06 vs. 0.07 ± 0.12). In the OD I group, no statistical difference was found between pregnant women and non-pregnant women in the number of oocyte donations (3.71 ± 0.11 vs. 3.89 ± 0.13), age (31.74 ± 0.73 vs. 30.25 ± 1.07), BMI (22.55 ± 0.48 vs. 23.12 ± 0.74), and AFC (0.09 ± 0.06 vs. 0.13 ± 0.13). Meanwhile, FSH (73.33 ± 5.48 vs. 67.06 ± 5.90), E2 (10.10 ± 1.42 vs. 9.68 ± 1.65), LH (35.15 ± 2.83 vs. 33.16 ± 3.21), and AMH (0.08 ± 0.06 vs. 0.01 ± 0.00) were not significantly different between the two groups. In the OD II group, data were comparable between pregnant patients and non-pregnant patients in the number of oocyte donations (3.83 ± 0.17 vs. 3.33 ± 0.33), age (31.17 ± 1.83 vs. 35.00 ± 2.52), BMI (21.57 ± 1.36 vs. 22.29 ± 2.23), and AFC (0.50 ± 0.22 vs. 0.33 ± 0.33). In the meantime, FSH (92.06 ± 11.95 vs. 50.16 ± 16.82), E2 (9.55 ± 4.672 vs. 18.46 ± 11.62), LH (52.30 ± 5.60 vs. 33.00 ± 11.12), and AMH (0.05 ± 0.03 vs. 0.04 ± 0.03) were not significantly different between the pregnant group and the non-pregnant group.

Baseline characteristics of pregnant patients in the OG group

There were 10 pregnancies among 88 women in the OG group. The detailed clinical information of each patient is listed in Table 3. Five patients were over 35 years old (patients 2, 3, 5, 6, and 10). Only four patients had pregnancies within one cycle (patients 2, 3, 4, and 6). Four pregnancies were obtained after the transfer of fresh embryos (patients 2, 3, 4, and 6) and six pregnancies after a frozen/thawed cycle. Additionally, eight patients achieved live births, including two twin births (patients 2 and 9). The basic serum FSH ranges from 25.37 to 138.4 IU/L, the basic serum E2 ranges from 1.36 to 73.1 pg/mL, the basic serum LH ranges from 6.39 to 68.33 IU/L, and the serum AMH ranges from 0.01 to 0.66 ng/mL. The number of AFC ranges from one to five.

Ovarian stimulation characteristics of patients with POI undergoing ET cycles

For the 10 pregnancies who used their own oocytes, the ovarian stimulation characteristics of each patient are listed in Table 4.

For patient 1, one embryo was frozen in the first PPOS cycle. One oocyte was obtained in the second PPOS cycle, while fertilization was abnormal. In the third PPOS cycle and fourth mild cycle, there were no oocytes retrieved. In the following E-P cycles, the woman got successfully pregnant and delivered.

For patient 2, six oocytes were obtained in the luteal-phase short-acting long protocol. After the transfer of two cleavage embryos, the patient obtained clinical pregnancy and delivered.

For patient 3, luteal-phase short-acting long protocol was administrated, and two oocytes were retrieved and then transferred.

For patient 4, similar to patient 3, she got five oocytes and then transferred two embryos and got pregnant.

TABLE 2 Baseline characteristics and hormone levels of patients in different group undergoing IVF /ICSI.

Variables	OG			OD I			OD II		
	pr egnant	non pr egnant	P value	pr egnant	non pr egnant	P value	pr egnant	non pr egnant	P value
Number of patients	10	78		35	16		6	3	
Number of oocytes donation	NA	NA		3.71±0.11	3.89±0.13	0.242	3.83±0.17	3.33±0.33	0.157
Age	33.40±1.30	32.64±0.55	0.678	31.74±0.73	30.25±1.07	0.237	31.17±1.83	35.00±2.52	0.283
BMI	22.58±0.59	21.98±0.30	0.382	22.55±0.48	23.12±0.74	0.556	21.57±1.36	22.29±2.23	0.275
AFC	1.70±0.58	1.08±0.15	0.259	0.09±0.06	0.13±0.13	0.921	0.50±0.22	0.33±0.33	0.655
Baseline hormone levels									
FSH (IU/L)	52.23 ±11.91	42.93±2.43	0.828	73.33±5.48	67.06±5.90	0.619	92.06 ±11.95	50.16±16.82	0.110
E2(pg/mL)	49.16 ±26.43	43.61±7.81	0.874	10.10±1.42	9.68±1.65	0.926	9.55±4.67	18.46±11.62	0.197
LH (IU/L)	20.00±7.01	22.76±1.78	0.086	35.15±2.83	33.16±3.21	0.847	52.30±5.60	33.00±11.12	0.197
AMH (ng/ml)	0.14±0.06	0.07±0.12	0.333	0.08±0.06	0.01±0.00	0.753	0.05±0.03	0.04±0.03	0.423
Type of infertility			1.000			0.942			0.117
Primary infertility	6	49		26	11		6	2	
Secondary infertility	4	29		9	5		0	1	
Duration of infertility	3.90±0.91	3.81±0.31	0.947	5.40±0.56	4.25±0.91	0.094	3.00±0.58	5.00±1.53	0.170
Number of previous pregnancies	1.20±0.57	0.81±0.14	0.709	0.20±0.07	0.31±0.12	0.380	0.00±0.00	0.33±0.33	0.157

Numbers are mean ± standard deviation; BMI, body mass index; FSH, follicle-stimulating hormone; E2, estradiol; LH, luteinizing hormone; AMH, anti-Mullerian hormone; AFC, Antral follicular count.

For patient 5, ovarian stimulation was made by using three different protocols: GnRH antagonist protocol, mild stimulation protocol, and PPOS protocol. The first cycle was canceled because of follicle dysplasia, while one and two embryos were frozen in the latter two cycles, respectively. Finally, this patient got pregnant after two embryos were implanted in the GnRH-a and E-P cycle.

For patient 6, she got one embryo and got pregnant successfully in the luteal-phase short-acting long protocol after IVA therapy.

For patient 7, no oocyte was obtained in the first mild stimulation cycle. The second PPOS cycle got one oocyte but failed to fertilize, and the third PPOS cycle also got no oocytes. Two embryos were frozen in the fourth PPOS cycle, and she got pregnant after one was transferred in the second E-P cycle.

For patient 8, one embryo was frozen in the first mild stimulation cycle. Unfortunately, there was no mature follicle in the second follicular-phase long-acting protocol cycle, and no oocyte was obtained in the third PPOS cycle. She canceled the fourth cycle and required FET. After transferring one embryo in the following E-P cycle, she got pregnant.

For patient 9, mild stimulation was used; two embryos were frozen in the first and fourth cycles, and the third cycle also got one oocyte. However, normal fertilization failed. Early ovulation was detected in the other two cycles. Finally, this patient got pregnant after two embryos were implanted in the GnRH-a and E-P cycle.

For patient 10, one embryo was transferred in the first luteal-phase short-acting long protocol cycle, but she failed to conceive. The following two mild stimulation cycles obtained three embryos in total, and then two embryos were transferred in the natural cycle and she conceived.

Ovarian stimulation characteristics of patients with POI undergoing ET cycles

In the patients with POI who had the chance of embryo transfer, we tried to find out the impact factor related to embryo transformation (Table 5). In the OG group, the results were comparable between pregnant patients and non-pregnant patients in basal endometrial thickness (3.83 ± 0.70 vs. 4.53 ± 0.48), endometrial thickness on ET day (9.00 ± 0.58 vs. 9.16 ± 0.29), number of embryos transferred (1.50 ± 0.22 vs. 1.42 ± 0.12), and number of good-quality embryos (1.50 ± 0.22 vs. 1.21 ± 0.15). In the OD I group, similar results were obtained between the two groups in basal endometrial thickness (3.81 ± 0.57vs 3.08 ± 0.74), endometrial thickness on ET day (10.71 ± 0.39 vs. 9.50 ± 0.66), number of embryos transferred (1.76 ± 0.07 vs. 1.68 ± 0.11), and number of good-quality embryos (1.35 ± 0.11vs 1.16 ± 0.19). In the OD II group, no statistical difference was found between pregnant

TABLE 3 Baseline characteristics of pregnant patients in OG group.

Serial number	Age	Type of infertility	Total number of cycles	Cycle type*	Protocol	Pregnant outcome	Duration of infertility	BMI	Baseline hormone levels				AFC	Number of previous pregnancy
									FSH (IU/L)	E2 (pg/mL)	LH (IU/L)	AMH (ng/ml)		
1	29	primary infertility	5	ET cycle	E-P protocol	singleton	4	22.9	50.38	32.26	21.97	0.01	0	0
2	36	secondary infertility	1	Fresh cycle	Luteal phase long protocol	twin	8	23.7	32.5	73.1	11.1	0.66	1	1
3	36	primary infertility	1	Fresh cycle	Luteal phase long protocol	singleton	6	21.8	27.73	19.65	7.57	0.31	6	0
4	29	primary infertility	1	Fresh cycle	Luteal phase long protocol	singleton	1	25.7	26.23	14.25	9.09	0.16	3	0
5	36	secondary infertility	4	ET cycle	GnRH-a + E-P protocol	abortion	4	25.5	25.37	21.16	8.38	0.06	0	3
6	36	secondary infertility	1	Fresh cycle	Luteal phase long protocol	singleton	2	21.48	138.4	1.36	68.33	0.01	2	5
7	27	primary infertility	6	ET cycle	E-P protocol	singleton	2	19.9	78.92	28.1	6.44	0.01	0	0
8	32	secondary infertility	5	ET cycle	E-P protocol	singleton	9	21.3	31.19	5	7.89	0.08	2	3
9	33	primary infertility	6	ET cycle	GnRH-a + E-P protocol	twin	2	22	85.56	28.5	52.81	0.01	1	0
10	40	primary infertility	4	ET cycle	Natural cycle	abortion	1	21.5	26.01	15.87	6.39	0.09	2	0

BMI, body mass index; FSH, follicle-stimulating hormone; E2, estradiol; LH, luteinizing hormone; AMH, anti-Mullerian hormone; AFC, antra follicular counts.
*cycle means the cycle when patients get pregnant.

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TABLE 4 Ovarian stimulation characteristics of pregnant patients in OG group.

Serial number	Cycle type	Protocol	E2 (pg/mL)	LH (IU/L)	P4 (ng/mL)	Basal endometrial thickness (mm)	Endometrial thickness on HCG (mm)	Length of stimulation n (d)	Total amount of Gn (IU)	Total oocytes retrieved	number of embryos transferred	cycle result
		PPOS	344.8	1.74	1.3	5	6	3	450	1	/	embryo frozen
		PPOS	267.2	8.13	0.46	5	7	3	450	1	/	Abnormal fertilization
	Fresh cycle	PPOS	362.4	3.37	0.38	5	9	3	450	/	/	No oocytes retrieved
		mild	73.79	13.45	0.45	5	8	9	1725	/	/	No oocytes retrieved
1	ET cycle	E-P	123.2	9.72	0.11	5	11	18	64	/	1	pregnant
2	Fresh cycle	short	2260	1.92	0.49	8	12	15	4500	6	2	pregnant
3	Fresh cycle	short	904.9	1.8	0.32	4	8	11	3300	2	2	pregnant
	Fresh cycle	short	1309	3.44	0.17	4	16	10	3000	5	2	pregnant
4		GnRH-a	25.37	24.86	0.07	2	2	7	2100	/	/	Follicle dysplasia
	Fresh cycle	mild	280.8	1.93	0.26	2	6	13	2250	1	/	embryo frozen
		PPOS	378.9	2.54	0.5	2	5	9	1425	2	/	embryo frozen
5	ET cycle	GnRH-a+ E-P	243.6	0.71	0.05	2	7	21	144	/	2	pregnant
	Fresh cycle	short	344.8	5.06	0.36	5	9	11	3300	1	1	pregnant
6*		mild	305.7	8.38	0.68	5	5	1	150	/	/	No oocytes retrieved
		PPOS	503.6	2.39	0.57	5	3	6	900	1	/	Abnormal fertilization
7		PPOS	1026	10.88	0.91	5	7	5	750	/	/	

(Continued)

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TABLE 4 Continued

Serial number	Cycle type	Protocol	E2 (pg/mL)	LH (IU/L)	P4 (ng/mL)	Basal endometrial thickness (mm)	Endometrial thickness on HCG (mm)	Length of stimulation n (d)	Total amount of Gn (IU)	Total oocytes retrieved	number of embryos transferred	cycle result
	Fresh cycle											No oocytes retrieved
		PPOS	1007	6.6	0.78	5	7	9	1350	2	/	embryo frozen
	ET cycle	E-P	156	69.61	0.64	5	9	9	28	/	/	not transfer
		E-P	184.2	56.99	0.05	5	9	17	78	/	1	pregnant
		mild	231.1	10.59	0.7	6	8	5	750	1	/	embryo frozen
	Fresh cycle	long	5	14.56	0.41	2	6	5	825	/	/	Follicle dysplasia
		PPOS	128.1	35.5	0.47	5	7	5	750	/	/	No oocytes retrieved
		antagonist	/	/	/	/	/	/	/	/	/	require FET
	ET cycle	E-P	209.2	12.33	0.05	6	8	18	84	/	1	pregnant
		mild	170.3	12.11	0.31	2	8	6	900	1	/	embryo frozen
8		mild	313.3	9	0.63	2	6	5	750	/	/	Follicle ovulation in advance
	Fresh cycle	mild	208.7	27.94	0.58	2	6	3	450	1	/	Abnormal fertilization
		mild	394.1	6.58	0.38	2	7	3	750	1	/	embryo frozen
		mild	558	5.44	0.39	2	5	7	1575	/	/	Follicle ovulation in advance
9	ET cycle	GnRH-a+ E-P	197	0.1	0.03	2	10	18	84	/	2	pregnant

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TABLE 4 Continued

Serial number	Cycle type	Protocol	E2 (pg/mL)	LH (IU/L)	P4 (ng/mL)	Basal endometrial thickness (mm)	Endometrial thickness on HCG (mm)	Length of stimulation n (d)	Total amount of Gn (IU)	Total oocytes retrieved	number of embryos transferred	cycle result
		short	573	5.9	0.69	3	9	19	5700	1	1	not pregnant
	Fresh cycle	mild	192.1	4.2	0.47	3	7	6	900	1	/	embryo frozen
		mild	211.8	4.57	0.42	3	8	7	1050	2	/	embryo frozen
10	ET cycle	natural	58.17	12.58	0.73	3	9	5	10	/	2	pregnant

E2, estradiol; P4, progesterone; LH, luteinizing hormone; ET, embryo transfer; on HCG, on the day of human chorionic gonadotropin administration; Gn, gonadotropin ; short, Luteal phase short-acting long protocol; long, Follicular phase long-acting protocol; PPOS, Progestin-primed ovarian stimulation; mild, Mild stimulation protocol; anti, GnRH antagonist protocol. *the patient accepted IVA therapy 156 days ago.

patients and non-pregnant patients in basal endometrial thickness (4.50 ± 2.08vs 2.57 ± 1.19), endometrial thickness on ET day (10.00 ± 0.82vs 11.71 ± 0.75), number of embryos transferred (1.83 ± 0.17vs 1 1.57 ± 0.20), and number of good-quality embryos (1.67 ± 0.21 vs. 1.43 ± 0.20).

Baseline characteristics and hormone levels of patients with POI with different cycle results

To investigate the factors associated with pregnancy outcomes, we further analyze the baseline characteristics and hormone levels of patients with POI with different cycle results (Table 6). The factors that resulted in non-pregnancy included no oocytes retrieved, follicle dysplasia, follicle ovulation in advance, no transferable embryo, and abnormal fertilization. A statistical difference was found between women with no oocytes retrieved and patients with follicle dyspepsia in serum E2 levels on HCG ($p < 0.001$, 323.68 ± 37.33 vs. 34.74 ± 10.85). Patients with follicle dysplasia and patients with follicle ovulation in advance showed a difference in AFC ($p = 0.011$, 0.58 ± 0.15 vs. 2.00 ± 0.72), serum E2 levels on HCG ($p < 0.001$, 34.74 ± 10.85 vs. 354.70 ± 46.72), and serum P4 levels on HCG ($p = 0.028$, 0.39 ± 0.06 vs. 0.66 ± 0.20). Patients with follicle dysplasia and patients with no transferable embryo also showed a difference in serum basal FSH level ($p = 0.034$, 39.23 ± 2.71 vs. 33.05 ± 0.36), serum E2 levels on HCG ($p < 0.001$, 34.74 ± 10.85 vs. 394.51 ± 102.25), and serum P4 levels on HCG ($p = 0.013$, 0.39 ± 0.06 vs. 0.62 ± 0.07). A significant difference was likewise identified between patients with follicle dysplasia and abnormal fertilization in serum E2 levels on HCG ($p < 0.001$, 34.74 ± 10.85 vs. 467.47 ± 97.76) and serum LH levels on HCG ($p = 0.014$, 14.85 ± 2.14 vs. 16.92 ± 6.11).

Discussion

Since the probability of spontaneous pregnancy in POI patients is only 5%–10%, infertility is a very challenging issue for these patients. The treatment of infertility in patients with POI, who suffer from premature follicular depletion and resistance to exogenous hormones, includes egg donation and IVF/ICSI, the latter of which is often hardly satisfactory (12).

In our study, we first compared the baseline characteristics and hormone levels between patients who used their own oocytes and patients who accepted oocyte donations. It is not difficult to find that the POI patients in the OD group had worse hormone levels and more years of infertility, which made them receive the oocyte donation directly. Next, we compared the baseline characteristics and hormone levels between pregnant patients and non-pregnant patients in the OG, OD I, and OD II groups and sought to identify factors that affect these pregnancy outcomes. However, compared with non-pregnant group, the results in the pregnant group were comparable. Obviously, the rapid treatment for POI with or without some IVF failures is oocyte donation.

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TABLE 5 Ovarian stimulation characteristics of patients with POI undergoing ET cycles.

ET cycle	OG			OD I			OD II		
	pregnant	not pregnant	P value	pregnant	not pregnant	P value	pregnant	not pregnant	P value
Number	6	16		35	16		6	3	
Number of ART cycles	6	19		35	22		6	7	
Basal endometrial thickness (mm)	3.83±0.70	4.53±0.48	0.436	3.81±0.57	3.08±0.74	0.482	4.50±2.08	2.57±1.19	0.596
Endometrial thickness on ET (mm)	9.00±0.58	9.16±0.29	0.818	10.71±0.39	9.50±0.66	0.503	10.00±0.82	11.71±0.75	0.088
Hormne levels on the day of endometrium transformation									
E2 (pg/mL)	169.23±27.43	384.78±127.56	0.445	157.52±9.24	189.54±15.42	0.054	373.97 ±187.03	496.35±223.70	0.886
P4 (ng/mL)	0.05±0.01	0.07±0.01	0.767	0.15±0.03	0.11±0.05	0.487	0.40±0.12	0.14±0.04	0.054
LH (IU/L)	15.41±8.62	17.07±4.10	0.567	20.91±2.31	19.63±2.24	0.593	41.80±8.57	23.45±8.53	0.158
Number of embryos transferred	1.50±0.22	1.42±0.12	0.739	1.76±0.07	1.68±0.11	0.528	1.83±0.17	1.57±0.20	0.327
Number of good-quality embryos	1.50±0.22	1.21±0.15	0.330	1.35±0.11	1.16±0.19	0.449	1.67±0.21	1.43±0.20	0.409

Numbers are mean ± standard deviation; E2, estradiol; P4, progesterone; LH, luteinizing hormone; ET, embryo transfer.

In recent years, many researchers have been seeking treatments to stimulate follicle development and elevate the oocyte function of POI patients. The advent of IVA brings a new light to POI patients (9, 13). *In vitro* studies have shown that the activators or inhibitors used in IVA may impair oocyte quality, and this safety uncertainty has implications for the application of IVA (14, 15). Nevertheless, IVA's use of *in vitro* ovarian cortical fragmentation instead of direct activation of follicles might have a higher efficiency and cause little impairment to the follicles (16). A study by Zhang et al. concluded that 13.75% of women obtained development of follicles after biopsy/scratch, and 1.25% of patients got pregnant and delivered (17). In one autoimmune POI case, *in vitro* maturation (IVM) is used to promote the development of oocytes in the sinus follicular stage into mature eggs, which results in a healthy live birth (18). While IVM is not sufficiently mature, the future holds the promise of maximizing the retention of immature oocytes for these to develop into mature ones for subsequent fertilization and embryo transfer processes.

TABLE 6 Baseline characteristics and hormone levels of patients with POI with different cycle results.

	No oocytes retrieved (1)	P value (2vs1)	Follicle dysplasia (2)	P value (2vs3)	Follicle ovulation in advance (3)	P value (2vs4)	No transferable embryo (4)	P value (2vs5)	Abnormal fertilization (5)
Number	28		26		7		6		8
Number of ART Cycles	31		29		9		7		9
Age	32.39±0.89	0.419	31.42±0.93	0.094	36.14±2.30	0.085	35.00±2.46	0.476	32.88±1.74
BMI	22.33±0.48	0.866	22.45±0.55	0.913	22.33±0.77	0.718	22.88±0.57	0.816	22.21±0.44
Baseline hormone levels									
FSH (IU/L)	46.68±5.33	0.842	39.23±2.71	0.930	42.70±8.06	0.034*	33.05±.36	0.570	46.34±7.84
E2 (pg/mL)	39.20±10.45	0.319	49.64±11.03	0.965	40.58±14.25	0.735	35.04±12.97	0.393	24.33±4.39
LH (IU/L)	21.61±3.09	0.319	24.30±2.66	0.234	19.37±6.54	0.227	15.55±4.20	0.516	22.70±6.55
AMH (ng/ml)	0.07±0.02	0.298	0.04±0.01	0.176	0.08±0.03	0.164	0.14±0.73	0.606	0.09±0.05
AFC	1.14±0.29	0.294	0.58±0.15	0.011*	2.00±0.72	0.101	1.83±0.79	0.174	1.25±0.45

(Continued)

TABLE 6 Continued

	No oocytes retrieved (1)	P value (2vs1)	Follicle dysplasia (2)	P value (2vs3)	Follicle ovulation in advance (3)	P value (2vs4)	No transferable embryo (4)	P value (2vs5)	Abnormal fertilization (5)
Baseline hormone levels									
Type of infertility		0.933		0.761		0.152		0.438	
Primary infertility	18		17		5		2		4
Secondary infertility	10		9		2		4		4
Years of infertility Hormone levels on HCG	4.36±0.54	0.155	3.38±0.48	0.607	3.00±1.02	0.941	3.33±1.15	0.168	4.50±0.91
E2 (pg/mL)	323.68±37.33	<0.001***	34.74±10.85	<0.001***	354.70±46.72	<0.001***	394.51±102.25	<0.001***	467.47±97.76
LH (IU/L)	19.23±5.15	0.387	14.85±2.14	0.089	9.33±1.32	0.208	15.98±6.64	0.014*	16.92±6.11
P4 (ng/mL)	0.53±0.08	0.057	0.39±0.06	0.028*	0.66±0.20	0.013*	0.62±0.07	0.548	0.63±0.09

Numbers are mean ± standard deviation; BMI, body mass index; FSH, follicle-stimulating hormone; E2, estradiol; P4, progesterone; LH, luteinizing hormone; AMH, anti-Mullerian hormone; AFC, antra follicular count * P<0.05 ** P<0.01 ***P<0.001.

Although oocyte donation is the better choice and many new treatments occur for POI patients, many of these infertile women are requesting IVF treatment despite the low success rate. In our study, we further analyze the details of pregnant patients in the OG group. For patients with POI undergoing ART, the ultimate goal is to obtain as many mature eggs as possible, although this goal is difficult to achieve and there is no consensus as to which protocol is optimal for this population. Compared to young women with a diminished ovarian reserve, older women with normal ovarian function had a higher embryo implantation rate, but there was no significant difference in live birth rates between the groups, suggesting a discrepancy in egg quality (19). To obtain maximal oocytes and embryos for patients with POI, many studies attempted to explore the optimal ovulation–stimulation protocols. In our study, we performed several protocols—four patients could obtain pregnancy only in the first IVF cycle through the luteal-phase short-acting long protocol and benefited from the better endometrial receptivity of this ovulation–stimulation protocols. However, we had to freeze embryos to transfer in thawed embryo transfer cycle in other ovulation–stimulation protocols.

In the study conducted by Kuang et al., they demonstrated that the PPOS protocol exhibited better suppression of premature LH peaks compared to the antagonist protocol but did not improve the cumulative live birth rate of patients for poor responders (20). We can obtain the same conclusion with this study. Obtaining the maximum number of oocytes through repeated ovarian stimulation and egg retrieval could improve the cumulative live birth rate for POI patients (21). Our study investigated if the patients have a choice to try luteal-phase short-acting long protocol to process fresh IVF cycle transfer, as it will take a shorter time and less cost to achieve pregnancy.

In our study, we also analyzed the ovarian stimulation characteristics of patients with POI in different groups. The results were comparable between the pregnant and non-pregnant patients who had a chance to undergo ET cycles, but we could see that the pregnant ones had more transferred embryos.

Researchers are also exploring various treatments to enhance the pregnancy outcomes of POI patients. In the study of Safak et al., the reproductive outcomes were significantly higher in the random start protocol with clomiphene citrate and gonadotropin treatment than a spontaneous folliculogenesis protocol in women with OPOI (22). Safak et al. also indicated that rescue of the developing oocyte at an early stage of stimulation combined with subsequent embryo transfer can reduce the cycle of retrieval and achieve a favorable outcome for the POI patients (23). Ishizuka et al. analyzed 466 patients with POI undergoing HRT with or without ovarian stimulation (OS), and the follicle growth rate was 48.3% (207/429) per patient in group OS and 5.4% (2/37) in group HRT, while the live birth rate was 5.8% (47/807) in group OS with IVF and no pregnancies occurred in group HRT (24). Moreover, some researchers have also tried using glucocorticoids in combination with high-dose Gn in order to obtain pregnancy in patients with POI (25). A retrospective study comparing the efficacy of glucocorticoids in POI showed that ovulation was achieved in six (20.7%) patients in the dexamethasone group compared to three (10.3%) in the control group, followed by two live births in the dexamethasone group (26). Some studies that attempted to induce ovulation by hHMG stimulation in POI patients and successfully obtained pregnancy were mainly in cases under estrogen replacement (26–28). In the future, we should combine the strengths from different studies to help POI patients obtain pregnancy outcomes in a short time.

Ultimately, we investigated the factors in IVF failures, the largest proportion being no oocytes retrieved and follicle dysplasia. The E2 levels on HCG day in follicle dysplasia is significantly lower than in no oocytes retrieved, follicle ovulation

in advance, no transferable embryo, and abnormal fertilization. The AFC is also the least in follicle dysplasia. The reason of follicle dysplasia is linked with POI, and there is no effective solution. Patients with follicle dysplasia should not try more IVF cycles and accept oocyte donation as soon as possible.

Generally, ovarian stimulation in POI women requires more cost and time. For POI patients with a stronger desire to have genetic offspring, luteal-phase short-acting long protocol may help them obtain pregnancy rapidly. If pregnancy cannot be generated within three to four cycles, it is suggested to discourage POI patients from ovarian stimulation and for them to accept oocyte donation.

Data availability statement

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

Ethics statement

The studies involving humans were approved by The Research Ethics Committee of the First Affiliated Hospital of Zhengzhou University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

BS: Data curation, Supervision, Writing – original draft, Writing – review & editing. LL: Data curation, Software, Writing – original draft. YZ: Conceptualization, Methodology, Visualization, Writing – review & editing. FW: Resources, Software, Visualization, Writing – review & editing. YS: Conceptualization, Investigation, Project administration, Writing – review & editing.

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Conflict of interest

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

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The late-follicular-phase progesterone to retrieved oocytes ratio in normal ovarian responders treated with an antagonist protocol can be used as an index for selecting an embryo transfer strategy and predicting the success rate: a retrospective large-scale study

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Objective: To determine whether the late-follicular-phase progesterone to retrieved oocytes (P/O) ratio during *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI) impacts pregnancy outcomes.

Design: 12,874 cycles were retrospectively categorized into four groups according to the P/O ratio percentile, with divisions at the 25th, 50th and 75th percentiles.

Results: The clinical pregnancy and live birth rates of fresh cycle embryos in Group D were significantly lower than those in the other three groups (45.1% and 39.0%, 43.2% and 37.2%, 39.6% and 33.5%, 33.4% and 28.2% in Group A, B, C, D, respectively; both $P < 0.008$). Multivariate logistic regression analysis revealed a significant negative correlation between the P/O ratio and live birth, particularly when the P/O ratio was ≥0.22 (OR = 0.862, 95% CI [0.774–0.959], P = 0.006).

Conclusions: The P/O ratio has certain predictive value for IVF/ICSI pregnancy outcomes and can be used for decision-making decision regarding fresh embryo transfer.

KEYWORDS

***in vitro* fertilization, late follicular phase, progesterone level, pregnancy outcome, antagonist protocol**

Introduction

According to the World Health Organization (WHO), infertility and sterility will be the third most serious condition worldwide in the 21st century. Furthermore, it is estimated that 15% to 20% (40–50 million) of women of reproductive age in China suffer from infertility, and 822,246 assisted reproductive technology (ART) cycles were conducted between 1981 and 2011 (1). Although IVF/ICSI is an effective method for treating infertility, overcoming the pregnancy rate bottleneck has always been a research hotspot in the field of reproductive medicine, and a premature surge in serum progesterone (P) in the late follicular phase may significantly influence the success rate of IVF/ICSI. Late follicular phase progesterone elevation (LFPE) is defined as a premature increase in serum P on the day of human chorionic gonadotropin (hCG) injection, and in controlled ovarian stimulation (COS) cycles, the incidence of LFPE ranges from 4.5 to 30% (2, 3). Whether LFPE affects the pregnancy outcomes of fresh IVF/ICSI cycles is still controversial. Some studies have shown that LFPE can reduce the pregnancy rate after embryo transfer in fresh cycles by affecting the quality of oocytes/embryos and endometrial receptivity (4–6). Conversely, another study revealed no correlation between LFPE length and pregnancy outcomes (7, 8).

Currently, the LFPE threshold is usually set according to the effect of LFPE on IVF/ICSI clinical pregnancy outcomes, and the threshold reported in the literature is 2.54–7.95 nmol/L (9, 10), which may be the main reason for the different results of previous studies. Furthermore, retrospective studies often fail to account for various potential confounders, including patient age, ovarian responsiveness, COS protocol, the quantity of oocytes and high-quality embryos, the day of embryo transfer, and the number of embryos transferred. These factors may have contributed significantly to the varying findings reported in previous studies.

The mechanism of LFPE is still unclear. One proposed theory is that in IVF cycles in which the pituitary is not downregulated, the concurrent elevation of luteinizing hormone (LH) levels and the abundance of LH receptor-expressing granulosa cells, attributed to multiple developing follicles, leads to intensified LH signaling and subsequently augmented P production (11, 12). However, LFPE has also been observed in pituitary-desensitized COS cycles (13). Another hypothesis concerns the gonadotropin (Gn) used in COS, and studies have shown that LFPE occurs when COS is induced using both human menopausal gonadotrophin (hMG) and purified follicle-stimulating hormone (FSH) preparations (containing less than 1% LH) (14, 15), even with varying FSH dosages (16). The authors of the latter studies postulated that the number of follicles is intricately linked to LFPE, suggesting that it is a reflection of the number of recruited follicles and is not necessarily indicative of a pathological condition. A retrospective study of 687 infertile women undergoing fresh IVF/ICSI treatment with a long agonist protocol revealed that the P/O ratio may be a valuable tool for predicting IVF outcomes when compared with serum P levels alone (17). The number of studies assessing the relationship between the P/O ratio and pregnancy outcomes among pregnant patients treated with the antagonist protocol is scarce. Thus, in this study, we aimed to explore the predictive value of the P/O ratio for pregnancy outcomes in IVF/ICSI cycles among normal ovarian responders treated with the antagonist protocol.

Materials and methods

Patients and study design

This was a retrospective cohort study. The data of patients who underwent fresh IVF/ICSI embryo transfer at Peking University Third Hospital between June 2016 and June 2021 were collected. The inclusion criteria were as follows: ① maternal age between 20 and 40 years without polycystic ovary syndrome (PCOS); ② normal ovarian responders who were undergoing their first IVF/ICSI cycles; ③ patients treated with the gonadotropin-releasing hormone (GnRH) antagonist protocol; and ④ patients who underwent a transfer of 2 fresh day-3 cleavage-stage embryos. The exclusion criteria were ① patients who had undergone preimplantation genetic testing or sperm or egg donation cycles; ② patients with conditions or diseases that may influence the pregnancy rate, including hydrosalpinx, intrauterine adhesion, submucous myoma, endometrial hyperplasia, uterine malformation, or endometrial polyps or an endometrial thickness ≤7 mm; ③ patients with uncontrolled endocrine diseases, such as hyperprolactinemia or thyroid dysfunction; or ④patients with incomplete data.

Based on the available data, the P/O ratio was calculated, and patients were categorized into four groups, with the 25th, 50th and 75th percentile set as the group boundaries and P/O ratios of 0.15, 0.22 and 0.32 set as the cutoff points. The four groups were defined as follows: P/O ratio <0.15 (Group A); 0.15 ≤ P/O ratio < 0.22 (Group B); 0.22 ≤ P/O ratio <0.32 (Group C); and 0.32 ≤ P/O ratio (Group D). According to the results of our previous prospective randomized controlled clinical studies (18, 19), it is recommended that the whole embryo be frozen when P levels are ≥6 nmol/L on the day of hCG injection. Therefore, all patients included in this study had P levels <6 nmol/L on the day of hCG injection.

Treatment process

Gn [FSH (Merck Serono Company of Switzerland or Merck of America)] and hMG (Livzon Pharmaceutical Company of Zhuhai) (150–225 U/day) were used to induce ovarian stimulation on the 2nd day of menstruation or withdrawal bleeding. The initial dose of Gn was determined according to the following information: antral follicle count (AFC); serum levels of FSH, LH, estradiol (E_2), P and anti-Müllerian hormone (AMH); age; and body mass index (BMI). After 4–5 days, follicular development was monitored by ultrasonography, and the dosage of Gn was adjusted accordingly. When the diameter of the follicle was larger than 14 mm, 0.25 mg of the antagonist (Merck Serono, Germany) was applied until the trigger day. When two follicles reached a diameter of ≥17 mm, endometrial thickness was measured on the same day, and venous blood was taken to measure LH, E_2 and P levels. Recombinant hCG (Merck Serono Company of Switzerland) 250 μg or hCG 10000 IU (Livzon Pharmaceutical Company of Zhuhai) was injected that

night. After 36–38 hours, the eggs were obtained by puncture under the guidance of vaginal B-ultrasound, and IVF or ICSI was selected according to the semen analysis results. Per the routine of our center, the 2 embryos with the best scores at the cleavage stage (D3) were transferred. Luteal support was started on the day of egg retrieval, and 90 mg of P gel (Merck Serono Company, Switzerland) was given qd, 20 mg of dydrogesterone (Abbott Healthcare Products Company, Netherlands) was given orally bid, or 20 mg of P was given by intramuscular injection qd (Zhejiang Xianxian Pharmaceutical Company).

According to morphology and level of fragmentation, day-3 embryos were divided into four grades (20): Grade 1: 4–6 cells on day 2 or 6–8 cells on day 3, with evenly sized blastomeres without cellular fragments and a smooth cytoplasm without vacuoles; Grade 2: 4–6 cells on day 2 or 6–8 cells on day 3, with <20% fragmentation, unevenly sized blastomeres and/or slightly granulated cytoplasm; Grade 3: >20% but <50% fragmentation, with blastomeres/cells of all sizes and/or heavily granulated cytoplasm or vacuoles; or Grade 4: >50% fragmentation. Embryos with D2 ≥2 cells and grade III or above and embryos with D3 blastomeres ≥5 cells and grade III or above were regarded as available embryos. The P/E_2 ratio was calculated with the following formula: P (in ng/mL) × 1,000/E_2 (in pg/mL) (21).

The serum β-hCG level was measured fourteen days after embryo transfer. A positive β-hCG level greater than 25 U/L indicated the likelihood of pregnancy. At 28 to 35 days after embryo transfer, gynecological ultrasonography was conducted. Clinical pregnancy was defined as a gestational sac or primitive cardiac pulsation on ultrasound. The patients were followed up by telephone until pregnancy termination. Miscarriage was defined as a clinical pregnancy that ended before 28 gestational weeks of gestation; early miscarriage was defined as a pregnancy that ended at ≤12 gestational weeks; and late miscarriage was defined as a pregnancy that ended between 13 and 28 gestational weeks. Live birth was defined as delivery of a newborn with vital signs at 28 weeks or more of gestation.

Observation indicators

The main observation index of IVF/ICSI outcomes was the live birth rate of transplantation cycles (the number of live births/number of transplantation cycles × 100%), and the secondary observation indices were the clinical pregnancy rate (the number of clinical pregnancies/the number of transplantation cycles × 100%), early abortion rate (the number of early abortions/the number of clinical pregnancies × 100%), and implantation rate (the number of intrauterine embryos/the total number of transplanted embryos × 100%).

Statistical analysis

The SPSS 26 software package was used for statistical analysis. The clinical data of the patients were collected by qualified personnel. Normally distributed data are expressed as the mean ± standard deviation ($\overline{\chi} \pm s$). Data that conformed to a nonnormal distribution are presented as the median (25th percentile, 75th percentile) [M (P25, P75)], and categorical data are presented as the rate (%). One-way analysis of variance or the Kruskal–Wallis nonparametric test with multiple comparisons and the χ^2 test were used to analyze continuous data and categorical data, respectively. To adjust for the influence of potential confounders on the live birth rate, binary logistic regression analysis with the likelihood ratio and backward regression was used to analyze related factors.

A two-sided P <0.05 was considered to indicate statistical significance among groups, and a two-sided P <0.008 was considered to indicate statistical significance between two groups of categorical data.

Results

Basic patient information

From June 2016 to June 2021, 18,813 women who experienced infertility underwent their first cycles of IVF/ICSI treatment with the antagonist protocol in our center. According to the inclusion and exclusion criteria, 12,874 cycles were included, and 5,939 cycles were excluded. The clinical characteristics of each group are shown in Table 1. Age was higher in Group D than in the other three groups (P<0.05). The AMH level in Group D was lower than that in the other three groups, but the baseline FSH level was higher than that in the other three groups (P<0.05). The AFC in Group D was lower than that in each of the other groups (P<0.05). The BMI of patients in Group D was lower than those in Group A and Group D (P<0.05). There were significant differences in the percentage of patients with primary infertility and the percentage of patients with infertility factors (Table 1).

Ovarian stimulation

The dosage of Gn, duration of Gn, LH, P and P/E_2 ratio on the day of hCG injection, and the P/O ratio were highest in Group D and lowest in Group A, with significant differences (P<0.05). The E_2 level on the day of hCG injection and endometrial thickness on the day of hCG injection in Group D were significantly lower than those in the other three groups (P<0.05). Among patients who underwent ICSI, the proportion of MII oocytes was significantly higher in groups D and C than in groups A and B (P<0.05). The ICSI fertilization rate in Group D was significantly lower than those in the other groups (P<0.008). The number of available embryos in Group D was significantly lower than that in the other three groups (P<0.05). The ratio of available embryos to retrieved oocytes was significantly higher in group D than in the other groups (P<0.008) (Table 2).

Pregnancy outcomes

The results of the IVF/ICSI procedures are presented in Table 3. The implantation rate of fresh embryos, clinical pregnancy rate and live birth rate decreased with increasing P/O ratio, with the lowest

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TABLE 1 Baseline characteristics of the patients in each group.

Items	Group A	Group B	Group C	Group D	χ2/F	P value
No. of patients	3038	3228	3436	3172		
Age (years)	31.1 ± 3.8	31.5 ± 3.9[a]	32.1 ± 3.9[ab]	32.9 ± 3.9[abc]	131.404	0.000
AMH (ng/ml)	2.8 (2.2–4.2)	2.8 (2.0–3.9) [a]	2.7 (1.9–3.5) [ab]	2.7 (1.8–3.2) [abc]	262.622	0.000
Baseline FSH (IU/L)	6.7 (5.5–7.3)	6.8 (5.5–7.5) [a]	6.9 (5.6–7.7) [ab]	6.9 (5.7–8.0) [abc]	78.380	0.000
Number of antral follicles	12 (10–15)	10 (9–14)[a]	10 (8–12)[ab]	9 (6–11)[abc]	981.794	0.000
BMI (kg/m^2)	22.5 (20.4–24.8)	22.2 (20.3–24.5) [a]	21.9 (20.0–24.1) [ab]	21.7 (19.8–23.9) [ab]	84.070	0.000
Primary infertility (%)	56.1	58.7	58.5	61.6	19.611	0.000
Infertility factors					79.174	0.000
Female factor	34.2	37.9	40.2	42.8		
Male factor	37.9	35.6	34.8	30.5		
Bilateral factors	22.1	21.4	20.8	22.9		
Unexplained factors	5.8	5.1	4.2	3.7		

a indicates P<0.05 compared with Group A; b indicates P<0.05 compared with Group B; c indicates P<0.05 compared with Group C.

rates in Group D (all P<0.008). Both the implantation rate and fresh cycle live yield in Group C were lower than those in Groups A and B, respectively (P < 0.008). The clinical pregnancy rate in Group C was lower than that in Group C (P < 0.008), while there were no significant differences among the remaining groups (P>0.008). There was no significant difference in the early spontaneous abortion rate among the groups (P>0.05).

A subgroup analysis was conducted on the P/O ratio at a significance level of 0.05. The results revealed a negative correlation between P levels and both the clinical pregnancy rate and live birth rate (Figure 1). Linear-by-linear association analysis demonstrated a significant decrease in the implantation rate, clinical pregnancy rate, and fresh-cycle live birth rate with an increase in the P/O ratio (*P*=0.000). Conversely, there was a

TABLE 2 Ovarian stimulation in the patients in each group.

Items	Group A	Group B	Group C	Group D	χ2/F	P value
No. of patients	3038	3228	3436	3172		
dosage of Gn (U)	1875 (1425–2400)	2100 (1650–2700) [a]	2325 (1800–2850) [ab]	2625 (2100–3150) [abc]	1198.753	0.000
duration of Gn (d)	10.0 ± 1.6	10.1 ± 1.6	10.2 ± 1.5[a]	10.3 ± 1.7[ab]	13.600	0.000
LH on hCG injection day (IU/L)	1.4 (0.9–2.2)	1.8 (1.1–2.8) [a]	1.9 (1.1–3.1) [ab]	2.1 (1.2–3.6) [abc]	454.564	0.000
E_2 on hCG injection day (pmol/L)	6834 (5110–9382)	6953 (5167–9963)[a]	7164 (5270–10235) [ab]	6498 (4574–9276) [abc]	90.621	0.000
P on hCG injection day (nom/L)	1.4 (1.1–1.7)	2.1 (1.6–2.5) [a]	2.6 (2.0–3.3) [ab]	3.2 (2.4–4.9) [abc]	5280.862	0.000
P/E_2 ratio	0.23 (0.17–0.31)	0.33 (0.24–0.44) [a]	0.40 (0.31–0.53) [ab]	0.55 (0.42–0.74) [abc]	4695.487	0.000
P/O ratio	0.11 (0.09–0.13)	0.18 (0.16–0.20) [a]	0.26 (0.24–0.29) [ab]	0.43 (0.37–0.54) [abc]	12072.402	0.000
Endometrial thickness on hCG day(cm)	10.8 ± 1.4	10.7 ± 1.4	10.6 ± 1.4[ab]	10.6 ± 1.5[ab]	18.199	0.000
No. of oocytes retrieved	13 (11–16)	12 (9–14) [a]	10 (8–13) [ab]	7 (5–9) [abc]	3928.658	0.000
MII oocyte rate (%)	76.3	76.6	79.4 [ab]	81.0[ab]	118.840	0.000*
ICSI rate (%)	43.9	41.3	41.5	38.4 [abc]	19.611	0.000*
Available embryo number	4 (3–7)	3 (2–5) [a]	3 (2–5) [ab]	2 (2–3) [abc]	988.474	0.000
Ratio of available embryos to retrieved oocytes (%)	35.9	35.8	36.7	40.9[abc]	106.512	0.000

a indicates P<0.05 compared with Group A; b indicates P<0.05 compared with Group B; c indicates P<0.05 compared with Group C. * indicates a significant difference between the two groups (P<0.008).

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TABLE 3 Pregnancy outcomes of the patients in each group.

Items	Group A % (n)	Group B % (n)	Group C % (n)	Group D % (n)	$\chi 2$	P value	Linear-by-linear association $\chi 2$	P value
Implantation rate	31.4 (1907/6067)	29.9 (1928/6456)	26.7 (1834/6872)[ab]	22.5 (1427/6344)[abc]	110.069	0.000*	95.731	0.000
Clinical pregnancy rate	45.1 (1371/3038)	43.2 (1395/3228)	39.6 (1362/3436)[b]	33.4 (1060/3172)[abc]	103.923	0.000*	97.615	0.000
Early spontaneous abortion rate	10.2 (140/1371)	10.2 (142/1395)	12.1 (165/1362)	12.7 (135/1060)	6.457	0.091	5.558	0.018
Fresh cycle live yield	39.0 (1185/3038)	37.2 (1202/3228)	33.5 (1151/3436)[ab]	28.2 (893/3172)[abc]	87.870	0.000*	72.448	0.000

a indicates P<0.05 compared with Group A; b indicates P<0.05 compared with Group B; c indicates P<0.05 compared with Group C. * indicates a significant difference between the two groups (P<0.008).

significant increase in the early spontaneous abortion rate with increasing P/O ratio (P=0.018, Table 3).

Multivariate binary logistic analysis of factors affecting live births

Univariate binary logistic regression analysis showed that age, BMI and P/O ratio were negatively correlated with live births (P<0.05), but AFC, the AMH level, the LH level and endometrial thickness on hCG injection day, the number of oocytes retrieved and available embryo number were positively correlated with live births (P<0.05), the basic FSH level, infertility factors, primary infertility, E_2 on hCG injection day, the dosage of Gn, duration of Gn and ICSI were not significantly correlated with live births (P>0.05) (Supplementary Table 1).

Multivariate binary logistic regression analysis with the likelihood ratio and backward regression was conducted to assess potential influencing factors, including age, baseline FSH, AMH, AFC, BMI, primary infertility status, infertility factors, duration of Gn, Gn dosage, LH and E_2 levels on hCG injection day, endometrial thickness, the number of available embryos, and the P/O ratio. Age, BMI and the P/O ratio were negatively correlated with the number of live births (P<0.05) but that the AFC, available embryo number, LH level and endometrial thickness on the hCG injection day were positively correlated with the number of live births (P<0.05) (Table 4).

Discussion

In this study, the linear-by-linear association results showed that the implantation rate, clinical pregnancy rate and fresh-cycle live birth rate decreased with increasing P/O ratio. Furthermore, multivariate binary logistic regression analysis confirmed these findings, indicating a significant negative correlation between the P/O ratio and live births, particularly when the P/O ratio was ≥0.22.

LFPE is influenced by numerous other factors. Recently, it has also been reported that the degree of P elevation varies with the type of ovarian response. Among the poor responders, the LFPE levels

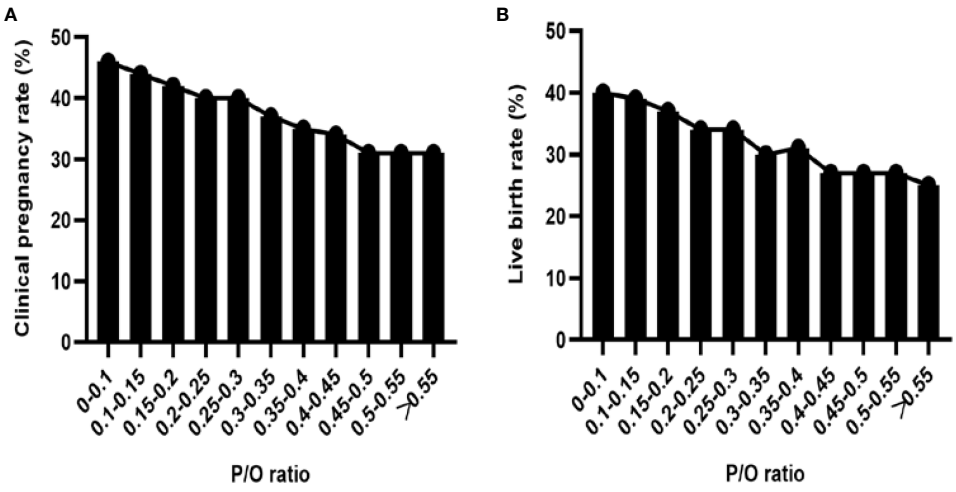

FIGURE 1
(A) Clinical pregnancy rate of subgroups with different P/O ratios. **(B)** Live birth rate in each subgroup according to the P/O ratio.

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TABLE 4 Multivariate binary logistic regression analysis of factors affecting live birth.

Influencing factor	P value	OR (95% CI)
Age (years)	0.000	0.957 (0.948–0.967)
AFC	0.009	1.012 (1.003–1.021)
BMI (kg/m^2)	0.000	0.978 (0.968–0.989)
P/O ratio	0.000	
P/O ratio <0.15		Reference
0.15 nmol/L ≤ P/O ratio <0.22	0.514	0.966 (0.869–1.073)
0.22 ≤ P/O ratio <0.32	0.006	0.862 (0.774–0.959)
P/O ratio ≥ 0.32	0.000	0.717 (0.637–0.806)
LH on hCG injection day (IU/L)	0.000	1.087 (1.067–1.107)
Endometrial thickness on hCG day (cm)	0.000	1.127 (1.098–1.157)
Available embryo number	0.000	1.064 (1.048–1.080)

were consistently lower, averaging 1.5 ng/ml. Intermediate responders had slightly higher levels, averaging 1.75 ng/mL, while high responders had the highest LFPE levels, averaging 2.25 ng/mL (22). In previous study, the ovulation induction protocol was shown to be is one of the factors. Compared to the antagonist protocol, the GnRH agonist protocol was found to be associated with a substantial increase in P levels (≥ 6.2 nmol/L) (23). To ensure the reliability of our findings, we limited our study to infertile patients with a normal ovarian response who underwent the antagonist protocol.

LFPE was found to be positively correlated with the E_2 level on the day of hCG injection but not with the pregnancy rate (24). Further study on the relationship between LFPE and E_2 revealed that there was no significant threshold value for the trigger-day P/E_2 ratio that was beneficial for predicting a live birth of GnRH antagonist cycles (25); however, in another study, P/E_2 > 0.55 affected the clinical pregnancy rate of women undergoing long agonist protocols and cleavage-stage but not blastocyst-stage embryo transfer (26). Given the positive correlation between E_2 and P levels, which may negatively impact endometrial receptivity, the predictive value of the P/follicle ratio for ART outcomes was evaluated in another study. In a group of 8649 normal responders, a LFPE-to-follicle ratio ≥14 mm was superior to the LFPE-to-follicle ratio alone in the prediction of clinical pregnancy (27). Due to potential variations in the interpretation of ultrasound examination results among observers, some authors have utilized the number of oocytes retrieved as a replacement for the number of follicles. In this study, the authors assessed the ability of the P/O ratio to predict ART outcomes in 687 infertile women undergoing treatment with long agonist protocols, fresh day-3 or day-5 embryos were transferred. The results indicated that the detrimental cut-off value for the P/O ratio was >0.15, with a sensitivity of 62% and specificity of 61%. Patients with a P/O ratio ≤0.15 had a significantly higher pregnancy rate (35.3%, [p < 0.001]) than did patients with a P/O ratio >0.15 (18.8%). A prospective study including 200 patients who underwent surgery with a long agonist protocol and whose embryos were transferred on day 3 or day 5 revealed that the P/MII oocyte ratio was significantly lower in patients who achieved clinical pregnancy than in those who could not, and using a cutoff value of 0.125, the sensitivity and specificity of the P/MII ratio in the prediction of no pregnancy in IVF/ICSI were 75.7% and 77.1%, respectively, with the area under the receiver operating curve (ROC-AUC) = 0.808 (28). We also found similar results in patients treated with the antagonist protocol in the present study. However, the results from a retrospective study including 6157 patients with agonist or antagonist COH revealed that in a multivariate analysis, the P/O ratio was not significantly associated with live birth but that P was independently associated, suggesting that the P/O ratio added no additional predictive value to the two variables separately and that the number of follicles or oocytes did not protect against the negative impact of P on live birth rates (29). This difference may be due to the older age of the patients (median age of 35 years), the percentage of embryos transferred on day 3 or day 5, and the number of embryos transferred. A previous prospective randomized controlled study in our center showed that the implantation rate, clinical pregnancy rate and live birth rate of fresh cycle embryo transfer in patients with an hCG injection day P ≥6 nmol/L were significantly reduced but that the pregnancy outcomes of fresh-cycle blastocyst transfers were significantly better than those of D3 embryo transfers (18). Therefore, in the current study, we focused solely on D3 embryo transfer data for our analysis.

FSH actively promotes P synthesis and output from granulosa cells without luteinization by upregulating the expression and increasing the enzymatic activity of 3β-hydroxysteroid dehydrogenase (3β-HSD), which converts pregnenolone to P (30). A correlation may exist between the number of hormonally active follicles and LFPE; thus, patients with more follicles usually have LFPE (9). However, from this study, we found the opposite result: in the group with the highest P/O ratio, the P level on the day of hCG injection was the highest, but the number of oocytes retrieved was the lowest, and vice versa. Interestingly, the numbers of retrieved oocytes and available embryos were lower in the group with the highest P/O ratio than in the other three groups; however, the percentages of MII oocytes were similar, and the ratio of available embryos to retrieved oocytes was the highest among the groups. These findings suggest that the group with the highest P/O ratio may have greater potential to develop into available embryos, which is consistent with the results of these studies (31–33). These authors reported that LFPE had no impact on oocyte/embryo quality. However, the implantation rate, clinical pregnancy rate and live birth rate of fresh-cycle pregnancies were significantly lower in the group with an increase in the P/O ratio than in the other groups, which supports the detrimental effect of LFPE on pregnancy outcomes via its effect on the endometrium (34). It was found that LFPE may change the endometrium from the proliferative phase to the secretory phase in advance, resulting in the unsynchronized development of the endometrium and embryo and subsequently affecting embryo implantation (35, 36). This was reinforced by evidence showing changes in endometrial gene

expression. There were 140 gene disorders in the endometrium on the day of hCG injection in the P>4.77 nmol/L group compared with the P<4.77 nmol/L group (37), and LFPE can inhibit HOXA10 by promoting the expression of miR-135a, thus changing the expression of related genes and affecting endometrial receptivity (38).

Previous studies have focused on the detrimental effects of LFPE on clinical pregnancy and live birth, but in our study, the relationship between the P/O ratio and early spontaneous abortion was explored. Although the early spontaneous abortion rate tended to increase with increasing P/O ratio, the difference was not significant, which corroborates previous observations (39).

The main strengths of this study include the large sample size and adjustment for potential confounders, such as patient age, ovarian response, COS protocol, day of embryo transfer, and number of embryos transferred. Moreover, PCOS is a prevalent endocrine disorder characterized by a diverse range of clinical phenotypes, including hyperandrogenemia, menstrual disorders and polycystic ovary morphology. Patients with PCOS exhibit increased sensitivity to Gn, leading to higher follicle production than in normal individuals (40), that is associated with adverse pregnancy outcomes, such as a low embryo implantation rate, clinical pregnancy rate, and live birth rate of fresh cycle embryo transfer, as well as an elevated miscarriage rate (41). Consequently, individuals afflicted with PCOS were excluded from this study. The main limitation arises from its retrospective nature. Despite the use of strict inclusion criteria regarding patient age and ovarian function, significant differences in basic characteristics persist among the groups. In the group with the highest P/O ratio, patients were older, had higher basic FSH levels, and had fewer antral follicles. These factors may account for the longer COS duration, higher Gn dosage, and lower number of retrieved oocytes. The age range of patients was too large, that may have an impact on pregnancy outcome. Additionally, this study did not differentiate the effects of hMG and purified FSH on pregnancy outcomes, which may impact the likelihood of LFPE (14, 15).

In conclusion, the rates of implantation, clinical pregnancy, and live birth in fresh-cycle embryo transfer decreased progressively with an increase in the P/O ratio, reaching significance when the ratio was ≥0.22. Based on these findings, we postulate that the P/O ratio has predictive value for pregnancy outcomes in IVF/ICSI procedures. Therefore, whether to carry out fresh embryo transfer in patients with LFPE and few retrieved oocytes should be carefully considered. However, due to the retrospective design of this study, randomized trials on potential biological mechanisms are necessary to further investigate the impact of the P/O ratio on embryo development and endometrial receptivity in the future.

Data availability statement

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

Ethics statement

The studies involving humans were approved by the Reproductive Medicine Ethics Committee of Peking University Third Hospital. Ethics No.: 2018SZ-001. 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

HZ: Data curation, Formal Analysis, Investigation, Methodology, Writing – original draft. SY: Formal Analysis, Methodology, Writing – review & editing. LC: Data curation, Writing – review & editing. CM: Writing – review & editing. PL: Writing – review & editing. JQ: Writing – review & editing. RL: Formal Analysis, Funding acquisition, Methodology, 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.2024.1338683/full#supplementary-material

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Trends in acupuncture for infertility: a scoping review with bibliometric and visual analysis

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Background: Unexplained recurrent implantation failure and the high cost of assisted reproductive techniques for those experiencing infertility have increasingly resulted in the use of acupuncture. However, the trends and research status of acupuncture on infertility resulting in natural conception have not been systematically summarized. This scoping review and knowledge graph analysis aimed to summarize existing clinical studies on acupuncture for infertility that resulted in natural conception.

Methods: Seven databases, namely, PubMed, Embase, the Cochrane Library, CNKI, VIP, Wanfang Data, and SinoMed, were searched up to August 2023 (updated on 1 April). Two authors independently identified related clinical studies and systematic reviews, and extracted data from included studies on acupuncture for infertility; any discrepancies were resolved by discussion or judged by a third author. A meta-analysis was conducted based on randomized controlled trials (RCTs), and data were synthesized using risk ratios with 95% confidence intervals.

Results: Of the 310 articles meeting the inclusion criteria, 274 were primary studies, 7 were systematic reviews, and 29 were case reports. Reported adverse events included mild ovarian irritation and early signs of miscarriage. Out of the 274 primary studies, there were 40 (14.60%) cases of male infertility and 234 (85.40%) cases of female infertility. Current research highlights on acupuncture for infertility focused on female infertility caused by polycystic ovary syndrome, ovulation disorder, and luteinized unruptured follicle syndrome (LUFS), while acupuncture for male infertility was a hotspot in the early research stage. The meta-analysis also suggested that acupuncture was more effective than human chorionic gonadotropin (HCG) [RR = 1.89, 95% CI (1.47, 2.42), 11 RCTs, 662 participants]. Acupuncture combined with HCG was comparable to HCG [RR = 2.33, 95% CI (1.53, 3.55), four RCTs, 259 participants]. Compared with no

treatment, acupuncture resulted in a higher pregnancy rate [RR = 22.12, 95% CI (1.39, 353.09), one RCT, 47 participants]. There was no statistical difference between acupuncture combined with HCG plus letrozole and HCG plus letrozole [RR = 1.56, 95% CI (0.84, 2.89), one RCT, 84 participants].

Conclusion: Current research highlights on acupuncture for infertility resulting in natural conception focused on female infertility caused by polycystic ovary syndrome, ovulation disorder, and LUFS, while studies on male infertility and female infertility caused by blockage in the fallopian tube, thin endometrium, and other factors were insufficient. Well-designed confirmatory clinical studies are still needed as the research hypotheses of most studies were unclear.

KEYWORDS

infertility, natural conception, acupuncture, scoping review, bibliometric and visual analysis

1 Introduction

Infertility is defined as the failure to become pregnant within 1 year of regular and unprotected intercourse, which may be related to a number of factors or unexplained reasons (1). As the problem may lead to a series of psychological distress, social stigmatization, economic strain, and even marital discord, it has been considered as a public health priority (2). Globally, the disability-adjusted life-years (DALYs) and the global disease burden of infertility also increased for both women and men throughout 1990 to 2017, and the age-standardized prevalence infertility for women and men increased by 14.96% and 8.22%, respectively, for this period (3). It has affected at least 180 million reproductive-aged couples worldwide (4).

In general, the prevalence of infertility among women is higher than among men; for example, in UK, the estimated prevalence of infertility was 12.5% for women and 10.1% for men (5). Recent research suggests that men are solely responsible for 20%–30% of infertility cases (4, 6). Female infertility can be caused by diseases such as pelvic lesions and ovulation disorders, while male infertility can also be caused by various diseases or factors, such as dysspermia and male sexual dysfunction (4). Conventional therapy for infertility involves drug treatment (clomiphene and gonadotropin-releasing hormone analogs) and assisted reproductive techniques (ARTs), such as *in vitro* fertilization (IVF), hormonal stimulation, and intracytoplasmic sperm injection (ICSI) (7), which can overcome male and female infertility. Even uterus transplantation has been proposed as a potential choice for uterine infertility, but it also brings further ethical challenges such as the selection of the donor, the impact on the recipient and offspring, and ethical and social challenges (8). The efficacy of a sole drug can be limited, although ART has advanced the outcomes of conception for these couples, and the live birth rates, through IVF or ICSI, are between 30% and 35% (9, 10). These technologies require strict conditions during their delivery, but can cause serious health problems including antepartum hemorrhage, congenital anomalies, preterm rupture of the membranes, low birth weight, perinatal mortality, preterm delivery, and gestational diabetes. Only 20% and 35% on initiated cycles and embryo transfer cycles result in the birth of a healthy baby, respectively (11–13). The adverse effects of ART in most countries, especially in low-income and middle-income countries, and the availability, accessibility, and quality of such infertility interventions remain a major challenge due to the lack of trained personnel and the necessary equipment and infrastructure (14).

Acupuncture has a long history for the treatment of female and male reproductive disease in China. Basic research has showed that acupuncture can affect gonadotropin-releasing hormone secretion and the menstrual cycle, and improve the blood flow of uterus (15). Previous systematic reviews have shown that acupuncture can decrease the rate of pregnancy loss (16), and there are also studies that have shown that acupuncture can improve fertility outcomes and mental health in both men and women (17, 18). Owing to a series of adverse events, unexplained recurrent implantation failure, or the high cost of ART, more patients tend to choose acupuncture as a conservative treatment (17). However, the benefits of acupuncture alone or combined with Western therapy on infertility have not been systematically summarized.

A scoping review aims to map types of evidence and identify current research gaps of an exploratory clinical research question (19).

Abbreviations: ART, assisted reproductive techniques; CI, confidence interval; CNKI, China Network Knowledge Infrastructure; HCG, human chorionic gonadotropin; ICSI, intracytoplasmic sperm injection; IVF, *in vitro* fertilization; LUFS, luteinized unruptured follicle syndrome; MD, mean difference; PCOS, polycystic ovary syndrome; RCTs, randomized controlled trials; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses; ROB, risk of bias; RR, risk ratio; SMD, standardized mean difference.

There is no comprehensive summary of acupuncture for infertility that resulted in natural conception; thus, this scoping review aimed to summarize existing clinical studies on acupuncture for infertility that resulted in natural conception among the population, the acupuncture method (including source of acupuncture, frequency, and course), and the consistency between research hypotheses and conclusions.

2 Methods

This study was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines extended edition for scoping reviews (20).

2.1 Eligibility criteria

2.1.1 Types of studies

Randomized controlled trials (RCTs), non-RCTs, cohort studies, case–control studies, case series studies, case reports, and secondary studies (such as systematic reviews and/or meta-analyses, overview of systematic reviews, expert consensus or clinical guidelines) were included in this review without limitation on language, date, and form of publication.

2.1.2 Types of participants

Individuals experiencing problems with infertility caused by either female factors such as pelvic lesions and ovulation disorders or male factors such as dysspermia, male sexual dysfunction, and unexplained infertility were included.

2.1.3 Types of interventions

The intervention group was defined as acupuncture therapy alone or acupuncture plus conventional drugs (such as ovulation induction medications and hormone therapy medications) or surgeries (such as laparoscopic surgery and varicocelectomy).

The exclusion criteria were as follows: (1) acupuncture combined with Chinese herbal medicines whether in the intervention group or the control group; (2) the control group receiving acupuncture; and (3) undergoing ART treatment (including artificial insemination, embryo transfer, IVF, ISCI, pre-implantation genetic diagnosis, embryo freezing and frozen embryo transfer technology, and *in vitro* maturation technology).

2.1.4 Types of outcomes

This study mainly focused on acupuncture for natural conception. Outcomes were pregnancy rate and live birth rate for women, and pregnancy rate (their partner) and semen parameters (including concentration, total number, vitality, and normal morphology) for men.

2.2 Search strategies

A systematic search of electronic databases was performed, including PubMed, Cochrane Central Register of Controlled Trials (CENTRAL; The Cochrane Library), Cochrane Database of Systematic Reviews (CDSR; The Cochrane Library), EMBASE, China Network Knowledge Infrastructure (CNKI), China Science and Technology Journal Database, Wanfang Data, and SinoMed (searched from onset until 5 August 2023, updated on 1 April 2024); there were no language and publication restrictions. The search strategy on PubMed is given in Additional File 1: Supplementary Table S1.

2.3 Data collection and analysis

2.3.1 Selection of studies

The studies were exported to EndNote software (V20) for management. Duplicate studies were removed independently by CYZ. The remaining studies were screened independently by TP and JJZ according to the inclusion/exclusion criteria. Firstly, the two researchers excluded unrelated studies by reading their titles and abstracts, then they acquired the full text for further screening. Any discrepancies were resolved by discussion or judged by a third author.

2.4 Bibliometric analysis and data extraction

The included literature was exported into CiteSpace 6.3.R1 software to convert the data format, then a collaborative network analysis on authors and institutions from literature, co-occurrence network analysis, and citation burst on keywords were conducted. The years covered were from 1994 to 2024, with a time slice of 2 years and a cutting method including pathfinder, pruning sliced networks, and pruning the merged network.

As for the characteristics of the included literature, the list was set up by CYZ using Microsoft Excel 2007. Data extraction was completed after cross-checking by YYH and ANS; FY and YM extracted the data independently. The following data were extracted according to PRISMA and the PICO (patient, intervention, comparison and outcome) framework: first author's name, publication year, title, journal, the type of literature, language, funding, design of research, objective, patients (sample size), disease/pathogenesis, the treatment of the control group, acupoint prescriptions and their rationale for selection, frequency and duration of acupuncture treatment, adverse events, follow-up, research hypotheses, outcomes, and main conclusions.

Descriptive analysis of data was carried out by calculating frequency and percentage, which was presented by tables and charts set up in Microsoft Excel 2007.

2.5 Quality assessment and data synthesis

When conducting meta-analysis on included RCTs, two reviewers (CYZ and SHY) independently assessed the quality of RCTs by using the Cochrane Risk of Bias tool (ROB), which included random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome

assessment, incomplete outcome data, selective reporting, and publication bias. Any discrepancies were resolved by discussion or judged by a third author.

Meta-analysis was performed using Review Manager 5.3. Risk ratio (RR) with 95% confidence interval (CI) was used to measure dichotomous variables, and mean difference (MD) or standardized mean difference (SMD) was used to measure continuous variables. When results were measured on the same scale, the outcomes were reported as MD; otherwise, the results were reported as SMD. Heterogeneity test was performed by chi-square test and presented as the value of I^2 statistics. When $I^2 \leq 50\%$ and $p \geq 0.10$, the fixed-effects model was used; otherwise, the random-effects model was used. The subgroup analysis was conducted to explore any factors that might explain the heterogeneity; if there was severe heterogeneity, sensitivity analysis was conducted to explore the potential sources of heterogeneity. Overall effects with a p-value below 0.05 were considered statistically significant.

2.6 Publication bias

A funnel plot was used to assess potential publication bias in a single meta-analysis involving 10 or more trials.

3 Results

3.1 Description of studies

Of the total of 16,899 articles identified in the search, 10,442 remained after duplicates were removed. A total of 9,650 articles were subsequently removed after screening by title and abstract. After full-text screening, 295 articles were included, and after supplementary searching on 1 April 2024, 15 articles were added; thus, there were 310 articles included. Data were extracted from 274 original studies and 7 meta-analyses. The remaining 29 case reports were just identified, but data were not extracted. For the study selection results, see Figure 1.

3.1.1 Basic information of included studies

The 274 studies included 192 (70.07%) RCTs, 73 (26.64%) case series, 5 (1.82%) non-RCTs, 3 (1.09%) cohort studies, and 1 (0.36%) matched controlled study (the characteristics of the extracted 274 studies are given in Additional File 1: Supplementary Tables S2, S3). Among them, 263 studies were in Chinese (95.99%) and 11 studies were in English (4.01%). The 274 studies consisted of 238 journal papers (86.86%), 34 master's theses (12.41%), and 2 conference papers (0.73%). The 238 journal articles were published in 114 different journals. There was an increasing overall trend in the number of publications year on year (seen in Figure 2).

3.1.2 Authors and institutions

There are a total of six authors who published three articles, while the other authors published less than three articles. The authors are all relatively stable but dispersed in small-scale cooperative networks, the contribution rate of different authors to the discipline varies depending on their publication volume, the core authors with higher contribution rates gather into a core author group, and no core author group has yet been formed in this field (see Figure 3). The top 10 institutions with the highest number of publications are shown in Table 1. Considering different institutions as nodes, nodes with a centrality exceeding 0.1 are referred to as key nodes, and due to the fact that the centrality of different institutions has not reached the target value, a cooperative network between different institutions has not been formed (see Figure 4).

3.2 Keyword analysis

Citation burst on keywords can reflect the research hotspots in this field at different times. This study has obtained 11 burst terms (see Figure 5). The burst term "male infertility" lasted up to 11 years, indicating that acupuncture for male infertility was a hotspot in the early research stage, while the burst term "Sanyinjiao" reflected that this acupoint was frequently used for infertility. Abdominal acupuncture, electroacupuncture, and moxibustion reflected the researchers' exploration of various acupuncture therapies at different times. In the recent 2 years from 2021 to 2023, the acupuncture for infertility caused by polycystic ovary syndrome (PCOS) was a hotspot research direction, mainly focusing on the impact of acupuncture on pregnancy outcomes and sex hormones.

Among the 274 primary studies, there were 40 (14.60%) cases of male infertility and 234 (85.40%) cases of female infertility. In the studies of male infertility, six kinds of diseases or causes were reported, including 24 (60.00%) idiopathic male infertility, 4 (10.00%) immune infertility, and 5 (12.50%) infertility caused by other diseases or causes, such as sexual dysfunction, varicocele, and genitourinary tract infection. Six studies (17.50%) did not report the specific disease or cause of male infertility and one (2.5%) study did not report in detail. As for female infertility, 20 kinds of diseases or causes were reported, with PCOS being the most common disease involving 80 (34.19%) studies. There were 19 (8.12%) studies about luteinized unruptured follicle syndrome (LUFS), 46 (19.66%) studies about ovulation disorder, 13 (5.56%) studies about block of fallopian tube, 8 studies about thin endometrium, 5 studies (2.14%) about luteal phase defect, and 28 (11.96%) studies about other diseases or causes such as heterotopia endometriosis, adenomyosis, hyperprolactinemia, decreased ovarian reserve function, ovarian insufficiency, premature ovarian failure, posterior uterus, serum anti-sperm antibody positive infertility, follicular dysplasia, immunological infertility, endocrine infertility, and unexplained infertility. Thirty studies (12.82%) did not report the specific disease or cause of female infertility and five (2.14%) studies did not report in detail.

The results of keyword co-occurrence analysis showed that the current research highlights on acupuncture for infertility focused on female infertility caused by PCOS, ovulation disorder, and LUFS, and the acupuncture interventions with high frequency included

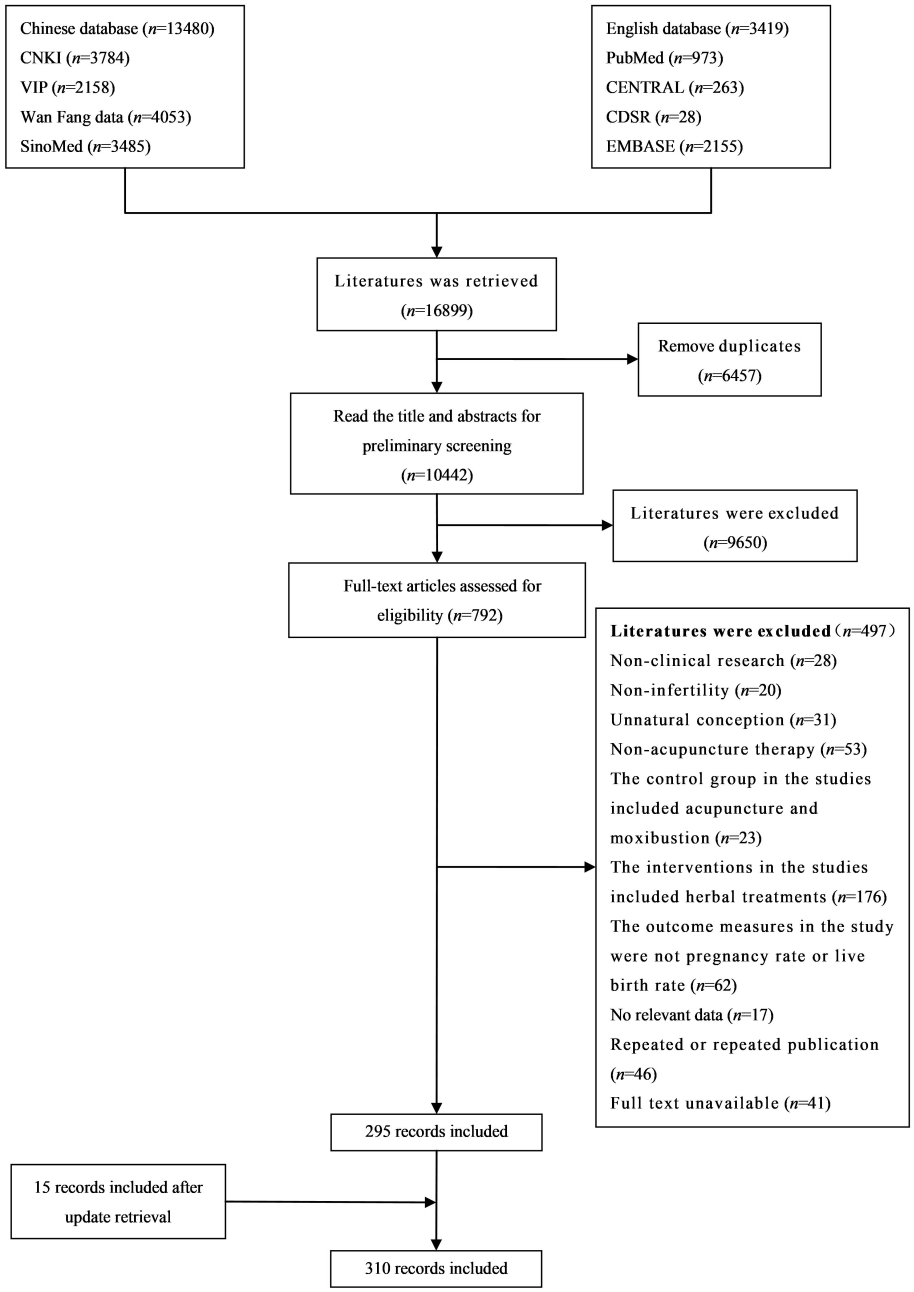


FIGURE 1
Flowchart of study selection.

acupuncture alone, moxibustion, acupoint catgut embedding, electroacupuncture, and warm acupuncture (see Table 2, Figure 6).

3.3 Interventions

3.3.1 Types of acupuncture vs. comparison

The types of acupuncture vs. comparison are listed in detail in Table 3. In 192 RCTs, the types of comparison were classified into 13 categories, among which the type of acupuncture combined with Western medicine vs. Western medicine was the most common type, with 96 studies (50%) in total. This was followed by 76 studies (39.58%) comparing acupuncture alone with Western medicine.

3.3.2 Acupoint prescriptions

Among 274 studies, the prescription of acupoint could be classified into three categories: (1) 202 (73.72%) studies with a fixed acupoint prescription (same acupoints for each patient); (2) 66 (24.09%) studies with a semi-fixed acupoint prescription (fixed acupoints combined with syndrome differentiation) according to the symptoms and characteristics of the disease; and (3) 6 (2.19%)

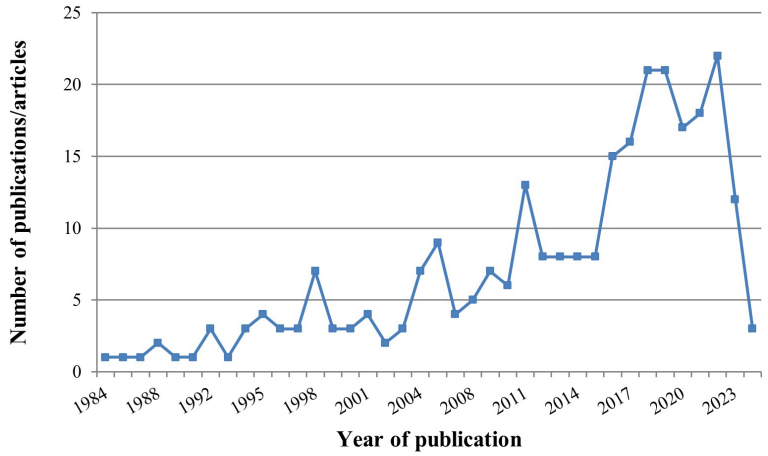


FIGURE 2
Chart of annual publications on acupuncture treatment for infertility.

studies with individualized selection acupoints, according to the characteristics of the disease.

There were four kinds of sources of acupoint prescription: (1) 9 studies (3.28%) were based on the relevant acupuncture textbooks; (2) 1 study (0.36%) was based on the relevant clinical guideline; (3) 14 studies (5.11%) were based on the experience of veteran TCM experts; and (4) 4 studies (1.46%) were based on personal clinical experience. A total of 246 studies (89.78%) did not explicitly report the source of the acupoint prescription.

3.3.3 Frequency and course of acupuncture

Among the 274 studies, 202 (73.72%) studies provided acupuncture three times or more per week, 22 (8.03%) studies provided acupuncture twice a week, 25 (9.12%) studies provided acupuncture once a week, and 27 (9.85%) studies did not clearly report the frequency of acupuncture. Of the 234 studies on female infertility, the course of acupuncture was 3 months or three menstrual cycles in 128 (54.70%) studies, 6 months or six menstrual cycles in 16 (6.84%) studies, and 56 (23.93%) studies did not explicitly report the course of acupuncture. Of the 40

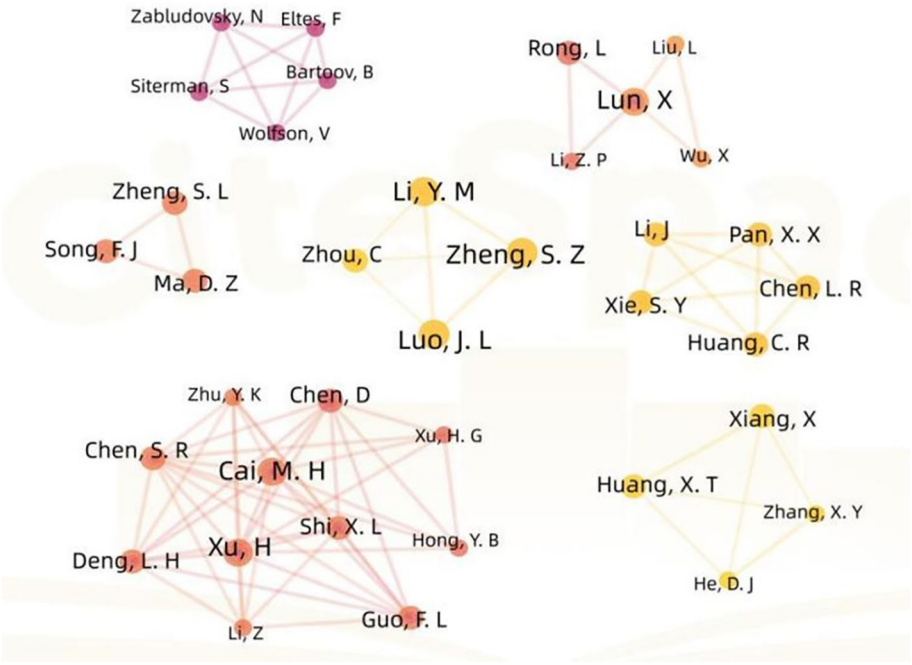


FIGURE 3
Cooperation network of authors in the field of acupuncture for infertility.

TABLE 1 Top 10 productive institutions in the field of acupuncture for infertility.

Number	Institution	Number of publications/articles
1	GZUCM	18
2	HLJUCM	5
3	SDUTCM	4
4	CDUTCM	4
5	The First Affiliated Hospital of GZUCM	4
6	Shenzhen Maternal and Child Health Care Hospital	4
7	FJTCM	3
8	Hubei Maternal and Child Health Care Hospital	3
9	The First Affiliated Hospital of TUTCM	3
10	Guangdong Hospital of Traditional Chinese Medicine	3

GZUCM, Guangzhou University of Chinese Medicine; HLJUCM, Heilongjiang University of Chinese Medicine; SDUTCM, Shandong University of Traditional Chinese Medicine; CDUTCM, Chengdu University of Traditional Chinese Medicine; FJTCM, Fujian University of Traditional Chinese Medicine; TUTCM, Tianjin University of Traditional Chinese Medicine.

studies on male infertility, 10 (25%) studies had a 3-month acupuncture course and 23 (57.5%) did not explicitly report this information.

3.4 Qualification of acupuncturist

There was just one RCT published in English that reported training the acupuncturist at the beginning of the study.

3.5 Outcomes and results

Of the 40 studies on male infertility, 12 studies (30%) defined pregnancy rates as outcome; 14 studies (35%) used sperm motility, quantity, and fragmentation as outcomes; and 14 studies (35%) included these two outcomes.

Of the 234 female studies, only 1 used both pregnancy rate and live birth rate as outcome; the remaining 233 studies only reported pregnancy rate as outcome.

As for the results, among 192 RCTs, 147 RCTs (76.56%) had positive results ($p < 0.05$) and concluded that the acupuncture group may have a better effect than the control group, and 28 RCTs (14.58%) reported negative results ($p > 0.05$) and concluded that there were no differences between acupuncture groups and control groups. There were 17 studies that did not report *p*-values. Of the five included CCTs, four studies reported positive results while the rest reported negative results. The three cohort studies reported positive results; one matched control study reported negative results.

3.6 Funding sources

Seventy-three studies reported funding sources, including 12 (16.44%) national projects, 54 (73.97%) provincial projects, and 7 (9.60%) college and university projects.

3.7 Adverse events

There were 34 studies providing adverse event reports, among which 8 studies reported no adverse events after acupuncture treatment and 26 studies reported adverse events including mild bleeding, sluggishness, dizziness, fatigue, nausea, vomiting, abdominal discomfort, mild ovarian stimulation, threatened abortion, weight gain, and visual disturbances.

3.8 Follow-up

Of the 274 studies, only 70 (25.55%) reported follow-up information after completion of acupuncture, with a minimum follow-up period of 3 weeks and a maximum follow-up period of 3 years. The remaining 204 (74.45%) studies did not mention follow-up after the trials.

3.9 Meta-analysis of acupuncture for LUFS infertility

Among the seven included meta-analyses, acupuncture for infertility caused by PCOS, ovulation dysfunction, and anovulatory have been published in the recent 4 years (21–24); the details about the seven included meta-analyses are provided in Supplementary Table S5. Except for LUFS, the number of RCTs for male infertility and female infertility caused by other diseases were less than three; therefore, we only conducted meta-analysis of acupuncture for LUFS infertility.

3.9.1 Characteristics of the included 17 RCTs

There were 17 included RCTs on female infertility caused by LUFS (25–41). All studies were published in Chinese. There were 1,099 patients involved, with 553 in the intervention group and 546 in the control group. The minimum sample size in the study

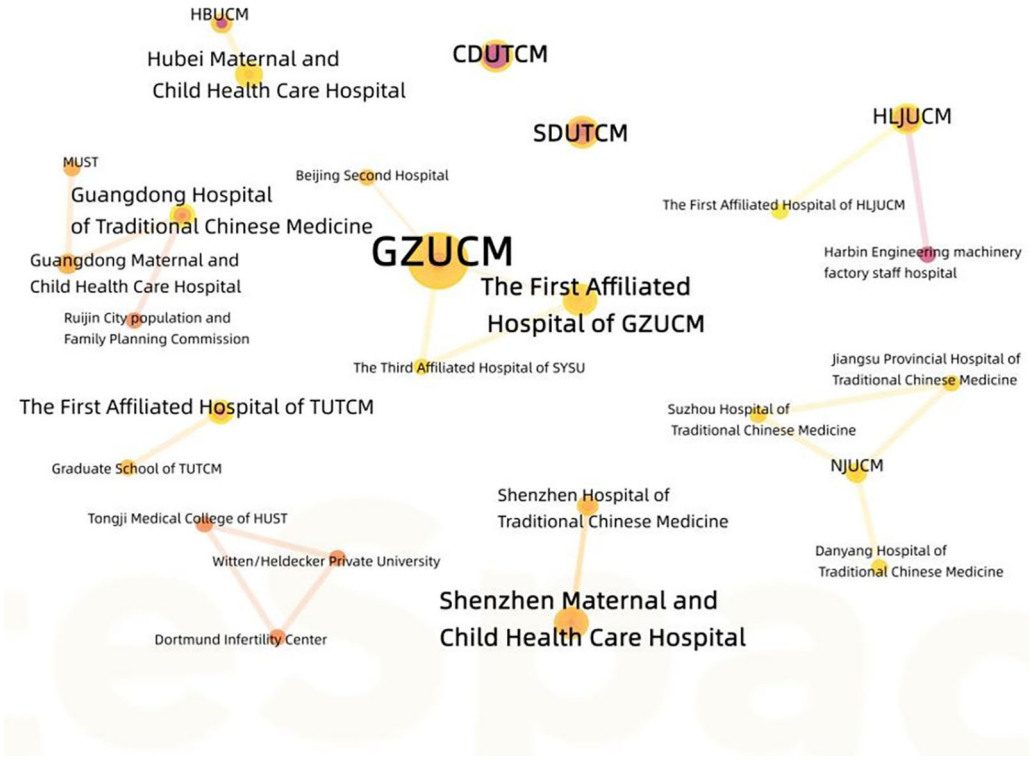
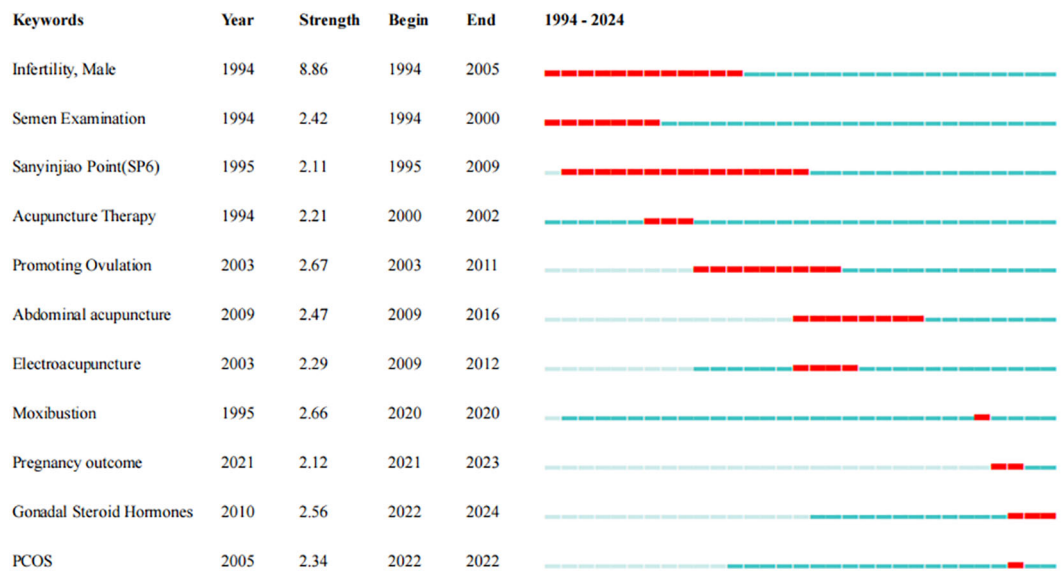


FIGURE 4
Cooperation network of institutions in the field of acupuncture for infertility.

was 50, and the maximum was 84. One study (37) did not report diagnostic criteria. Only three studies (29, 30, 32) reported follow-up time, while the remaining studies did not report follow-up information. The basic characteristics of the included RCTs are shown in Table 4.

3.9.2 Quality assessment of the included RCTs

The risk of bias on primary outcome in 17 RCTs is summarized in Figures 7 and 8. Nine RCTs used the random number table and computer software to generate random sequences, which was considered to have a low risk of bias. Eight RCTs (25, 26, 29, 31,



Keywords	Year	Strength	Begin	End	1994 - 2024
Infertility, Male	1994	8.86	1994	2005	
Semen Examination	1994	2.42	1994	2000	
Sanyinjiao Point(SP6)	1995	2.11	1995	2009	
Acupuncture Therapy	1994	2.21	2000	2002	
Promoting Ovulation	2003	2.67	2003	2011	
Abdominal acupuncture	2009	2.47	2009	2016	
Electroacupuncture	2003	2.29	2009	2012	
Moxibustion	1995	2.66	2020	2020	
Pregnancy outcome	2021	2.12	2021	2023	
Gonadal Steroid Hormones	2010	2.56	2022	2024	
PCOS	2005	2.34	2022	2022	

FIGURE 5
Citation burst on keyword.

TABLE 2 Top 14 keywords.

Number	Keywords	Count	Centrality
1	Infertility, female	141	0.56
2	Acupuncture therapy	141	0.83
3	Polycystic ovary syndrome	71	0.16
4	Clinical research	43	0.16
5	Anovulation	40	0.06
6	Infertility, male	25	0.15
7	Moxibustion	20	0.07
8	Clomiphene	18	0.02
9	Acupoint catgut embedding	17	0.08
10	Electroacupuncture	16	0.03
11	Luteinized unruptured follicle syndrome	14	0.03
12	Promoting ovulation	11	0.03
13	Endometrial receptivity	10	0.03
14	Warm needling	10	0

32, 36, 37, 39) only mentioned "random" but did not report the details, and the risk of bias was considered to be unclear. Three RCTs reported the use of opaque envelopes as their random sequence concealment methods (27, 28, 34), which were considered to have a low risk of bias. Others did not report this information and were considered to have unclear risk of bias. All studies did not report the use of blinding, and the risk of bias was considered to be unclear. As for incomplete outcome data, one RCT (26) reported that the number of dropouts and deletions exceeded 20% of the total sample size, indicating a high risk of bias. Other studies were evaluated as having a low risk of bias. Four RCTs (30, 31, 35, 39) did not report predetermined outcomes and were evaluated as having a high risk of bias; three RCTs (25, 33, 36) did not report the study protocol and registration information and were considered to have unclear risk of bias; the remaining 10 RCTs were considered to have a low risk of bias. Because of insufficient reporting on items such as random allocation methods, random sequence concealment, and implementation of blinding in the included studies, as well as a high risk of methodological bias, the overall methodological quality of the included RCTs was not high.

3.9.3 Pregnancy rate of acupuncture for LUFS infertility

3.9.3.1 Acupuncture vs. human chorionic gonadotropin

The fixed-effects model meta-analysis showed that compared with HCG therapy, acupuncture therapy a resulted in a higher pregnancy rate [RR = 1.89, 95% CI (1.47, 2.42), 11 RCTs, 662 participants, $p < 0.00001$] (see Figure 9).

3.9.3.2 Acupuncture + HCG vs. HCG

The fixed-effects model meta-analysis showed that compared with HCG therapy alone, acupuncture combined with HCG therapy resulted in a higher pregnancy rate [RR = 2.33, 95% CI (1.53, 3.55), four RCTs, 259 participants, $p < 0.0001$] (see Figure 10).

3.9.3.3 Acupuncture vs. no treatment

One RCT (27) reported that compared with no treatment, acupuncture resulted in a higher pregnancy rate [RR = 22.12, 95% CI (1.39, 353.09), one RCT, 47 participants, $p = 0.03$].

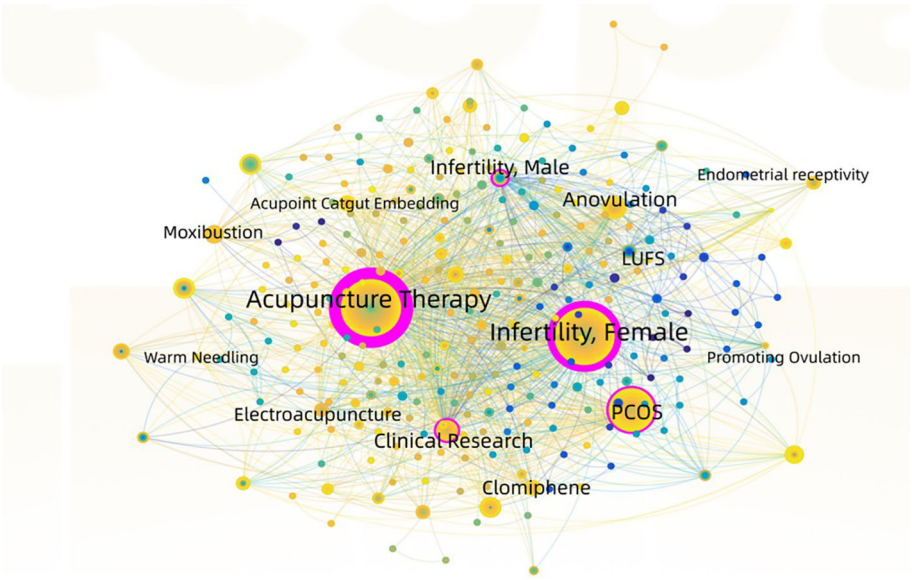


FIGURE 6
Co-occurrence map of keyword.

TABLE 3 Comparison types of acupuncture versus control group.

Study types	Comparison types	Number of studies
RCT	Acupuncture combined with Western medicine vs. Western medicine	96
	Acupuncture vs. Western medicine	76
	Acupuncture combined with surgery vs. Surgery	5
	Acupuncture combined with Western medicine vs. Western medicine vs. Acupuncture	4
	Acupuncture vs. Sham acupuncture	3
	Acupuncture vs. Western medicine vs. Condom isolation	1
	Acupuncture combined with Western medicine vs. Fake acupuncture combined with Western medicine vs. Acupuncture	1
	Acupuncture combined with behavioral therapy combined with Western medicine vs. Western medicine	1
	Acupuncture combined with infrared irradiation vs. Blank	1
	Acupuncture combined with Western medicine and endovascular therapy vs. Western medicine	1
	Acupuncture combined with guidance vs. General treatment	1
	Acupuncture combined with lifestyle intervention and Western medicine vs. lifestyle intervention and Western medicine	1
	Acupuncture combined with core muscle rehabilitation exercise and Western medicine vs. Western medicine	1
CCT	Acupuncture combined with Western medicine vs. Western medicine	2
	Acupuncture vs. Western medicine	1
	Acupuncture combined with hysterosalpingography vs. Hysterosalpingography	1
	Western medicine A combined with acupuncture vs. Western medicine A combined with B vs. Western medicine A combined with Western medicine C	1
Matched control study	Acupuncture combined with Western medicine vs. Western medicine	1
Cohort study	Acupuncture vs. Western medicine	1
	Acupuncture combined with Western medicine vs. Western medicine	2

Western medicine: clomiphene citrate, letrozole, human chorionic gonadotropin, urinary gonadotropin, human menopausal gonadotropin, diethylstilbestrol, estradiol valerate, deoxyprogesterone, progesterone, progesterone, progesterone acetate, progesterone, estradiol/estradiol flexor progesterone, methylprednisolone, prednisone, metformin, bromocriptine mesylate, and aspirin. Operation: pelvic adhesiolysis, tubal fluid opening, tubal umbrella endoplasty, tubal umbrella endostomy, and ultrasonic crystal oxygen salpingography.

TABLE 4 Characteristics of included RCTs of acupuncture for luteinized unruptured follicle syndrome.

Included studies	Sample size	Age	Treatment group interventions	Control group interventions	Course of treatment	Outcome
(41)	T: 36 C: 36	T:/ C:/	Acupuncture + HCG	HCG	4 weeks	Pregnancy rate
(40)	T: 30 C: 30	T: 32.51 ± 2.75 C: 31.79 ± 3.62	Acupuncture	HCG	3 menstrual cycles	Pregnancy rate
(38)	T: 36 C: 36	T: 23–38 C: 22–38	Acupuncture + HCG	HCG	3 menstrual cycles	Pregnancy rate
(39)	T: 43 C: 41	T:/ C:/	Acupuncture + HCG + letrozole	HCG + letrozole	3 menstrual cycles	Pregnancy rate
(35)	T: 30 C: 30	T: 29.30 ± 3.67 C: 28.37 ± 3.79	Acupuncture	HCG	No report	Pregnancy rate
(37)	T: 33 C: 33	T: 23–40 C: 23–40	Acupuncture	HCG	3 menstrual cycles	Pregnancy rate

(Continued)

TABLE 4 Continued

Included studies	Sample size	Age	Treatment group interventions	Control group interventions	Course of treatment	Outcome
(36)	T: 40 C: 40	T: 25.8 ± 2.56 C: 24.8 ± 2.16	Acupuncture	HCG	3 menstrual cycles	Pregnancy rate
(34)	T: 30 C: 30	T: 28.27 ± 3.84 C: 28.85 ± 2.13	Acupuncture	HCG	3 menstrual cycles	Pregnancy rate
(33)	T: 32 C: 32	T: 29.59 ± 3.44 C: 31.22 ± 12.79	Acupuncture	HCG	3 menstrual cycles	Pregnancy rate
(32)	T: 30 C: 30	T: 31.87 ± 3.58 C: 31.77 ± 3.63	Acupuncture + HCG	HCG	1 month	Pregnancy rate
(31)	T: 30 C: 30	T:/ C:/	Acupuncture	HCG	No report	Pregnancy rate
(29)	T: 33 C: 32	T: 29.39 ± 4.39 C: 29.22 ± 4.15	Acupuncture	HCG	3 months	Pregnancy rate
(30)	T: 30 C: 26	T: 30.27 ± 3.44 C: 30.07 ± 2.89	Acupuncture	HCG	3 menstrual cycles	Pregnancy rate
(28)	T: 25 C: 25	T: 30.48 ± 4.03 C: 29.71 ± 3.05	Acupuncture	HCG	3 menstrual cycles	Pregnancy rate
(27)	T: 25 C: 25	T: 30.88 ± 3.37 C: 31.60 ± 4.56	Acupuncture	No treatment	No report	Pregnancy rate
(26)	T: 40 C: 40	T: 29.18 ± 2.63 C: 29.00 ± 2.71	Acupuncture + HCG + endovascular therapy	HCG	3 menstrual cycles	Pregnancy rate
(25)	T: 30 C: 30	T:/ C:/	Acupuncture + HCG	HCG	3 menstrual cycles	Pregnancy rate

T, treatment group; C, the control group; HCG, human chorionic gonadotropin.

3.9.3.4 Acupuncture + HCG + letrozole vs. HCG + letrozole

For the comparison between acupuncture combined with HCG plus letrozole and HCG plus letrozole, the result showed no significant difference on the pregnancy rate (39) [RR = 1.56, 95% CI (0.84, 2.89), one RCT, 84 participants, $p = 0.16$].

3.9.4 Publication bias

Funnel plots were drawn to explore the possibility of publication bias for the 17 RCTs. The scattered point distribution in the funnel plots was basically symmetrical (see Figure 11).

4 Discussion

4.1 Main findings

Acupuncture can be used for infertility caused by various male and female factors. Current research highlights on acupuncture for infertility focused on female infertility caused by PCOS, ovulation disorder, and LUFS, while studies on male infertility and female infertility caused by blockage in the fallopian tube, thin endometrium, and luteal phase defect, among others, were insufficient. Current existing evidence of acupuncture on infertility indicated that the RCTs were still the main study type, but the information on the funding source for these RCTs was not evident. There was a lack of studies with large sample sizes and the acupoints tended to follow a fixed protocol (73.5%) (but the source of the acupoint protocol was not clear). The frequency and course of acupuncture varied from once to thrice a week and from 3 months to more than 6 months, respectively. Adverse events reported included mild ovarian irritation and potential early signs of miscarriage (some uncomfortable local pain). The contribution rate of different authors to this discipline varies depending on their publication volume, the core authors with higher contribution rates gather into a core author group, and no core author group has yet been formed in this field. Because the centrality of different institutions has not reached the target value 0.1, a cooperative network between different institutions has not been formed.

As for the acupuncture intervention itself, it is a complex intervention; the acupoints, frequency, and course of intervention differ for different patients according to different conditions. In particular, when it comes to pregnancy safety, professional qualified acupuncturists are required. No study focused on health economic analyses, and most RCTs with positive results did not explicitly propose their hypothesis (superiority) but concluded that the acupuncture intervention group was more clinically effective than the control group. For the large sample size confirmatory clinical studies, examination of statistical differences between two groups is critical to substantiate the results and it is important to establish an appropriate hypothesis and aim at the design stage of the study. Superiority, non-inferiority, or equivalence clinical trials should be clearly described for each study in order to correctly interpret the results (42).

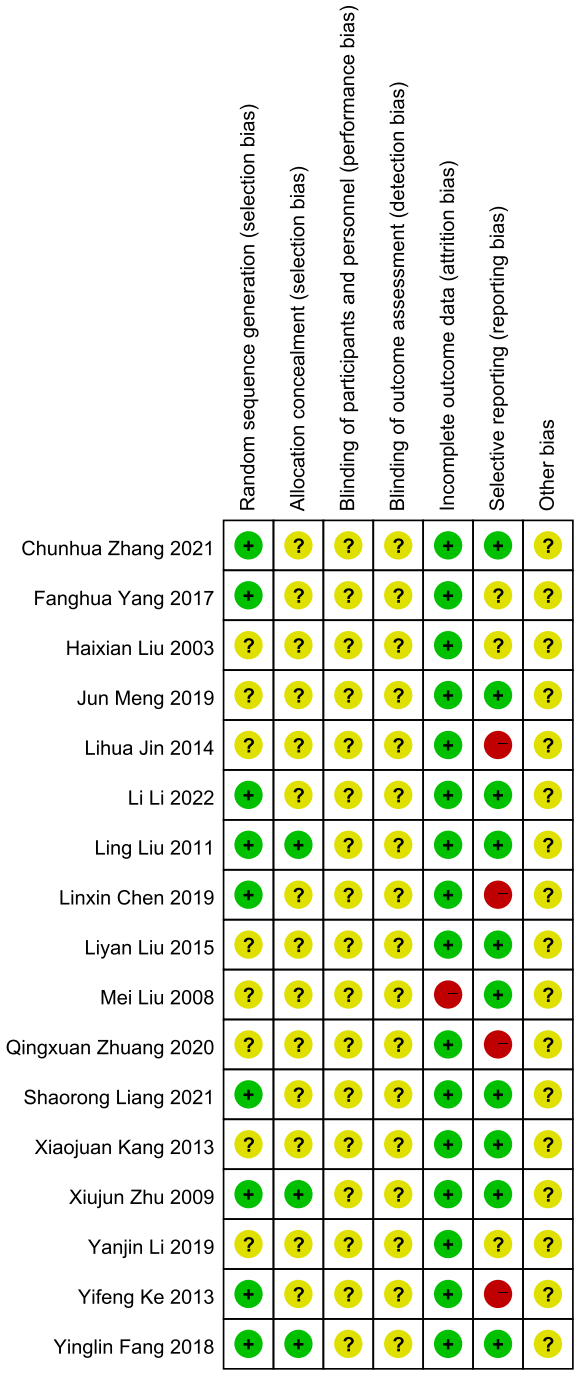


FIGURE 7
Risk of bias 17 included RCTs.

4.2 Limitations of this study

This study was conducted according to the guidelines of scoping reviews. The quality of included studies was not assessed as this is not required for scoping reviews. A previous scoping review on acupuncture for IVF has been published; thus, we excluded studies that combined acupuncture with ART, and the outcomes were limited to pregnancy rate, live birth rate, or semen parameters. We only searched seven databases, namely, three English databases and four Chinese databases, and we will consider including additional relevant databases such as Web of Science to enhance the comprehensiveness of this study when we update this scoping review in the coming years. This summary of current evidence does not reflect the effectiveness of acupuncture on other parameters such as menstruation, ovulation disorder, and sperm motility. Other exclusions involved studies that combined acupuncture and herbal medicine or used herbal medicine as the control group, while herbal medicine is also an alone or adjuvant treatment for infertility in clinical practice.

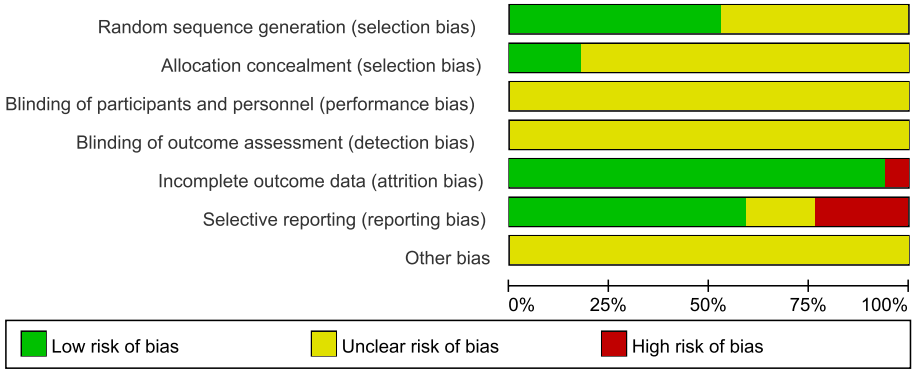


FIGURE 8
Risk of bias summary of 17 included RCTs.

4.3 Implications for future research

Acupuncture is widely used all over the world, and the increase in funding for acupuncture clinical studies has provided opportunities for current researchers to produce more high-quality primary clinical evidence (43). As we have summarized in this manuscript, there are currently at least 25 kinds of conditions or causes about male and female infertility clinical studies has been published, are they all the dominant populations of acupuncture? Pilot studies are still required to screen acupoint regimens, frequency, and course of acupuncture intervention and even dominant populations based on syndrome pattern differentiation and its treatment of acupuncture. As for real-world studies with a large sample size, researchers should consider the heterogeneity between participants. Future studies on acupuncture should not ignore the complexity of acupuncture, its different forms of delivery, techniques, dependence on expertise, use of combined therapies, and highly personalized treatment regimens (44). Researchers

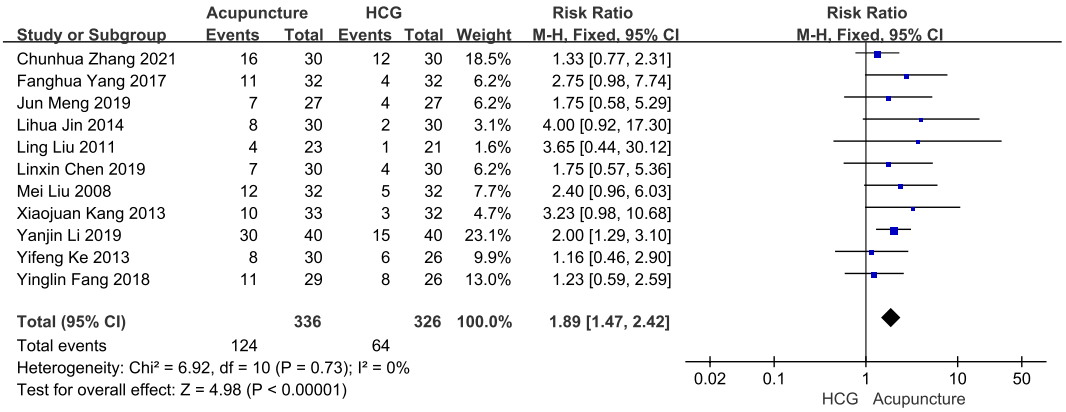


FIGURE 9
Forest plots of the pregnancy rate comparison between acupuncture and HCG.

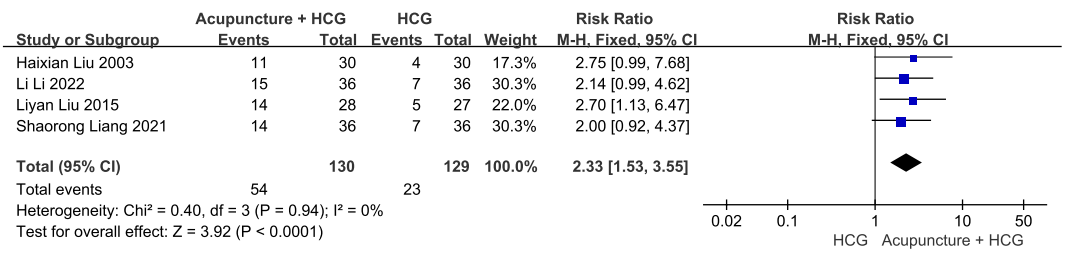


FIGURE 10
Forest plots of the pregnancy rate comparison between acupuncture combined with HCG and HCG alone.

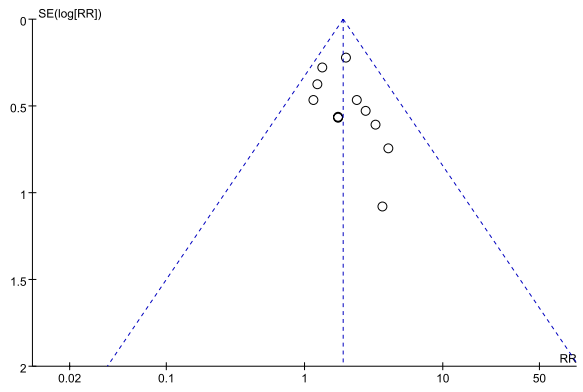


FIGURE 11
Funnel plot.

should also consider the inclusion of a qualitative interview in their study design. Meanwhile, health economics analysis of acupuncture on infertility could identify whether there are cost savings if the intervention was more widely available. Different countries have different medical systems and policies. Appropriate research design should be considered such as superiority, non-inferiority, or equivalence clinical trials. Moreover, researchers should also follow the reporting guidelines according to different study types.

5 Conclusions

This study identified and summarized the current clinical evidence of acupuncture for infertility. Acupuncture may potentially be used for treating various male and female infertility factors. Current research highlights on acupuncture for infertility focused on female infertility caused by PCOS, ovulation disorder, and LUFS, while studies on male infertility and female infertility caused by blockage in the fallopian tube, thin endometrium, and other factors were insufficient. Despite the large number of RCTs in this field, larger confirmatory clinical studies with appropriate hypothesis evidence on acupuncture for infertility resulting in natural conception are still lacking as the research hypotheses for most studies were unclear and most studies were exploratory in nature with small sample sizes.

Data availability statement

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

Author contributions

ZT: Conceptualization, Methodology, Project administration, Writing – original draft. CZ: Data curation, Software, Visualization, Writing – original draft. XL: Writing – review & editing, Methodology, Supervision. SY: Formal analysis, Writing – review & editing. YH: Data curation, Writing – review & editing. AS: Data curation, Writing – review & editing. FY: Data curation, Writing – review & editing. TP: Data curation, Writing – review & editing. JZ: Data curation, Writing – review & editing. YM: Writing – review & editing, Data curation. NR: Conceptualization, Writing – review & editing. PB: Conceptualization, Writing – review & editing, Project administration. WG: Conceptualization, Methodology, 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.2024.1351281/full#supplementary-material

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Puberty suppression in adolescents with gender dysphoria: an emerging issue with multiple implications

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Controversy exists over puberty suppression (PS) in adolescents with gender dysphoria (GD). PS is preferentially achieved with GnRH analogues. By preventing the development of secondary sex characteristics, PS may improve psychological functioning, well-being, quality of life, emotional and behavioral (especially internalizing) problems and depressive symptoms, thus decreasing suicidality. PS can also extend the diagnostic period and give transgender adolescents time to explore their gender identity. GnRHa may also decrease the need for feminization/masculinization surgery. However, 2-year treatment with GnRHa may result in bone mass accrual retardation (decrease in BMD/BMAD z-scores), growth velocity deceleration (decrease in height SDS), increase in fat mass, temporary pause in oocyte/sperm maturation. The most common side effects of GnRHa are hot flashes, mood fluctuations, fatigue and headache. They are usually mild and rarely lead to GnRHa discontinuation. Based on current scientific evidence, PS could be recommended to adolescents who meet the diagnostic criteria of gender incongruence (by DSM-5 and/or ICD-11) and have long-lasting intense GD, which aggravates with puberty onset. Before initiating PS, possible mental issues should be addressed and informed consent (by the adolescent/caregiver) should be given, after counseling on probable reproductive effects of GnRHa. GnRHa can only be started after the adolescent has entered Tanner stage 2. Nevertheless, published studies are inadequate in number, small in size, uncontrolled and relatively short-term, so that it is difficult to draw safe conclusions on efficacy and safety of GnRHa. Large long-term randomized controlled trials are needed to expand knowledge on this controversial issue and elucidate the benefit and risks of PS.

KEYWORDS

puberty suppression, gender dysphoria, transgender, GnRH, transgender and fertility, transgender and bone, transgender and mental health, gender incongruence

Introduction

Individuals whose gender identity differs from the sex assigned at birth have probably existed since ancient times. Rapid progression on diagnosis and management of gender dysphoria (GD) in adolescents has been made over the last decades, leading to an increase in the number of individuals seeking or referred for endocrine care (1, 2). The first clinic for treatment of transgender youth opened in Amsterdam in 1987 and since then gender centers have proliferated.

Gender dysphoria/incongruence is defined according to ICD-11 and/or DSM-5-TR (Table 1) classifications as a marked persistent incongruence between one's experienced and assigned gender. The discordance of primary and/or developed/anticipated secondary sex characteristics with the expressed gender "often leads to a desire to 'transition'" in adolescence and adulthood (1, 3). Distress is not a required indicator of the ICD-11 classification of gender incongruence (1).

Prevalence of GD between 0,6 and 1,7% has been reported in children and adolescents (2) and varies greatly based on definition, country, year, source of data (from health-system records or self-reports) etc., with higher rates reported in recent years and most estimates obtained from survey studies conducted in schools in the United States or Western Europe (1, 2). The etiology of GD is largely unknown; however current research suggests that psychosocial and biological factors play a role in the development of gender identity (2).

Adolescence is accompanied by rapid physical, emotional, cognitive maturation. Body changes, sexual maturation and increased growth rate during puberty may worsen the distress of adolescents with GD. Puberty suppression (PS) has been proposed as a means of preventing unpleasant development of sex characteristics, thus alleviating the distress they frequently cause. However, PS in transgender adolescents is a subject of debate. Scientific controversies exist regarding safety and necessity, while legal and ethical barriers also exist.

Goal of PS in GD

Goal of early PS in adolescents with GD is to prevent further permanent development of undesirable endogenous secondary sex

TABLE 1 Diagnostic criteria for GD.

DSM-5 criteria for GD	ICD-11 criteria for gender incongruence
A. A marked incongruence between one's experienced/expressed gender and assigned gender of at least 6 months in duration	marked and persistent incongruence between an individual's experienced gender and the assigned sex
- in adolescents and adults, at least 2 of the following: 1. A marked incongruence between one's experienced/expressed gender and primary and/or secondary sex characteristics (or in young adolescents, the anticipated secondary sex characteristics) 2. A strong desire to be rid of one's primary and/or secondary sex characteristics because of a marked incongruence with one's experienced/expressed gender (or in young adolescents, a desire to prevent the development of the anticipated secondary sex characteristics) 3. A strong desire for the primary and/or secondary sex characteristics of the other gender 4. A strong desire to be of the other gender (or some alternative gender different from one's designated gender) 5. A strong desire to be treated as the other gender (or some alternative gender different from one's designated gender) 6. A strong conviction that one has the typical feelings and reactions of the other gender (or some alternative gender different from one's designated gender)	Adolescence and Adulthood: desire to 'transition', in order to live and be accepted as a person of the experienced gender, through hormonal treatment, surgery or other health care services to make the individual's body align, as much as desired and to the extent possible, with the experienced gender. ◦ The diagnosis cannot be assigned prior the onset of puberty. ◦ Gender variant behaviour and preferences alone are not a basis for assigning the diagnosis
- in children: at least 6 of the following (one of which must be the first criterion): 1. A strong desire to be of the other gender or an insistence that one is the other gender (or some alternative gender different from one's assigned gender) 2. In boys (assigned gender), a strong preference for cross-dressing or simulating female attire; or in girls (assigned gender), a strong preference for wearing only typical masculine clothing and a strong resistance to the wearing of typical feminine clothing 3. A strong preference for cross-gender roles in make-believe play or fantasy play 4. A strong preference for the toys, games or activities stereotypically used or engaged in by the other gender 5. A strong preference for playmates of the other gender 6. In boys (assigned gender), a strong rejection of typically masculine toys, games, and activities and a strong avoidance of rough-and-tumble play; or in girls (assigned gender), a strong rejection of typically feminine toys, games, and activities 7. A strong dislike of one's sexual anatomy 8. A strong desire for the physical sex characteristics that match one's experienced gender	Pre-pubertal childhood: incongruence must have persisted for about 2 years. ◦ a strong desire to be a different gender than the assigned sex; ◦ a strong dislike on the child's part of his or her sexual anatomy or anticipated secondary sex characteristics and/or ◦ a strong desire for the primary and/or anticipated secondary sex characteristics that match the experienced gender; and ◦ make-believe or fantasy play, toys, games, or activities and playmates that are typical of the experienced gender rather than the assigned sex. • Gender variant behaviour and preferences alone are not a basis for assigning the diagnosis.
B. The condition is associated with clinically significant distress or impairment in social, occupational, or other important areas of functioning. ■ Specify if the condition exists with a disorder of sex development	

characteristics, which could lead to substantial distress (3). Such features include Adam's apple, deep voice, brow and mandible prominence, tall stature and facial hair in those assigned male at birth (AMAB), and breasts, female body shape, and relative short stature in those assigned female at birth (AFAB).

PS can prolong the diagnostic period and offer transgender adolescents the time needed to explore their gender identity (1, 3), before deciding to proceed to partially reversible (testosterone/estrogen) and irreversible (surgical) treatments. During PS the adolescents can think over and verify their decision, discuss hesitations, doubts and fears with their health care professionals (HCP) and parents/guardians. They can also go through social transition and try living like adolescents of the experienced gender. If the adolescents regret treatment, reversible GnRHa can be discontinued and puberty usually recommences a few months later.

GnRHa mechanism of action and formulations

GnRH agonists (GnRHa) are recommended to suppress puberty (3). At the onset of puberty, GnRH pulsatile secretion by hypothalamus begins. Intermittent GnRH release is required for normal gonadotropin secretion, while continuous GnRH administration results in downregulation of GnRH receptors on anterior pituitary and suppression of gonadotropins and gonadal steroids (after an initial transient increase). Half-life of GnRH is about 3-6 minutes, because it is not bound to serum proteins, thus it is rapidly degraded by proteases. GnRH analogs are less vulnerable to proteolysis, have longer half-life and higher affinity for GnRH receptor due to substitution of amino-acids of natural GnRH, mainly at position 6 (4).

Leuprolide, triptorelin, goserelin, histrelin and nafarelin are available GnRHa that can be administered subcutaneously (or intramuscularly) for PS (5) (Table 2).

Diagnosis of GD in adolescence and criteria for GnRHa treatment

Endocrine society advises that diagnosis of GD/gender incongruence in adolescents should be made only by mental health professionals (MHP) who can use DSM and/or ICD, who are skilled in diagnosis of psychiatric disorders and can distinguish between GD and psychiatric conditions with similar manifestations (e.g. body dysmorphic disorder) and know the criteria for PS and other gender-affirming treatment in adolescents (3). DSM–5 clearly articulates that "gender non-conformity is not in itself a mental disorder".

World Professional Association for Transgender Health (WPATH) recommends that HCP only recommend gender-affirming treatments requested by transgender adolescents when "the adolescent meets the diagnostic criteria of gender incongruence" (DSM-5, ICD-11) (1). The Endocrine Society suggests that "adolescents who meet diagnostic criteria for GD/gender incongruence, fulfill criteria for treatment, and are requesting treatment should initially undergo treatment to suppress pubertal development" (3).

WPATH and Endocrine Society suggest some eligibility criteria for GnRHa treatment of adolescents (3). It is recommended that a trained MHP confirms diagnosis of (suppressed or expressed) intense persistent GD, which deteriorated with puberty onset. The MHP establishes that possible coexisting mental or physical health or social problems, which might interact with therapy and jeopardize compliance, have been managed, and that the adolescent's condition and functioning are stable. The adolescent should be informed about the efficacy and possible adverse effects of GnRHa (including potential temporary impact on fertility) and about choices for fertility preservation. Informed consent should be given by the adolescent, provided that he/she has adequate cognitive and emotional maturity and mental capacity (assessed by the MHP). The parent(s)/guardian should give consent before and support during GnRHa, if the adolescent is younger than the age of legal consent. A pediatric endocrinologist (or pediatrician trained in growth assessment) confirms the indication for GnRHa, the absence of contraindications and the first signs of puberty (Tanner stage 2).

Persistence of childhood GD and recommended time for GnRHa initiation

Gender diversity/incongruence can be expressed in prepubertal children; however, it frequently desists into adolescence, which may

TABLE 2 Available GnRH analogs for PS.

GnRH analog	Dose
Leuprolide (acetate)	3,75-7,5 mg monthly im or sc 11,25 mg im every 3 months im or sc 22,5 mg sc every 3 months, 30 mg sc every 4 months 45 mg sc every 6 months
triptorelin	3,75 mg every 4 weeks (every 2 weeks during the first month) im or sc 11,25 mg every 3 months 22,5 mg every 6 months
goserelin	3,6 mg every 4 weeks sc 10,8 mg every 3 months sc
histrelin	50 mg (delivering 65 mcg/d), yearly sc (implant surgically inserted in the upper inner arm)
nafarelin	1600 to 1800 μg 4 times per day intranasally (nasal spray)

be a critical period for gender identity development (1, 3). A study of 139 boys referred at a mean age of 7,49 years (range 3,33–12,99) for assessment of GD, showed that GD persisted in only 12,2% during follow-up (until mean age 20,58 years) (6). Another study of 127 adolescents, referred for GD before the age of 12, showed that persistence of childhood GD in adolescence was more likely in AFAB and older children and in those who had experienced a social role transition (7). The Endocrine Society recommends "against puberty blocking and gender-affirming hormone treatment in prepubertal children with GD/gender incongruence" (3).

Thus, persistence of GD should be evaluated after the first signs of puberty (3), which consist of "the breast bud stage" in girls (elevation of breast and papilla "as small mound" and expansion of areolar diameter) and testes volume greater than 4 ml in boys (accompanied by slight increase in penis length and scrotum size and change in the texture and reddening of scrotum skin). These first changes of puberty typically occur at the age of 9-14 in boys and 7-13 in girls. The discomfort transgender adolescents feel after the first physical changes of puberty can contribute to diagnosis confirmation of GD persistence. Therefore, Endocrine Society and WPATH suggest that HCPs begin PS in eligible transgender adolescents "only after they first exhibit physical changes of puberty (Tanner stages G2/B2)" (1, 3).

Measurement of FSH/LH and estradiol/testosterone (by ultrasensitive assays) in early morning blood samples may supplement clinical examination and confirm hypothalamus-pituitary-gonads (HPG) axis activation at puberty onset (3).

GnRHa administration could probably be initiated in an adolescent at Tanner stage 1 only in cases of constitutional delay in growth and puberty. In these cases GnRHa may be added soon after beginning estrogen or testosterone (1).

GnRHa can be administered at later stages of puberty (Tanner 4 or 5) to deter further breast development in AFAB and facial hair growth or further voice deepening in AMAB, although secondary sex characteristics will not regress completely. GnRHa can also be used in late puberty to suspend erections in AMAB or halt unpleasant menses in AFAB (alternatively, lynestrenol or medroxyprogesterone can be administered to cause amenorrhea) (3).

However, it is uncertain if GnRHa initiation before completion of puberty influences further gender identity development or if it contributes to GD persistence (8). There are concerns that PS may deter adolescents with GD from feeling comfortable with birth-assigned gender. By suppressing gonadal hormones, GnRHa may restrict sexual desire, thus preventing adolescents from having age-appropriate (socio-) sexual experiences, which affect gender identity development.

A retrospective cohort study of 434 transgender adolescents (71,9% AFAB) of mean age 15,4 years (at first visit) showed that GnRHa did not increase the likelihood of subsequent gender-affirming hormone therapy (GAHT) use. GnRHa treatment was associated with longer time between the first visit and GAHT initiation. In multivariate analysis GnRHa use was independently associated with lower risk of GAHT initiation. These associations still existed among 54 adolescents 10 to 13 years old (at first visit), suggesting that GnRHa could be offered to young transgender adolescents without worry for affecting their decision to proceed to GAHT treatment. Adolescents between 14 and 17 years old at first visit received GnRHa less frequently, but were more likely to begin GAHT and after a shorter time, as compared to those between 10 and 13 years old (9).

Effectiveness of GnRHa in gonadal and puberty suppression

Preliminary results of the first 21 adolescents, treated (according to the Dutch protocol) with triptorelin (3,75 mg monthly) for at least 2 years, showed sufficient suppression of gonadotrophins and gonadal steroids (estradiol in AFAB, testosterone in AMAB) to prepubertal levels. Puberty did not develop. Testicular volume reduced in AMAB (10).

GnRHa are effective in HPG axis suppression in transgender adolescents, similarly to central precocious puberty (CPP). A retrospective review of medical records of 60 youth (30 transgender, 30 CPP) showed that reduction in FSH/LH and testosterone/estradiol was not significantly different during GnRHa treatment between transgender and CPP youth. FSH levels were lower after histrelin treatment of transgender adolescents compared with leuprolide treatment (11).

The efficacy of GnRHa in PS was also shown in a prospective study of 116 (57,8% AFAB) transgender adolescents. Gonadotropins and estradiol were suppressed within 3 months of triptorelin. Testosterone levels were also suppressed in AMAB adolescents. No adjustment in GnRHa treatment was necessary in anyone due to insufficient suppression. GnRHa resulted in testicular volume reduction in 88% (43 of 49) AMAB, in 33 of them it decreased from 13,9 at baseline to 8,6 mL at 12 months. Among four AFAB with breast Tanner stage 2 at presentation, one manifested complete regression of breast development after 6 months of GnRHa, while menses ceased in those who had had menarche usually after a withdrawal bleed (12).

In a prospective observational study in the UK of triptorelin monotherapy, gonadotropins were suppressed by 6 months and remained suppressed thereafter in all 44 transgender adolescents studied. Most AFAB were at stage 4 and post-menarcheal (at triptorelin initiation) and secondary amenorrhea occurred in all AFAB during the first 3 months of GnRHa (13).

Another prospective study of 36 transgender adolescents in Italy confirmed reduction in gonadotropins by GnRHa. FSH in AMAB and LH in AFAB decreased nonlinearly and more rapidly during the first months of triptorelin. Testosterone declined in AMAB in the first half of triptorelin treatment and stabilized afterwards. Estradiol levels fell significantly and approximately linearly in AFAB in the first 10 months of triptorelin. Statistically significant transition to earlier Tanner stage was reported after GnRH in 9 patients (25%). Menstruation ceased at T3 in all 20 AFAB who had menarche. Hair growth and acne severity receded in all (especially AMAB) during triptorelin (14).

In order to evaluate adequate HPG axis suppression, Endocrine Society suggests measurement of FSH/LH and estradiol/testosterone at baseline and every 6-12 months during PS. If there are clinical (menses, erections, hair growth) and/or laboratory evidence of insufficient HPG suppression, GnRHa dose can be raised or the interval between the doses can be shortened (3).

Mental health in adolescents with GD

Development of primary and/or secondary sex characteristics during puberty can cause adolescents with GD serious psychological distress (3). Distress can hinder activities of daily living, resulting in depression and suicidal ideation.

The role of GD as a stressor could be explained by the minority stress model, which highlights the "excess exposure to social stress faced by sexual minority populations due to their stigmatized social status" (15). Many children have to confront social stigmatization and bullying at school. Nahata et al. reported that 58,2% of 79 young individuals with GD had documented school victimization (16).

Many studies have pointed out high rates of mental health concerns in untreated transgender adolescents. In a retrospective medical record review of 79 young individuals with GD, 78,5% were diagnosed with depression, 63,3% with anxiety, 74,7% reported suicidal ideation, 55,7% exhibited self-harm and 30,4% made suicide attempt(s) (15). Tordoff et al. noted that 56,7% of 104 transgender and nonbinary youth had moderate to severe depression, 50% had moderate to severe anxiety and 43,3% reported self-harm or suicidal thoughts before receiving gender-affirming treatment (17).

However, de Vries et al. suggested that the majority (67,6%) of adolescents with GD don't have concurrent psychiatric disorders. Anxiety occurred in 21%, mood disorders in 12,4% and disruptive disorders in 11,4% of the adolescents (18).

Many adolescents with GD are referred for treatment due to poor psychological functioning. In a cross-sectional study in Amsterdam, 272 adolescents referred to a specialized gender identity clinic manifested more behavioral and emotional (especially internalizing) problems, more self-harm/suicidality, and poorer peer relations before gender-affirming treatment compared with cisgender controls from the general population (19).

Therefore, HCPs should "undertake a comprehensive biopsychosocial assessment of adolescents" who present with GD and request "transition-related care", which is recommended only when "the adolescent's mental health concerns (if any) that may interfere with diagnostic clarity, capacity to consent, and/or gender-affirming medical treatments have been addressed". Moreover, "it is critical to differentiate gender incongruence from specific mental health presentations, such as obsessions and compulsions,…, broader identity problems,…". Treatment of transgender youth in multidisciplinary clinics in close cooperation with MHPs is beneficial (1).

Haltering the stress that adolescents may experience during puberty by hormonal suppression and allowing them some time to explore their gender identity can provide some relief to the distress from the development of secondary characteristics (Table 3).

In Trans Youth Care–United States (TYCUS), a prospective, observational study of 315 transgender and nonbinary youth (mean age 16), participants who had not experienced considerable endogenous puberty changes had better psychosocial functioning. Twenty adolescents who started GnRHa at Tanner stage 2-3 and 4 adolescents who initiated GAHT without previous GnRHa treatment at stage 3 (due to relatively late onset of puberty) had greater positive affect and less anxiety before starting GAHT than those who had started GAHT in later puberty (thus experiencing gender-incongruent puberty). These differences were statistically significant only among 20 early-pubertal AMAB (20).

Transgender children (aged 3–12 years) with early social transition don't manifest increased levels of depression, but have slightly increased levels of anxiety, with their rates of internalizing problems being considerably lower than children with GD living as their birth-assigned gender (28). Access to gender-affirming therapy may help social transition, potentially reducing the rates of distress and school victimization.

Individuals who received PS have been found to have better psychological outcomes, improved well-being and higher quality of life. Adolescents with GD had significantly better psychosocial functioning after one year of GnRHa treatment than those who received psychosocial support alone (21). In a Dutch longitudinal observational cohort study of 70 adolescents, general functioning improved during PS, so that no one discontinued GnRHa and all proceeded to GAHT (22).

The first longer term study of 55 young transgender adults, who had received PS during adolescence, showed that psychological functioning steadily improved (especially in transmen), objective and subjective well-being (post-treatment) was comparable to peers in general population and none reported regret during PS (23).

However, Carmichael et al. found no significant change in psychological functioning during 3 years of triptorelin treatment (started at late puberty for the majority) of 44 adolescents with GD, although the majority reported positive changes (e.g. feeling happier, better relationships with family and peers) (13).

Depression has been shown to decrease during PS. De Vries et al. and Fisher et al. found a reduction in depressive symptoms in adolescents with GD during GnRHa treatment (14, 22, 23). TYCUS study showed less depression in transgender youth, whose puberty was suppressed or started later (20). In a prospective observational cohort study, treatment of 104 youth with puberty blockers or GAHT was associated with 60% lower odds of moderate to severe depressive symptoms during the first year, while significant increase in moderate to severe depression was noticed in youth who had not received treatment (17).

PS may decrease suicidality. Tordoff et al. showed that PS or GAHT was associated with 73% lower odds of self-harm or suicidal thoughts in youth (17). Van der Miesen et al. found that among 178 transgender on puberty blockers, self-harm/suicidality was less frequent than in clinic-referred transgender and similar with non-clinical peers (19). Khatchadourian et al. noted a reduction in suicide attempts (from 12% to 5%) after GnRHa treatment of 27 youth with GD in Canada (24). A cross-sectional survey of 20619 transgender adults (aged 18 to 36 years) showed that those (n=89) who had received PS during adolescence had lower odds of lifetime suicidal ideation (in comparison with those who wanted, but did not receive PS) (25). Fisher et al. found lower repulsion by life leading to lower suicidal tendency during triptorelin treatment, which was associated with the decrease in FSH/LH, in waist circumference and in acne severity in AMAB (14). However, Carmichael et al. found no significant change in self-harm during triptorelin treatment (13).

Emotional and behavioral, especially internalizing, problems may improve after GnRHa treatment. In the study by van der

TABLE 3 Effect of PS on mental health.

	Positive effect of PS on mental health	Negative/neutral effect of PS on mental health
Chen D et al. 2023 (20)	• Higher scores for appearance congruence, positive affect and life satisfaction, • lower scores for depression and anxiety (in AMAB who started GnRHa at early puberty)	
Costa R et al. 2015 (21)	• better psychosocial functioning after 1 year PS (compared with psychological support only)	
De Vries A et al. 2011 (22)	• Improvement in general functioning, • reduction in depressive symptoms, • reduction in behavioral and emotional problems and in internalizing and externalizing behavior T-scores	No significant change in anxiety, anger, body satisfaction and GD
De Vries A 2014 et al. (23)	• Steady improvement in psychological functioning (especially in AFAB), decrease in depression, objective and subjective well-being • decrease (not statistically significant) in anxiety only in AFAB (during and longer after PS)	Persistence in GD and body image difficulties AFAB reported more dissatisfaction with secondary sex characteristics after PS, (GD resolved after subsequent of GAHT and gender-reassignment surgery)
Tordoff D et al. 2022 (17)	• 60% lower odds of moderate to severe depressive symptoms during 1st year of PS or GAHT	no association between PS or GAHT and moderate to severe anxiety
Van der Miesen A et al. (19)	• lower frequency of self-harm/suicidality (than clinic-referred GD) less emotional and behavioural problems, with lower score on internalizing (emotional) problems • lower score on peer relations (than clinic-referred GD)	higher score on peer relation problems (than cisgender controls from general population)
Khatchadourian K et al. 2014 (24)	• reduction in suicide attempts (from 12% to 5%)	
Turban J et al. 2020 (25)	• lower odds of lifetime suicidal ideation	
Becker-Hebly I et al. 2021 (26)	• improved ("within the norm mean") mental and physical health-related quality of life (lower than age-matched controls, statistical significance not tested)	Persistence in internalizing problems during 2 years follow-up after GnRHa
Kuper L et al. 2020 (27)	• Improvement in body dissatisfaction during gender-affirming treatment (including 10 adolescents receiving PS only)	No decrease in social anxiety symptoms
Carmichael P et al. 2021 (13)	• Experience/satisfaction: positive (or mixed) changes for the majority (e.g. feeling happier, better relationships with family and peers) • Mood changes at 6–15 months: improved mood (49%), mixed changes (feeling happier but with some mood swings; 15%) • predominantly positive or neutral change in family (feeling closer/accepted) and peer (feeling more sociable or confident, widening circle) relationships • At 6–15 months, 66% reported positive changes in gender role (feeling more feminine/masculine, living in preferred gender identity in more/all areas of life, feeling more secure in gender identity), no negative change	no significant change in psychological functioning and in self-harm during 3 years of triptorelin (started at late puberty for the majority) 24% reported negative mood changes at 6-15 mths
Fisher A et al. 2023 (14)	• significant reduction in externalizing and internalizing problems (lower scores on scales related to thought and social problems, somatic complaints, and anxious–depressive symptomatology) • improvement in body uneasiness lower repulsion by life component of suicide tendency scale less depressive symptoms (lower self-rating scale in Beck Depression Inventory) • lower levels of anxiety (lower self-rating scale in Beck Anxiety Inventory)	no significant improvement in aggressive behavior, rule-breaking behavior and attention problems

Miesen et al., transgender adolescents receiving PS had less emotional and behavioral problems, as they scored lower on internalizing (emotional) problems, in comparison to cisgender peers from the general population as well as to transgender clinic-referred adolescents. Regarding peer relations, transgender adolescents on PS scored less than referred transgender but higher than cisgender adolescent controls (19). De Vries et al. noticed decreased behavioral and emotional problems and lower internalizing and externalizing behavior t-scores during PS (22, 23). Fisher et al. found that externalizing and internalizing problems decreased significantly after GnRHa treatment of trans-adolescents (14). However, in a small German cohort study of 11 adolescents with GD (73% AFAB), internalizing problems persisted during 2 years (average) follow-up after GnRHa treatment (26).

Nevertheless, the latter study showed that GnRHa treatment resulted in improved ("within the norm mean") mental and physical health-related quality of life, which was lower than age-matched controls pretreatment, although statistical significance was not tested (26).

Anxiety may not decrease significantly during PS. Tordoff et al. found no association between PS or GAHT treatment of youth with GD and moderate to severe anxiety (17). Kuper et al. found that social anxiety symptoms did not decrease during PS (27). De Vries et al. (2011) reported that anxiety and anger did not change during GnRHa treatment of 70 adolescents with GD (22), although the longer term study by de Vries et al. (2014) noted a decrease (not statistically significant) in anxiety only in transmen during and later after PS (23). Moreover, Fisher et al. found a reduction in anxiety during triptorelin treatment of 36 transgender adolescents, which was associated with the decrease in FSH (14).

PS may not amend body dissatisfaction. De Vries et al. (2011) noticed that body satisfaction and GD did not change significantly during GnRHa treatment of 70 adolescents (22). The longer-term study by de Vries et al. (2014) showed that GD and body image difficulties persisted during PS (with transmen reporting more dissatisfaction with secondary sex characteristics after PS), although GD resolved after subsequent administration of GAHT and gender-reassignment surgery (23).

However, other studies have showed that body changes during PS may have a positive psychological effect on trans-adolescents. In TYCUS study, transgender youth who had not gone through significant physical changes of endogenous puberty had greater appearance congruence, which was associated with better psychosocial functioning, although less improvement in appearance congruence was noticed after two years of gender-affirming treatment in youth treated since early puberty than those starting GAHT in later puberty (20). Kuper et al. reported that body dissatisfaction of youth improved during gender-affirming treatment (including 10 adolescents who received PS only, starting early at mean age of 13,7 years) (27). Fisher et al. concluded that gender-affirming physical changes (e.g. reduction in WHR and acne severity in AMAB) induced by triptorelin treatment of 36 transgender adolescents could account for improvement in body uneasiness and in psychological functioning (14).

The overall positive impact of PS on mental health highlights the need for worldwide availability of gender-affirmative care for transgender adolescents to help alleviate possible psychological problems, reducing them to rates indistinguishable from the general population.

However, there is a limited number of studies focusing on the psychological effects of PS on adolescents with GD (Table 3), which highlights the importance of the need for further prospective studies in this field. Furthermore, the small number of participants in the existing studies, the variety in studied outcomes, the use of different tools and the different time periods between initial assessments and follow-up prevents us from drawing firm conclusions.

A matter of discussion is the ethical and legal issues around medical treatment of children with GD. Judicial Review for 'Bell V's Tavistock Case' in the UK ruled that children younger than 16 years are incapable of giving informed consent to medical interventions for GD; however, the Court of Appeal subsequently reversed High Court's decision, concluding that determination of a child's competence to consent should be decided on an individual basis by clinicians and parents (29). However, such judgements cannot be generalized, since laws vary across the globe.

GD persistence into adulthood and high rate of post-GnRHa GAHT use

A recent cohort study of 720 transgender (69% AFAB) individuals, who began GnRHa treatment in adolescence (median age at GnRHa start 14-16 years) for a minimum duration of 3 months (before adding GAHT) showed that 98% of the study population continued gender-affirming hormones at follow-up into adulthood (30).

In a retrospective study in Canada, transition to testosterone or estrogen was made for 14 of 15 AFAB and for 5 of 11 AMAB (respectively) receiving GnRHa, which had been started at Tanner stage 4-5 for the majority (59%) (24).

A retrospective study in a gender clinic in the Netherlands showed that the majority of adolescents who had started GnRHa subsequently continued treatment with GAHT and only a minority discontinued GnRHa. Among 143 adolescents (73,4% AFAB) who began GnRHa treatment (at a median age of 15-16), 87% started GAHT after a median duration of 0,8 years on GnRHa. Nine (6%) adolescents (8 of whom AFAB) stopped GnRHa after a median of 0,8 years, five of whom no longer wanted gender-affirming treatment, while three adolescents discontinued due to possible side effects (31).

GnRHa administration may reduce the necessary concurrent doses of GAHT

A retrospective review of outpatient medical records of 83 transgender adolescents (73% AFAB), 17 of whom received GnRHa (median age at GnRHa start 14,5 years for AMAB, 13,9 for AFAB), showed that initiation of GnRHa before GAHT was associated with significantly lower average dose of oral estradiol/subcutaneous testosterone. Frequency of adverse effects of GAHT was not significantly different between those taking and those not taking GnRHa (32).

GnRHa may reduce the need for feminization/masculinization surgery

Transgender individuals who had received PS at early stages may not need to undergo most of the non-genital surgeries. In a retrospective study of youth with GD in Canada one (out of 15) AFAB did not need chest surgery, because GnRHa was started early and prevented breast growth (24). Mastectomy with chest reconstruction (and liposuction) in AFAB, facial feminization surgery and chondrolaryngoplasty in AMAB may be unnecessary,

because development of biological sex characteristics has been impeded. Penile inversion vaginoplasty may be impracticable in AMAB who had received PS early, because GnRHa may result in penoscrotal hypoplasia (leaving inadequate amount of penile tissue), in these cases intestinal vaginoplasty is more appropriate (33).

Alternatives to GnRHa in late-pubertal transgender adolescents

Proandrogenic and antiandrogenic progestins are a cheap oral alternative in late-pubertal AFAB and AMAB, respectively, who have already developed secondary sex characteristics.

Lynestrenol is an androgenic progestin, converted to norethisterone, which reduces LH, SHBG, total testosterone, while free testosterone may rise slightly (33). In a retrospective analysis of data from 45 AFAB adolescents at Tanner stage B4 or further treated with lynestrenol monotherapy (and in combination with testosterone for at least 6 months) showed than mean LH and E2 (not FSH) levels decreased during the first 6 months and remained stable in the next 6 months of lynestrenol monotherapy, while LH and FSH were fully suppressed after combination therapy. SHBG and total testosterone fell significantly, although free testosterone increased non-significantly in the first 6 months of lynestrenol and remained unchanged in the next 6 months. The most common side effects of treatment were headaches, hot flushes, and fatigue. Hematocrit rose significantly in the first 6 months of monotherapy and combination but remained stable thereafter. Acne increased non-significantly during lynestrenol monotherapy and appeared more frequently during the first 6 months of combination. Metrorrhagia was mainly reported in the first 6 months, but was significantly reduced in the following 6 months of monotherapy and increased slightly during combination. Weight and BMI significantly increased in the first 6 months and returned to baseline after 12 months of lynestrenol monotherapy, while significant weight gain was noticed after combination. Triglyceride levels did not change, although mean HDL fell and mean LDL increased significantly in the first 6 months of lynestrenol, the latter did not change during combination. Glucose levels, HbA1c and HOMA were not significantly altered during treatment (34).

Cyproterone acetate (CA) has been used in late-pubertal AMAB due to its antiandrogenic effects, resulting mainly through competitive inhibition of binding to androgen receptor. According to a retrospective analysis of data from CA treatment of 27 AMAB (presenting at Tanner stage G4), more than half of the studied youth reported decreased facial hair growth (reduced shaving frequency) and some reported less spontaneous erections. Breasts developed during CA monotherapy (29,6% to Tanner B2-B3) and further (66,7% to Tanner B3, 9,5% reached Tanner B4) during subsequent combination treatment (CA with estrogen), although breast size was small in most cases. Fatigue was the most common side effect, reported in 37% of AMAB during CA monotherapy, receding in the majority after addition of estrogens. Other relatively common side effects of CA (monotherapy) include emotionality (11,1%) and breast tenderness (7,4%). Growth was significantly less compared with age-matched peers, because all adolescents had already reached at least Tanner G4. Weight gain during CA monotherapy was small, although it was greater than in age-matched male peers, but rise in height was also more than in controls, so that BMI was not significantly altered. CA did not have a significant effect on glucose levels, HbA1c, HOMA index and LDL, while triglycerides declined. FSH levels were slightly reduced, but not suppressed, during the first 6 months of CA. CA resulted in a significant progressive reduction in testosterone levels (below male, but not within female, reference interval). Prolactin increased during CA, but none of the treated AMAB manifested galactorrhea. Neither increase in PRL nor decrease in testosterone were associated with breast development (35).

CA and lynestrenol treatment for approximately one year can induce body composition changes in line with the desired gender appearance. However, CA may restrain pubertal bone mass accrual (mainly at LS), while lynestrenol has probably no significant impact on normal bone development. In a prospective study of 21 AMAB and 44 AFAB at Tanner stage G4 or G5 treated with CA or lynestrenol (respectively) for 10,6 months (mean), mean total testosterone and estradiol reduced, FSH and LH decreased only during lynestrenol, free testosterone and total testosterone-to-estradiol ratio decreased during CA and SHBG declined (raising free testosterone) during lynestrenol. PINP reduced significantly during lynestrenol and CA, reduction was greater (by 46,5%) during CA, which also induced a s-CTX drop by 17,1%. During lynestrenol, lean mass increased significantly, resulting in reduced body fat percentage (compared with age-matched female peers), weight and waist/hip ratio (WHR) increased, muscle (not fat) area at left lower leg and at nondominant forearm and grip strength also increased. Thus, lynestrenol induced a more masculine body composition and musculature. During CA, fat mass significantly increased, lean mass decreased, muscle area decreased significantly, while grip strength was not significantly altered, leading to decreased z-scores (compared to male peers). Total hip aBMD (absolute values and z-scores) increased in AFAB and decreased in AMAB. In AFAB femoral neck (FN) and lumbar spine (LS) aBMD absolute values increased significantly, without significant change in z-scores, indicating bone development similar to female peers. However, FN and LS aBMD z-scores decreased during CA (with stable aBMD absolute values). Trabecular volumetric BMD at radius increased in AFAB (similarly as in age-matched control female), while it decreased in AMAB. Periosteal circumferences z-scores of radius and tibia reduced during CA, indicating significantly less periosteal expansion (36).

A cumulative dose-dependent association has been found between CA use and meningioma. CA is a progestogen and meningiomas express progesterone receptors. EMA recommended (in 2020) restrictions in CA use of daily doses of 10 mg or more, only for androgen-dependent conditions, when lower doses or other treatments have failed (37).

Spironolactone can promote feminization, because it is a moderate androgen receptor antagonist, which also partially inhibits 17α-hydroxylase/17,20-lyase, reducing thus androgen synthesis, while it also has a weak estrogen receptor agonist (in the absence of endogenous estrogen) as well as a partial

progesterone receptor agonist effect (37). Spironolactone has been used as an antiandrogen in gender-diverse adolescents (38).

In a retrospective study of 330 transwomen, those requesting breast augmentation had used spironolactone more frequently in comparison with other antiandrogens (CA, finasteride, dutasteride). The most common adverse effect of antiandrogens was depression, which was more frequent than with GnRH analog. CA was significantly correlated with depression (8,3%) (39).

Bicalutamide has been tested in a few AMAB as a second-line puberty blocker (alternatively to GnRHa). Bicalutamide antagonizes the androgen receptor, resulting in increased testosterone, which is aromatized to estrogen. A retrospective study of 23 transgender female adolescents (mean age 16) received bicalutamide (50 mg daily), six individuals received estrogen concomitantly. Breast development (≥ Tanner stage III) was noticed within 6 months in 84,6% of the study population, acne and frequency of shaving decreased. Estradiol levels were above 20 pg/dl (except in one subject) (40).

Medroxyprogesterone inhibits HPG axis and it has been used as a contraceptive. A retrospective study of 14 adolescents with GD supported the efficacy of medroxyprogesterone for menstrual cessation in AFABs and for puberty delay in AMABs. None stopped treatment due to adverse effects, three discontinued in order to return to assigned gender (due to psychosocial reasons). Seven AMAB adolescents, presenting at puberty stages 2-4, were treated with oral medroxyprogesterone (4 of whom for 3 years), with initial dose 10–30 mg per os bid and increasing doses until age 15-16 (thereafter dose decreased, while estradiol was added). One AMAB stopped treatment after 6 months. Complete suppression of testosterone was not always achieved with medroxyprogesterone, greater suppression was feasible when starting treatment in early puberty. Six postmenarchal AFAB (Tanner 5) received depot intramuscular medroxyprogesterone (initial dose 150 mg every 3 months, then every 2 months), to stop menses. One AFAB presenting at Tanner 2a received per os medroxyprogesterone (20 mg bid), with subsequent regression of breast tissue (41).

Effect of PS on bone metabolism

Growth spurt, time of the most rapid height velocity, occurs between Tanner stage 2 and 3 in girls and between stages 3 and 4 in boys. During puberty, bone mass and mineralization increase rapidly. Peak bone mass, which is achieved in early adulthood, reflects bone strength and predicts later development of osteoporosis. Gonadal steroids (estrogen and androgen) and growth-hormone/IGF1, which increase significantly during puberty, have a significant impact on skeletal development. Androgen increase periosteal apposition, expanding bone size and strength in male. Estrogen decrease periosteal apposition and endocortical resorption, so that a calcium reservoir is made for pregnancy and lactation. Estrogen inhibits chondrocyte proliferation at growth plate, leading to epiphyseal fusion and cessation of linear growth (42).

Limitations exist in the normal values used as reference for interpretation of bone density measurements in transgender individuals, because PS results in gonadal hormone reduction, which may differentiate the pattern of bone growth from that expected for the birth-assigned gender. According to International Society for Clinical Densitometry (ISCD) 2019 official positions, "z-scores should be calculated using the normative database that matches the gender identity" of transgender individuals. Because transgender female have lower BMD than cisgender male (before GnRH and GAHT treatments), female reference database in transgender female may be more reliable to evaluate z-score, although it may lead to its overestimation, missing cases of low BMD (for age) and ignoring the need to search for secondary causes of osteoporosis. Male reference database may be appropriate for estimation of z-score in transgender men, because their BMD is close to that of cisgender men. Construction of a reference database for bone density of transgender may be an aim for future research (43).

The Trans Youth Care Study showed high prevalence of low BMD before (or no more than 2 months after) GnRHa initiation among 63 transgender adolescents in early puberty. Low (lower than -2) areal or volumetric BMD z-score was found in 30% of AMAB and 13% of AFAB. These rates are significantly higher than those expected (2,3%) in a normal distribution. AFAB had higher mean BMD z-scores than AMAB, with statistically significant difference at the hip. A negative association was found between age at puberty blocker placement and total hip BMD z-scores, explained (at least in part) by the later puberty onset in males. AFAB and transgender youth with normal BMD reported statistically significantly higher physical-activity scores than AMAB and youth with low BMD, respectively (44).

There is concern that puberty blockers may delay normal bone development and affect peak bone mass (Table 4) by inhibiting normal endogenous production of gonadal steroids.

Most studies showed that rate of bone development during 2-year GnRHa treatment of transgender adolescents is lower than in age-matched peers. Initial results of 21 adolescents treated with triptorelin for at least 2 years (according to the Dutch protocol) showed that bone density (in LS, non-dominant hip and total body) did not change significantly, while z-scores decreased significantly (10).

Similarly, a prospective observational study of triptorelin monotherapy in 44 adolescents with GD showed that bone development was retarded, with BMD stable at the hip and increased at the LS (after 2 years), but at a slower pace than in age-matched peers. At baseline, most AMAB were at Tanner stage 3 and most AFAB were at stage 4 and post-menarcheal, while no participant was at stage 2. Hip BMD did not change at 12 and 24 months. LS BMD did not change significantly at 12 months, but it increased after 24 months of GnRHa (compared to baseline). LS BMC was greater than baseline at 12 and 24 months. LS and hip BMD z-scores decreased after 12 and 24 months of GnRHa (13).

Another study in the Netherlands analyzed BMD development in 34 transgender after triptorelin monotherapy (for median 1,3-1,5 years), which was initiated at late puberty in the majority, followed by GAHT with continuation of GnRHa until gonadectomy. In AMAB, BMD did not change, while z-score was reduced, but not significantly, during GnRHa monotherapy. In AFAB both LS and

TABLE 4 Effect of GnRHa treatment on bone density.

	BMD/BMAD LS		BMD/BMAD hip		BMD/BMAD LS z-score		BMD/BMAD hip z-score	
	AFAB	AMAB	AFAB	AMAB	AFAB	AMAB	AFAB	AMAB
Klink et al. 2015 (45)*	↓*	↔ *	↓ (FN) *	↔ (FN) *	↓ *	↔ *(decrease, but not significant)	↓ (FN) *	↔ (FN) * (decrease, but not significant)
Navabi et al. 2021 (46)	ND	ND	ND	ND	↓ (+LS BMAD z-score↓)	↓	↓ (TH)	↓ (TH)
Vlot et al. 2017 (47)	↓ (BA≥14)** ↔ (BA<14)**	↔**	↓ (BA≥14)** ↔ (BA<14)**	↔**	↓**	↓ (BA<15)** ↔ (BA≥15)**	↓ (BA≥14)** ↔ (BA<14)**	↔**
Schagen et al. 2020 (48)	↓ (Tanner 4-5) ↔ (Tanner 2-3)	↔	↓	↓ (Tanner 4-5) ↔ (Tanner 2-3)	↓	↓	↓	↓ (Tanner 4-5) ↔ (Tanner 2-3)
Joseph et al. 2019 (49)	↔	↔	↔	↔	↓ ¶ (BMD+BMAD)	↓ ¶ (BMD+BMAD)	↓	↓
Carmichael et al. 2021 (13)	↔*	↔*	↔*	↔*	↓* (12-24 mths) ↔* (36 mths)	↓* (12-24 mths) ↔* (36 mths)	↓* (12-24 mths) ↔* (36 mths)	↓* (12-24 mths) ↔* (36 mths)
Stoffers et al. 2019 (50)	↓	ND	↓	ND	↓	ND	↓	ND

↔ no statistically significant change during GnRHa treatment; BA, bone age; FN, Femoral Neck; ND, No data / Not determined; TH, Total Hip.
*BMD, ** BMAD.
¶ greater decrease after 1 year GnRHa, lower decrease during the 2nd year.
assigned female at birth (AFAB), assigned male at birth (AMAB).
↓, decrease.

FN absolute BMD and z-scores decreased significantly during GnRHa monotherapy (45).

A decrease in BMD z-scores after one year of leuprolide monotherapy was also found in a retrospective study of 116 transgender youth (69% AFAB) in Canada. At baseline more than 80% were at Tanner stage 4-5 and aBMD values were lower in AMAB than in AFAB. LS and left total hip aBMD z-scores decreased significantly after GnRHa treatment. LS bone mineral apparent density (BMAD) z-scores fell significantly among AFAB (46).

A retrospective cohort study of 70 transgender (60% AFAB) adolescents showed decreased BMAD z-scores mainly in the LS (except in "old" AMAB) without significant alteration in BMAD absolute values during GnRHa treatment. During GnRHa, (LS and hip) BMAD and hip BMAD z-score decreased only in "old" [bone age (BA) 14 years or more] AFAB, while LS BMAD z-score decreased in AFAB and in "young" (BA < 15) AMAB adolescents (47).

A significant decrease in z-scores during GnRHa was also evident in an observational prospective study of 29 early-pubertal (Tanner 2-3 at GnRHa start) and 92 late-pubertal (Tanner 4-5) transgender adolescents. During 2 years of GnRHa treatment, LS and hip BMD and BMAD z-scores decreased significantly (except for hip BMAD z-score of early-pubertal AMAB, whose decline was not statistically significant). BMAD was not significantly altered in the LS of AMAB and early-pubertal AFAB and in the hip of early-pubertal AMAB, while a small, but statistically significant, decrease was found in hip BMAD of AFAB and of late-pubertal AMAB and in LS BMAD of late-pubertal AFAB after 2-years GnRHa treatment (48).

A decrease in BMD z-scores during GnRHa, rapid during the first year, was also found in a retrospective review of 70 adolescents with GD. Most AFAB (94,9%) were in mid-late puberty and had menarche, while most AMAB (57%) were early-pubertal (G2-G3). Z-scores were lower in AFAB than AMAB. Z -scores of hip and LS BMD and of LS BMAD decreased significantly after the first year of GnRHa, a lower drop was noticed in hip and LS BMD and LS BMAD z-scores after the second year. However, no significant change was noticed in absolute values of hip or LS BMD or LS BMAD during GnRHa (49).

Another retrospective study of 62 AFAB adolescents showed that LS and hip BMD and BMD z-scores after at least 6 (median 8) months GnRHa monotherapy (at testosterone initiation) were lower than at GnRHa initiation (50).

Reduced bone turnover probably accounts for the decrease in z-scores during PS. Vlot et al. found that levels of bone formation and resorption markers (especially in younger transgender adolescents) reduced during GnRHa treatment in accordance with decreased BMAD z-scores. P1NP (bone formation marker) decreased significantly in young transgender adolescents during triptorelin treatment, a smaller reduction was also found in old AMAB. Osteocalcin was not affected by GnRHa in most adolescents, except an increase found in old AFAB. A decrease of ICTP (resorption marker) was noticed in all groups except old AFAB (47). In the study by Schagen et al. markers of bone formation (P1NP, P3NP and osteocalcin) and of bone resorption (1CTP) fell significantly in AMAB and in early-pubertal AFAB adolescents

after 2 years of GnRHa, especially within the first year (during which BMD was stable). In late-pubertal AFAB, P3NP and 1CTP decreased less but significantly during GnRHa (48).

BMD z-scores probably stabilize relatively and do not decrease further after the second year of GnRHa treatment. Carmichael et al. found that LS and hip BMD z-scores (and absolute hip BMD) did not decrease after 36 months of triptorelin monotherapy of 44 transgender adolescents (although LS BMC increased) (13). Schagen et al. showed that aBMD did not change significantly in a few (4 AFAB, 11 AMAB) adolescents treated with GnRHa for 3-4 years (started at mean age 12,6-12,7 years). No further reduction in aBMD z-scores was found in most subjects during the third or fourth year of triptorelin. However, BMD z-scores were lower at 36 months (than before GnRHa start) in the hip of AMAB and in the LS of AFAB (although the decrease was noticed mainly during the first year) (48).

BMD increases during GAHT (following GnRHa) treatment. Delemarre-van de Waal et al. found that hip and LS BMD z-scores increased during GAHT treatment (administered after triptorelin) of 21 transgender adolescents (10). Stoffers et al. noticed that BMD increased during testosterone (especially during the first 6 months) treatment of 62 AFAB adolescents (50). In the study by Klink et al. GAHT treatment (combined with GnRHa) after triptorelin monotherapy increased absolute LS and FN aBMD. LS aBMD z-score improved (not statistically significantly) in AFAB, while it did not increase in AMAB during GAHT (45). In the study by Vlot et al. hip BMAD and z-scores increased in AFAB and did not change in AMAB adolescents after 24 months of GAHT treatment (added to triptorelin at age 16), while LS BMAD absolute values and z-scores increased in all groups (47).

However, increase in BMD during GAHT may not compensate for the decrease in z-scores during PS, especially in AMAB. Vlot et al. observed that z-scores did not return to pre-GnRHa levels in most transgender adolescents after 2-year GAHT treatment (47). Stoffers et al. found that, after 1-2 years of testosterone treatment, LS and left hip BMD and BMAD were not significantly different than at GnRHa initiation, while BMD and LS BMAD z-scores remained lower than before GnRHa (50). Klink et al. showed significantly lower LS BMD z-score (for birth-assigned gender) at age 22 than at GnRHa initiation in AMAB and a trend for decrease in AFAB. At age 22 years, 6 AMAB (40%) had a LS BMD z-score lower than -2. Duration of GnRHa monotherapy was not correlated with BMD and z-scores at age 22 (45). Van der Loos et al. found that BMD z-scores in transgender individuals who had received PS caught up with pre-GnRHa levels after long-term (around 11 years) of GAHT, except for LS in AMAB (where z-scores remained lower than pretreatment values) (51). Thus, concerns are raised about possible delay or attenuation in acquisition of peak bone mass by GnRH treatment of adolescents.

Apart from bone density, GnRHa treatment of transgender adolescents during early puberty may also affect bone geometry development. A retrospective cohort study in Amsterdam of 322 transgender adolescents (67% AFAB, 86% of whom were in late puberty at study entry) showed that the alterations in hip subperiosteal width and endocortical diameter were similar with the reference curve of the experienced gender in study subjects who began GnRHa treatment in early puberty, while they remained within the reference curve for birth-assigned gender in adolescents who initiated GnRHa in mid- or late puberty (52).

However, data on fracture risk during or after GnRHa treatment of transgender adolescents are lacking.

Because long-term (>1 year) hypogonadism may affect bone density, baseline BMD testing is indicated for transgender individuals prior to initiation of hormone therapy that lowers endogenous gonadal steroid levels for a significant period. Follow-up BMD testing in transgender should be done, when the results are likely to influence patient management, for example in individuals receiving PS (44). BMD measurement (using DXA) is recommended by Endocrine Society every 1–2 years during PS. Normal calcium intake and exercise may be beneficial in preserving bone health in transgender adolescents during PS (3).

Effect of GnRHa on growth and height

Height SDS frequently decreases during the first 2 years of GnRHa treatment of transgender adolescents. Early experience with the Dutch protocol showed that height velocity (HV) of 21 adolescents with GD decelerated during triptorelin treatment for at least 2 years, especially in younger individuals (BA < 13 in girls, < 15 in boys), whose height SDS declined, while sitting-height:height ratio did not change (10). Schagen et al. (2016) observed that height SDS decreased significantly during the first two years of GnRHa treatment and did not change in the third year (12). Carmichael et al. found decreased height z-score with increased height during triptorelin monotherapy (started usually at stage 3-4) of 44 adolescents with GD (13).

TYCUS study of 55 transgender youth (52,7% AFAB) treated with GnRHa (84% histrelin, 16% leuprolide) for at least 10 months showed that initiation of GnRHa at early puberty resulted in growth rates similar with prepubertal controls, while beginning GnRHa later in puberty resulted in reduced HV. The majority (61,8%) of transgender adolescents started GnRHa at Tanner stage II. Median HV (5,1 cm/year) of transgender youth treated with GnRHa since stage II or III (n=50) for one year was not significantly different from HV of pre-pubertal age-matched cisgender controls. Starting GnRHa at stage IV was correlated with lower HV (1,6 cm/year). Age at GnRHa initiation was negatively associated with HV even when controlled for Tanner stage (53).

A recent retrospective cohort study of 146 AFAB treated with triptorelin before age 16 for at least 6 (mean 37) months showed that GnRHa does not have a significant negative effect on adult height, although it may raise it slightly above predicted, when started at a younger age. The cohort was subdivided into the pubertal (BA ≤ 14 before GnRHa initiation) and the postpubertal group (BA > 14). During GnRH, predicted adult height (PAH) increased by 2,4 cm, while mean height SDS decreased by 0,2. Height SDS of AFAB with BA > 12 (at GnRHa start) decreased more compared with those with BA ≤ 12 (whose height SDS was not reduced) during GnRHa, although height SDS at start of GAHT (after 3 years of GnRHa) was similar (between BA > 12 and ≤ 12). GnRHa also resulted in deceleration of bone maturation (increase in the difference between bone and chronological age), causing a

delay of -1,9 years. Growth accelerated and height increased (by 5 cm) during subsequent testosterone treatment. Adult height SDS was comparable to height SDS at GnRHa start. Adult height in the pubertal group was higher than in the postpubertal (difference 3 cm) and younger BA (at GnRHa start) was associated with significantly greater adult height compared with PAH (1,2 cm/y), although the difference between adult and mid-parental height was not significant (in pubertal compared with postpubertal group) (54).

Another retrospective cohort study of 161 AMAB treated with triptorelin or pamorelin for 2,4 years (mean) and subsequently with estradiol (concurrently with GnRH until gonadectomy) showed that, although GnRHa and GAHT affect growth velocity (GV), their effect on adult height is not important. The cohort was divided into the pubertal group, with BA < 16 years (at GnRHa start), and the postpubertal group, with BA ≥ 16 and those who had completed growth clinically (without BA measurement). During GnRHa, PAH increased by 1,5 cm, while height SDS reduced continuously (–0,37/year). Average GV declined (by -1,9 cm) from 5,3 cm/year in the first year to 3,5 cm/year in the second year of (GnRHa) treatment. Bone maturation slowed down during GnRHa, which caused a delay in BA by 1,6 years, with more prolonged GnRHa management related to longer delay of BA (–0,5 years/year of GnRHa). During estrogen treatment, GV accelerated (height SDS increased). When GnRHa was followed by regular-dose (2 mg/d) 17β-estradiol, adult height was slightly lower than PAH at GnRHa initiation and close to target height. Growth reduction (adult height lower than PAH by 4,8) was achieved with high-dose (100-200 μg) ethinyl estradiol (EE). High-dose (6 mg/d) 17β-estradiol did not significantly reduce growth. Adult height was slightly below target height in AMAB treated with high-dose 17β-estradiol and EE. In the postpubertal group adult height was 2.7 cm lower than in the pubertal on regular-dose estradiol. The difference between adult and target height in 42 postpubertal AMAB was significantly larger compared to pubertal AMAB who received regular-dose estradiol (55).

Similarly, another retrospective study of 32 trans-adolescents showed that early PS (started at mean age 12,4-13) and GAHT do not affect final height. The difference between final and target height (for gender assigned at birth) was not statistically significant, which suggests that final height is closer to gender assigned at birth, rather than to experienced gender (56).

Overall, data at this point suggest that GnRH results in decrease of height SDS, with minimal effect on adult height. According to Endocrine Society Guidelines, "during treatment, adolescents should be monitored for negative effects of delaying puberty, including a halted growth spurt and impaired bone mineral accretion". Measurement of height, weight, sitting height is recommended every 3–6 months during PS. Monitoring of BA by X-rays of the left hand can also help in growth evaluation (if clinically indicated) (3).

Effect of GnRHa on body composition, body fat and weight

GnRHa may increase body weight and body fat (Table 5). Nokoff et al. observed higher percent body fat in transgender youth treated with GnRHa since early puberty (59). Van de Waal et al. noticed that fat mass percentage rose significantly during the first year of triptorelin treatment and stabilized afterwards, while lean body mass (LBM) fell significantly during the first year and did not change further (10).

Another retrospective study of 548 transgender adolescents (69% AFAB) showed that LBM z-scores decreased and fat mass z-scores increased gradually throughout 3 years of PS in AMAB and during the first year of PS in AFAB (and stabilized afterwards). Decline in LBM z-scores was greater after 3 years of triptorelin in late pubertal adolescents (58).

A retrospective study of 192 transgender adolescents (63% AFAB) treated with GnRHa showed changes of body composition and body shape toward the experienced sex at age 22. GnRHa was started at least at Tanner stage 2 (female) or 3 (male). In AMAB, a statistically significant enlargement in waist and hip circumference with reduction in WHR and a significant elevation in total body fat (TBF) and in body fat percentage in the android and gynoid region with a significant decline in LBM percentage were noticed. In AFAB, waist and hip circumference, WHR and LBM percentage increased significantly, while percentage of TBF and of gynoid fat decreased, without significant change of android fat. Alterations in body fat and LBM were not different at 22 years after adjustment for Tanner stage at GnRHa initiation, although beginning treatment at an earlier stage achieved a larger similarity of body shape to the experienced gender at 22 years in AFAB, a comparable tendency was observed in AMAB. SDS for WHR, body fat and LBM at age 22 in AMAB were more similar with ciswomen (than with AFAB), while in AFAB they were between reference values for ciswomen and cismen (57).

A retrospective cohort study of 192 transgender individuals (63% AFAB) showed that BMI and LDL increased during GnRHa monotherapy (started at Tanner stage 4 or 5 in most). BMI was also higher at age 22 (as compared with GAHT start), with higher prevalence of obesity in AMAB (9.9%) and AFAB (6.6%) than in controls (60).

Schagen et al. (2016) found that BMI SDS did not significantly change in the first year of triptorelin treatment and increased (by 0,13) in the second year in AMAB, while it increased in AFAB (by 0,17) in the first year and did not significantly change thereafter. LBM percentage significantly decreased during the first year of treatment, whereas fat percentage and absolute fat mass significantly increased (12).

In the recent study by Carmichael et al. treatment of 44 transgender adolescents with triptorelin caused no significant change in weight and BMI z-scores at 24 months, although a rise was observed at 36 months (13).

Joseph et al. observed a gradual rise in height and weight during 3-year GnRHa treatment of 31 adolescents with GD, with a larger upsurge in the height of AFAB and in the BMI of AMAB (49).

Navabi et al. (2021) found that BMI z-score did not change significantly after GnRHa, although BMI increased only in AFAB. TBF and gynoid fat increased significantly in all. In AFABs android (fat percentage) and LBM also rose significantly. LBM z-score decreased significantly and TBF (%) z-score increased in AMAB, while they did not change significantly in AFAB (46).

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TABLE 5 Effect of GnRHa on total body fat (TBF), gynoid/android fat, LMB and BMI.

	TBF		Gynoid fat		Android fat		LBM		BMI	
	AFAB	AMAB	AFAB	AMAB	AFAB	AMAB	AFAB	AMAB	AFAB	AMAB
Navabi B et al. 2021 (46)	↑ (+2,2%) TBF z-score ↔ (+0,13%)	↑ (+5,4%) TBF z-score ↑ (+1,05%)	↑ (+1,83%)	↑ (+7,17%)	↑ (+2,75%)	↔	↑ (+ 1,05 Kg), LBM z-score ↔	↔, LBM z-score↓ (–0,73)	↑ (+1,36 mean) (BMI z-score ↔)	↔ (+0,57 mean) (BMI z-score ↔)
Schagen S et al. 2016 (12)	↑ (+4,4% after 1 year of GnRHa)	↑ (+4,5% after 1 year of GnRHa)	ND	ND	ND	ND	↓ (-3,8% after 1 year of GnRHa)	↓ (-3,7% after 1 year of GnRHa)	↑ (+1,1 mean) after 1 year GnRHa, BMI SDS: ↑ by 0.17 (after 1 year of GnRHa), ↔ after 1st year	↑ (+0,9 mean) after 1 year GnRHa, BMI SDS ↔ (after 1 year of GnRHa), ↑by 0.13 (at 2nd year)
Klaver M et al. 2018 (57)	↑(+3% during GnRHa alone), ↓ (-3% at age 22 vs GnRHa start)	↑ (+6,6% during GnRHa alone, +10% at age 22 vs GnRHa start)	↑ (+3% during GnRHa alone), ↓ (-5% at age 22 vs GnRHa start)	↑ (+7% during GnRHa alone, +11% at age 22 vs GnRHa start)	↑ (+4% during GnRHa alone), ↔ (+1% at age 22 vs GnRHa start)	↑ (+5% during GnRHa alone, +9% at age 22 vs GnRHa start)	↓ (-3% during GnRHa alone), ↑ (+3% at age 22 vs GnRHa start)	↓ (-6% during GnRHa alone, -10% at age 22 vs GnRHa start)	↑ (+0,9 during GnRHa alone, +2,3 at age 22 vs GnRHa start)	↑ (+1,1 during GnRHa alone, +3 at age 22 vs GnRHa start)
Boogers LS et al. 2023 (58)	Z-scores ↑ + 0,47 during 3-year PS (+0,31 at 1 year)	z-scores↑ (+1,06) during 3 years of PS	ND	ND	Android/ gynoid fat ratio↑ (+ 0,07) during 3- year PS	Android/ gynoid fat ratio stable during 3-year PS	z-score ↓ (- 0,32) at 1 year PS (stable at 2nd-3rd year)	z-score↓ (-1,13) during 3 years of PS	↑ + 2 kg/m^2 (stable BMI SDS) during 3 years of PS	↑ + 1,8 kg/ m^2 (stable BMI SDS) during 3 years of PS

↔ no statistically significant change, ↑ increase, ↓ decrease; ND, no data.

Effect of GnRHa on blood pressure, lipids and glucose

GnRHa may increase blood pressure (BP), especially in AFAB. Diastolic BP percentiles increased significantly after GnRH treatment of 15 AFAB adolescents (at Tanner stage 4 or 5), although BP levels remained within the normal range for age and did not meet criteria for hypertension. Diastolic BP percentiles decreased to baseline after adding testosterone. Systolic BP did not change significantly (61). Klink et al. reported three cases of AFAB adolescents presenting arterial hypertension after triptorelin treatment. BP normalized after discontinuation of GnRHa in two of these cases, reoccurrence of hypertension after GnRHa restart was noticed in one case (62). Klaver et al. (2020) found that diastolic BP increased (by 4 mm Hg) in AMAB during GnRHa monotherapy (60). However, Nokoff et al. (2021) found lower (not statistically significant in AFAB, statistically significant in AMAB, in comparison with cisgender females and males, respectively) mean systolic BP among transgender adolescents on GnRHa (59). Stoffers et al. noticed non-significant increase in diastolic BP and decrease in systolic BP during GnRHa treatment of 62 transgender adolescents (50). Possible mechanism of hypertension during GnRH in AFAB is hypoestrogenism, as estrogen induces vasodilation (60). Therefore, BP monitoring is recommended before and every 3-6 months during GnRHa treatment by Endocrine Society (3).

Data on the effect of GnRHa on glucose metabolism are scarce. A small study of 17 transgender youth treated with GnRHa showed lower insulin sensitivity (higher HOMA-IR), more frequent dysglycemia (higher HbA1c), higher leptin and percent body fat than cisgender controls. In AFAB total (not percent) lean mass was lower (than matched cisgender females), percent lean mass was lower in AMAB. Insulin sensitivity (assessed by 1/fasting insulin) was inversely correlated with percent fat and with BMI percentile (59). However, Klaver et al. detected no significant change in glucose and HOMA-IR during GnRHa monotherapy of 192 transgender adolescents (60). Carbohydrate metabolism was not affected by GnRHa treatment of 21 adolescents in Amsterdam (10). No significant change in HbA1c was noticed after triptorelin treatment of trans-adolescents in the studies by Fisher and by Stoffers et al. (14, 50). In a multicenter analysis no statistically significant difference was found in the odds of dysglycemia in transgender youth receiving GnRHa monotherapy (63).

Limited evidence exists on the effect of GnRHa on lipids. Lipid metabolism did not differ after 2-year triptorelin treatment of 21 adolescents (10). Fisher found no statistically significant changes in lipid levels during triptorelin treatment, except a slight HDL increase in AMAB (14). Klaver et al. (2020) noticed that LDL increased (by 0,2 mmol/l) during GnRHa monotherapy, it increased further during combination GnRHa-GAHT treatment only in AFAB, while LDL was not significantly different at the age of 22 in transgender in comparison with cisgender peers (60). Stoffers

et al. observed non-significant increases in total cholesterol (by 0,44 mmol/l), LDL (by 0,15 mmol/l), HDL (by 0,12 mmol/l) and triglycerides (by 0,01 mmol/l) during triptorelin treatment of 62 adolescents with GD (50). No association was found between GnRHa monotherapy of transgender youth and dyslipidemia in the recent analysis by Valentine et al. (63), although those treated with a combination of testosterone and GnRHa (or with combined oral contraceptive pills) had higher odds of dyslipidemia.

The latter multicenter retrospective, cross-sectional study analyzed cardiometabolic parameters in transgender and gender-diverse youth. GnRHa alone was not associated with significantly higher odds of cardiometabolic-related diagnoses. Norethindrone or medroxyprogesterone prescription for menstrual suppression in AFAB and antiandrogen spironolactone prescription for AMAB had no significant impact on cardiometabolic outcome, while AFAB receiving combined oral contraceptive pills had higher odds of overweight/obesity (63).

Effect of GnRHa on fertility

Initiation of GnRH analogs during early puberty temporarily pauses germ cell maturation through suppression of gonadotropins and gonadal hormones.

Rise in both gonadotropins is the main drive for spermatogenesis initiation as well as for the pubertal proliferation of Sertoli cells, which provide the cytoarchitectural environment and the nutrients necessary for germ cell development. Spermatogenesis begins usually at Tanner stage 3 or (in around 20% of boys) at stage 2 (42). Therefore, at Tanner stage 2-3 some spermatogonia (possibly less spermatocytes as well) may be found in testes before starting PS in AMAB, while mature spermatozoa are absent.

Histological and immunohistochemical analyses of testicular tissue (obtained during orchiectomy) were carried out in a cohort study of 214 AMAB treated with triptorelin (since adolescence) or CA (in adulthood) in combination with estrogen (since age 16 or after 18) and orchiectomy (at the age of 18 or older, after at least one year on estrogen). Full spermatogenesis was noticed in 10 transwomen (4,7%), who had begun GnRHa at Tanner stage 4 or further. Immature germ cells (60-70% spermatogonia) were found in all transwomen who started GnRHa at Tanner stage 2 or 3 (64).

Suppression of testosterone halts spermatogenesis. In a prospective cohort study of 97 AMAB treated with CA plus estrogens, immunohistochemical staining of testicular tissue (obtained during gonadectomy) for 4 germ cell differentiation markers showed no spermatogenesis in 77,3% and partial spermatogenesis in 22,7% of the participants. In all AMAB with adequately suppressed serum testosterone levels (within female reference range), spermatogenesis was fully suppressed (65).

Oocytes go through meiotic maturation just before ovulation, which starts after menarche. Primary oocytes remain in meiotic arrest (at prophase I) until puberty. Positive feedback of estradiol on pituitary, which is necessary for midcycle LH surge and ovulation, occurs at mid-puberty before menarche. After preovulatory LH surge, meiosis I is resumed and enters metaphase I, during which conversion of primary to secondary oocyte takes place and formation of first polar body occurs (before ovulation). Meiotic maturation continues, until oocyte enters metaphase II. A second short meiotic arrest (at metaphase II) lasts until fertilization (42).

Consequently, PS before menarche and prevention of puberty completion in AFAB probably deters oocyte maturation, thus fertilization may be negatively affected. Therefore, for transgender men who received GnRHa since Tanner stages 2 or 3, there is probably no need for menstrual suppression or for contraception measures, as they have likely not achieved the maturity necessary for pregnancy, similar to congenital hypogonadotropic hypogonadism (33).

A mouse model, which mimicked AFAB youth receiving PS (followed by testosterone), showed absence of corpora lutea, suggestive of continued anovulation, at day 21 after GnRHa implantation, in parallel with suppressed HPG axis suppression and decreased ovarian and uterine weight. The number of primary follicles was significantly lower during GnRHa treatment compared with controls (66).

Delaying or temporarily discontinuing GnRHa to allow gamete maturation could be suggested, but it is usually not selected by transgender, due to development of secondary sex characteristics (3). Suppression of HPG axis by GnRHa is reversible and there is no published data that GnRHa harms ovarian function, ovulation or fertility permanently. Thus, sperm (or oocytes) may be collected at some time after GnRHa discontinuation (before GAHT initiation).

Studies in CPP children show only temporary reproductive effect of PS, which returns to normal after GnRHa discontinuation. In a small study of 7 boys with CPP treated with GnRHa for 5,6 years (mean), pubertal response of FSH/LH to GnRH test was noted within 1,5 years and spermarche 0,7 to 3 years after cessation of GnRHa (with normal semen analysis) (67). In a study of 87 girls with CPP ovarian volume decreased and uterine length remained unchanged during triptorelin, while they increased, with completion of ovarian and uterine development, appearance of menarche and rise of FSH and LH peaks (at LHRH test) about one year after the end of GnRHa treatment (68). In TAP-144-SR Japanese Study of 76 children with CPP treated with leuprorelin, menarche/remenarche appeared in more than 95% of girls at (mean) 17,5 months (with all idiopathic and half of organic CPP cases having ovulatory menstrual cycles) and serum testosterone raised to normal adult levels in all boys at 11 months (mean) after the end of GnRHa (69).

A prospective study of 15 girls with CPP (or early puberty) showed that AMH (a marker of ovarian reserve) decreased significantly (by 51%) after the first 3 months of leuprolide and remained partially suppressed after 12 months. AMH returned to pretreatment levels 6 months after stopping leuprolide, suggesting that GnRHa probably does not have a negative impact on future reproductive function (70).

However, no studies have examined the effect of GnRHa on long-term reproductive function of transgender adolescents and conclusions from studies of GnRHa use in CPP girls cannot be generalized to AFAB, because CPP girls eventually complete puberty, while AFAB may never complete it (as they usually start GAHT without discontinuing GnRHa).

Mature spermatozoa may be present at Tanner stages 3-4, thus sperm cryopreservation (before starting PS) is feasible in late

puberty. According to de Nie et al., cryopreservation of mature spermatozoa (collected through TESE or during orchiectomy) may be feasible for 10% of AMAB starting GnRHa at Tanner stage 4-5, while it is not possible for those starting GnRHa at Tanner stage 2-3. Cryopreservation of testicular tissue (obtained during genital gender-affirming surgery) containing spermatogonial stem cells may be a future option for fertility maintenance for all transwomen starting GnRHa at Tanner stage 2-3 as well as for 90% of those starting GnRHa at Tanner stage 4-5 (64).

Another future experimental fertility option for transgender female may be uterus transplantation, which could improve dysphoric symptoms and offer the satisfaction of pregnancy and parenthood. However, its efficacy and safety in transgender women has not been proven and additional research is needed (71). There may also be legal barriers to its application.

Oocyte (or embryo) cryopreservation is feasible in late-pubertal perimenarchal AFAB. Oocyte retrieval requires ovarian stimulation by gonadotropins and transvaginal GD. Cryopreservation of ovarian tissue (usually of the cortex containing primordial follicles), which is obtained via biopsy or oophorectomy, is an option for fertility preservation, that could be useful in early-pubertal AFAB. *In vitro* growth of follicles (obtained from preserved ovarian tissue) is a promising method, which permits maturation of oocytes until metaphase II (71).

In conclusion, it is recommended that HCP inform transgender adolescents (before starting treatment) of the reproductive effects of gender-affirming treatments, including the potential loss of fertility (1). Endocrine Society recommends that clinicians "counsel all individuals seeking gender-affirming medical treatment regarding options for fertility preservation prior to initiating PS in adolescents" (3). Adolescents may not be competent enough to decide about fertility and may not fully realize the possible impact of gender-affirming treatments on fertility, thus consent and discussion should involve parent(s)/guardian(s) and the MHP.

Effect of GnRHa on cognitive function

A study of 40 adolescents with GD (55% AFAB) showed that those receiving PS with GnRHa (n=20) did not develop brain functioning similar to the experienced gender, on the contrary sex differences in neural activation resembled their birth-assigned gender. GnRHa did not harm executive functioning, since it had no significant effect on ToL (Tower of London) performance (reaction times and accuracy) scores (an executive functioning task), although AMAB who received GnRHa had significantly lower accuracy scores. In AMAB who received GnRHa, a greater activation was found in functional MRI in some brain regions (in bilateral dorsolateral prefrontal cortex, left rostrolateral prefrontal cortex and left precuneus) during task load ToL performance (compared to female controls), while in AFAB treated with GnRHa bilateral precuneus activation was less (than male control) (72).

During normal puberty brain maturation occurs, which is associated with increases in sex steroids. For example, testosterone affects neural axon radial growth. There are concerns that suppression of sex hormones during puberty may prevent maturation of brain. A case report of a 11-year-old adolescent AMAB stated that brain white matter did not change during 28 months of GnRHa treatment and that operational memory decreased (by 9 points) after 22 months and remained stable after 28 months (of GnRHa) (73).

Expression of GnRH receptors has been found in the hippocampus, which regulates some cognitive functions, such as spatial orientation, learning and memory. Longtime peri-pubertal GnRHa treatment is related to alterations in mRNA expression of genes, involved in hormone signaling (GH, ESR1) and synaptic plasticity (VGF, NCAM1, LHX5) within the hippocampus of sheep (74). GnRHa does not significantly affect spatial orientation (74, 75). In a study using an ovine model, GnRHa did not affect maze traverse times of male sheep. Blockade of GnRH and testosterone affected the manner in which animals moved through a maze, increasing their emotional reactivity, which was restored with testosterone replacement. Blockade of GnRH signaling impaired long-term spatial reference memory, this effect was not restored with testosterone replacement (75).

Other GnRHa adverse effects

In a prospective study adverse events were mild and frequent (in 22-25%) during the first two years of GnRHa treatment of adolescents with GD, while they were less common after 12 months (13). Hot flushes or mild headaches were the most common side effects (in 47,7% of participants during the first year). In two AFABs very small doses of 'add-back' estradiol decreased headaches and hot flushes. Mild fatigue was reported by 5–8% during the first two years, with no one reporting moderate or severe fatigue. Mild sleep problems, mood swings and weight gain were reported rarely (2-3%).

Similarly, hot flushes, mood swings, weight gain, and fatigue were the most common adverse effects of GnRHa, noted in 65% (11 out of 17) transgender adolescents, in a retrospective study (32). The first 11 AFAB treated (according to the Dutch protocol) with triptorelin (starting at pubertal stages 4-5) experienced numerous hot flashes, which occurred less often as months passes by (10).

Withdrawal bleeding may occur soon after GnRHa initiation (due to estrogen fall) before menses cease (12).

A few transgender adolescents have discontinued GnRHa due to side effects. In a small retrospective study of youth with GD in Canada, one (out of 27) transgender adolescent decided to cease GnRHa treatment because of emotional lability. In one AFAB leuprolide was changed to triptorelin due to sterile abscesses. One AFAB manifested leg pains and headaches during GnRHa, which receded. One young patient (with BMI above 85 percentile before treatment) gained 19 kg within 9 months of GnRHa (24).

In a retrospective study of 143 adolescents in the Netherlands, 3 subjects discontinued GnRHa due to possible side effects (mood disturbances/swings, suicidal thoughts, nausea/weight loss). One of

them (AFAB) discontinued GnRHa after 4 months due to hot flushes, increase in migraine, fear of injections and stress (partly owing to school problems) and restarted GnRHa restart 5 months later (31).

Discussion

PS can only be recommended to adolescents who meet the diagnostic criteria of GD (by DSM-5 and/or ICD-11) and have long-lasting intense GD, which aggravate with puberty onset. Possible mental issues should be addressed before treatment and informed consent (by the adolescent/parent) should be given, after counseling on possible reproductive effects of GnRHa.

The main aim of GnRHa treatment of adolescents with GD is the prevention of development of secondary sex characteristics (e.g. facial hair and voice deepening in AMAB), which aggravate distress. GnRHa can be administered only after the adolescents have entered Tanner stage 2. GnRHa are effective in arresting puberty and reversibly stop its progression to the following Tanner stages. GnRHa result in testicular volume reduction in AMAB, regression of breast development and menses cessation in AFAB. GnRHa may decrease the need for feminization/masculinization surgery (e.g. mastectomy in AFAB).

By alleviating the distress caused by puberty physical changes, GnRHa may improve psychosocial functioning, well-being, quality of life, emotional and behavioral (especially internalizing) problems and depressive symptoms, thus decreasing suicidality. However anxiety, body dissatisfaction and GD may not ameliorate.

PS can extend the diagnostic period and give transgender adolescents time to explore their gender identity before proceeding to GAHT and/or surgery. GnRHa administration may also reduce the necessary doses of GAHT.

However, PS may retard normal bone mass accrual. Most studies showed that BMD (and BMAD) z-scores (mainly in the LS) decrease during the first two years (rapidly during the first year) (49) of GnRHa treatment (and may stabilize during the third or fourth year) (48), especially in AFAB adolescents. Absolute BMD (and BMAD) values did not change significantly after GnRHa in the majority of the studies, although a reduction was noticed in a few studies (45, 48) particularly in late-pubertal AFAB. The decrease in z-scores is probably due to reduced bone turnover, as bone formation (mainly P1NP) and bone resorption (e.g. ICTP) markers decreased significantly during GnRHa, especially in younger adolescents (47, 48). Bone density increases during subsequent GAHT treatment, although z-scores may not reach levels before GnRHa start, especially in LS of AMAB (45, 47). However, most studies examined the effect of GnRHa initiated in late puberty on bone density. Evidence on the consequences of GnRHa started in early puberty on bone density in GD individuals (especially in AFAB) is relatively limited, although much bone has already developed in late puberty. Furthermore, inadequate data exist regarding the effect of GnRHa treatment for more than 2 years. CA may also result in decreased aBMD z-scores (mainly in LS), while lynestrenol probably does not affect normal bone development significantly (36). It is unknown whether GnRHa (or CA) increases the risk of fractures either in adolescence or later in life.

Height SDS decreases during the first two years of GnRHa treatment (10, 12, 54, 55), with HV lower than prepubertal controls, when GnRHa is started at Tanner stage 4 (53). Bone maturation may decelerate during GnRHa, which may cause a delay in BA by 1,6-1,9 years (54, 55). GnRHa does not seem to have a significant effect on adult height. However, adult height may be increased above predicted in AFAB, when GnRHa is initiated at a young BA. Adult height may be below predicted in AMAB, when GnRHa is followed by high-dose EE.

GnRHa, especially when started early at puberty, may cause changes in the body phenotype of transgender adolescents (mainly AFAB) resembling the desired sex (56). After GnRHa TBF, gynoid and android fat percentage increased, although Klaver et al. found that TBF and gynoid fat decreased (android fat increased non-significantly) in AFAB (57). LBM percentage (and LBM z-score) decreased in AMAB (12, 46, 57) (especially during the first year), while LBM increased in AFAB (12, 46) (without change in LBM z-score). Waist and hip circumference increase during GnRHa, with WHR increased in AFAB and decreased in trangirls (57). BMI z-score probably does not change significantly during GnRHa (46), although BMI (or BMI SDS) may increase during the first year of GnRHa treatment in AFAB (12, 46) and stabilize thereafter (12).

Through gonadotropin and gonadal steroid suppression, GnRHa initiation during early puberty reversibly halts normal pubertal gametes maturation, thus fertilization may be negatively affected. Therefore, transgender adolescents should be counselled about the possible reproductive effects of PS and about options for fertility preservation before starting treatment (1). Fertility preservation can be achieved via oocyte (or embyo) cryopreservation in late-pubertal AFAB and by sperm cryopreservation for late-pubertal AMAB.

GnRHa may cause hypertension, especially diastolic, mainly in AMAB adolescents, thus BP monitoring is recommended. The most common side effects of GnRHa are hot flushes, mood fluctuations, fatigue and headache. They are usually mild and rarely lead to GnRHa discontinuation.

A major drawback in examining the consequences of PS on transgender adolescents is the absence of large comparative studies. Limited evidence exists regarding long-term effect of PS on fertility, bone density and fracture risk, and neurocognitive development of transgender. There are no randomized controlled trials examining the efficacy and safety of GnRHa in adolescents with GD. The existing studies are limited in number, of small sample size, uncontrolled, observational, usually short-term, potentially subject to bias, so that evaluation of the evidence needs to be done with cautiousness. Moreover, conduct of the studies mainly in a limited number of tertiary referral centers and absence of research in low-income populations prevents extrapolation of their results. Gender-affirming treatment is more readily available in the western world, while in many less developed countries much more work is needed in these areas due to limited access to treatment and prejudice surrounding this topic.

Conclusion

The inadequate published evidence from relatively small, uncontrolled studies suggests that PS (preferentially by GnRHa) can be beneficial in adolescents at Tanner stage 2 of further, who meet DSM-5/ICD-11 criteria of GD and give informed consent, after being counselled about potential reproductive effects, without having unaddressed mental health concerns. Advantages of PS are prevention of development of secondary sex characteristics and improvement of psychosocial functioning, especially decrease in emotional problems and depression. Disadvantages of GnRHa are the possible deceleration in bone mineral accrual and in height/growth velocity and the temporary pause in gametes maturation. Long-term randomized controlled studies with large study populations are needed in adolescents with GD to prove the positive and elucidate the possible negative effects of PS (on bone, growth, reproduction, cognition). Further research is needed to identify adolescents who might benefit, and those who are at greater risk.

Author contributions

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Derivation and validation of the first web-based nomogram to predict the spontaneous pregnancy after reproductive surgery using machine learning models

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Objective: Infertility remains a significant global burden over the years. Reproductive surgery is an effective strategy for infertile women. Early prediction of spontaneous pregnancy after reproductive surgery is of high interest for the patients seeking the infertility treatment. However, there are no high-quality models and clinical applicable tools to predict the probability of natural conception after reproductive surgery.

Methods: The eligible data involving 1013 patients who operated for infertility between June 2016 and June 2021 in Yantai Yuhuangding Hospital in China, were randomly divided into training and internal testing cohorts. 195 subjects from the Linyi People's Hospital in China were considered for external validation. Both univariate combining with multivariate logistic regression and the least absolute shrinkage and selection operator (LASSO) algorithm were performed to identify independent predictors. Multiple common machine learning algorithms, namely logistic regression, decision tree, random forest, support vector machine, k-nearest neighbor, and extreme gradient boosting, were employed to construct the predictive models. The optimal model was verified by evaluating the model performance in both the internal and external validation datasets.

Results: Six clinical indicators, including female age, infertility type, duration of infertility, intraoperative diagnosis, ovulation monitoring, and anti-Müllerian hormone (AMH) level, were screened out. Based on the logistic regression model's superior clinical predictive value, as indicated by the area under the receiver operating characteristic curve (AUC) in both the internal (0.870) and external (0.880) validation sets, we ultimately selected it as the optimal model. Consequently, we utilized it to generate a web-based nomogram for predicting the probability of spontaneous pregnancy after reproductive surgery. Furthermore, the calibration curve, Hosmer–Lemeshow (H-L) test, the decision curve analysis (DCA) and clinical impact curve analysis (CIC) demonstrated that the model has superior calibration degree, clinical net benefit and generalization ability, which were confirmed by both internal and external validations.

Conclusion: Overall, our developed first nomogram with online operation provides an early and accurate prediction for the probability of natural conception after reproductive surgery, which helps clinicians and infertile couples make sensible decision of choosing the mode of subsequent conception, natural or IVF, to further improve the clinical practices of infertility treatment.

KEYWORDS

reproductive surgery, spontaneous pregnancy, predictive model, online nomogram, individualized medicine, machine learning

Introduction

During the last decades, the number of infertile couples caused by different etiologies has gradually increased worldwide since 1990 (1), resulting in a substantial medical and social burden. Nowadays, reproductive surgery and *in vitro* fertilization and embryo transfer (IVF-ET) are two main treatment strategies for infertility (2). Reproductive surgery is a minimally invasive technology that aims to restore the functional anatomy and accomplish fertility preservation to enhance the chance of natural or assisted pregnancy. The definition of spontaneous pregnancy refers to the process of achieving pregnancy without the use of assisted reproductive technologies or additional interventions, which is important for both spouses, such as saving time and expense and reducing the risk of low birth weight and birth defects in newborns. Compared with IVF, a successful reproductive operation could offer patients the opportunity for natural conception monthly and avoid the complications of IVF, such as ovarian hyperstimulation syndrome and multiple pregnancies (3). Even without spontaneous pregnancy after a 1~2-year postoperative period, endoscopic surgical procedures could provide comprehensive evaluations including anatomy and function of the reproductive organs to improve pregnancy outcome in subsequent IVF (4).

In the era of precision medicine, early prediction of the reproductive surgery outcomes, such as spontaneous pregnancy, is of high interest for the women seeking the infertility treatment. However, there is still lacking of a high-quality model and clinical applicable tool to predict the probability of natural conception after reproductive surgery. On the one hand, due to the heterogeneity of operational quality control, the longer learning curve of surgical skill, and the absence of verification of conception rates following surgery, the majority of available literature regarding postoperative pregnancy outcomes consists of small single-institution retrospective cohort studies. On the other hand, the assessment of women's potential for fertility after operation primarily relies on the clinical experience generated by physicians, hence it is really difficult to give an individualized opinion since every patient has a unique situation. Some patients blindly adhere to attempt natural pregnancy after surgery, missing the golden time of IVF therapy, especially when the recurrence of endometriosis or hydrosalpinx requiring a second operation comes. Therefore, in order to make informed decisions regarding natural fertilization or IVF as early as possible, it is critical to timely anticipate the likelihood of spontaneous pregnancy after surgical reconstruction of reproductive function.

Notably, the prediction model derived from machine learning (ML) algorithms is a reliable and widely used statistical tool (5) that can consider various factors simultaneously to provide a probability of a specific outcome, especially in medicine (6). Nevertheless, as far as we know, there has been no research that has developed a forecasting model for the probability of natural pregnancy after reproductive surgery, and the key predictors are also under discussion. Hence, the objective of this research was to derivate and validate an analytical model based on multiple typical ML algorithms to ascertain the crucial clinical factors and provide an early personalized evaluation of probability of postoperative spontaneous pregnancy.

Materials and methods

This prediction model study is reported in accordance with the Transparent Reporting of a Multivariable Prediction Model for Individual Prognosis or Diagnosis (TRIPOD) checklist (7). The study was approved by the Ethics Committees of Yantai Yuhuangding Hospital (YT2023–054) and Linyi People's Hospital [LYRMYY (2023–04-036)]. Since this research was a retrospective cohort study, the data was made anonymous and there was no need for informed consent.

Study population

Between June 2016 and June 2021, a total of 2049 individuals underwent surgical procedures for infertility at the Department of Reproductive Medicine, Yantai Yuhuangding Hospital, China. Data on demographic, preoperative clinical assessment, surgical procedure

details, operative diagnosis and blood biochemical parameters were retrospectively collected from an electronic medical record system (Jiahe Meikang Information Technology, Beijing, China), which were utilized for the derivation and internal validation of the prediction model. In the external validation cohort, 363 infertile couples were hospitalized at the Department of Reproductive Medicine, Linyi People's Hospital, from January 2019 to June 2021. Data about pregnancy of follow-up evaluations was recorded by phone call or review of outpatient clinic revisit records. The follow-up period was 2 years. The data is reviewed, extracted, and cross-checked by the expert clinical team, with two separate clinicians who were unaware of the recorded results conducting the verification. Any disagreements were resolved by roundtable consensus.

Inclusion and exclusion criteria

The eligibility requirements were as follows: (1) age ≤38 years; (2) patients having an almost menstrual cycle (counting from the first day of one menstrual period to the first day of the next cycle) is 21 to 35 days and lasts from 3 to 7 days duration with volume of blood loss 50–80 ml; (3) spouse's roughly normal semen quality; (4) couples' normal sexual life; (5) patients obtaining at least one grossly functionally normal fallopian tube after surgery; (6) patients holding intentions to get a natural pregnancy after surgery during at least 2-year observation period. In contrast, the analysis did not include patients with a history of unsuccessful IVF and pathology requiring surgical treatment before the next IVF. Patients who converted to IVF treatment due to personal reasons within a 2-year follow-up period were excluded from this study. In addition, we excluded patients who needed for preimplantation genetic diagnosis and lacked primary measured data. All participants included in this research were of Han descent, and had no history of psychiatric or neurological illness, and no history of alcohol or drug abuse, and no recent history of smoke.

Dependent variable

As a primary outcome, clinical pregnancy was defined as observation of one or more intrauterine gestational sacs by a transvaginal ultrasound scan during follow-up period after reproductive surgery. The pregnancies from artificial insemination and IVF were not taken into account, meanwhile the ectopic pregnancy was regarded as a failure.

Independent variables selection and definition

Independent variables were selected based on the known clinically risk factors and availability in the electronic medical record system (Jiahe Meikang Information Technology, Beijing, China), which include: female age, body mass index (BMI, kg/m^2, <20; 20–24.9; 25.0–29.9; ≥30.0), infertility type (primary or secondary), duration of infertility, history of previous pelvic surgery, and tubal patency test by hysterosalpingography (HSG, mild, moderate or severe altered tubal patency). Women voluntarily had a baseline serum AMH measurement by an ultrasensitive two-site ELISA (AnshLabs, Webster, TX, USA) (8) on the first day of hospitalization before surgery. In clinical terms, preoperative AMH was categorized into three grades based on the following criteria: low (≤1.2 ng/ml), normal (1.2–4.0 ng/ml), and high (≥4.0 ng/ml). The reference data for these grades are derived from previous literature sources in conjunction with our empirical generalizations (9–11). In addition, some patients experienced ovulation monitoring using transvaginal ultrasounds (≥2 times per menstrual cycle) to clearly define ovulation time after surgery in our or other clinics.

To assess the patient's physical condition, common serum biochemical parameters were determined on the first day of hospitalization, as following: carbohydrate antigen 125 (CA125), total cholesterol (TC), triglyceride (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), alanine aminotransferase (ALT), aspartate transaminase (AST), creatinine (Cr), fasting insulin (INS) and fasting glucose (Glu).

All included subjects had undergone diagnostic or operative laparoscopy combined with hysteroscopy routinely. According to intraoperative dominant manipulation, the main operative diagnoses were categorized into seven subgroups, as mentioned in the studies by Ban Frangez, H., et al. (3) and Premru-Srsen, T., et al. (12). These subcategories encompass diagnostic surgery, mild to moderate endometriosis, severe endometriosis, intramural fibroids, unilateral tubal factor, bilateral tubal factor and miscellaneous cases. See attached Additional File 1: Supplementary Table 1 for more details.

Screening independent risk factors

Firstly, covariates with a P value less than 0.2 from the univariate logistic analysis were chosen for the binary multivariate logistic regression analysis, which was used to determine which predictors independently associated with spontaneous pregnancy according to the backward stepwise selection with the Akaike information criterion (AIC). Odds ratios (OR) with 95% confidence interval (CI) were calculated.

To ensure accuracy of predictive factors selection, the least absolute shrinkage and selection operator (LASSO) analysis was also employed to identify the most significantly independent features from the training dataset (6), augmented with ten-fold cross-validation.

Model construction

Six common machine learning algorithms, namely logistic regression, decision tree, random forest, support vector machine (SVM), k-nearest neighbor (KNN), and extreme gradient boosting (XGBoost), were utilized to construct the predictive model in the training cohort. Additionally, we assessed the robustness and generalization ability of the above predictive models by comparing their performance parameters including the area under the curve

(AUC) of the receiver operating curve (ROC), accuracy, precision, sensitivity, and specificity in the internal and external validation sets.

Evaluation and validation of the nomogram

We ultimately selected the logistic regression as the optimal model due to its superior clinical predictive value in both internal and external validation sets (refer to the Results section for more information). Subsequently, the nomogram was constructed using the findings from the analysis of multivariate logistic regression. In order to support their integration into the clinical setting, a *Shinyapp.io* application (https://www.shinyapps.io/) was utilized to create an interactive web-based dynamic nomogram.

To evaluate the nomogram's prediction accuracy, the AUC of the ROC with the bootstrapping method was used to determine the discrimination of the proposed model (7). Further, the calibration curves were plotted to test the goodness-of-fit of the model concurrently accompanied with the Hosmer-Lemeshow test (13). The clinical usefulness of this nomogram was evaluated through decision curve analysis (DCA), which aimed to identify the prediction's net benefit threshold. The nomogram's clinical effective rate was evaluated using the clinical impact curve (CIC) (14). Last but not least, the sensitivity analyses were performed to assess how the prediction performance change with univariable models compared with that of our final nomogram from the perspective of AUC and DCA.

Statistical analysis

R software (version 4.2.3, available for download https://www.rproject.org/) was utilized to perform all statistical analysis. Various specific packages such as "pROC", "rms", "ggplot2", "dca", "DynNom", "tidyverse" and "mlr3" were employed. Descriptive statistics were used to summarize baseline characteristics. Continuous variables were presented as mean (standard-deviation). A complete randomized analysis of variance was used to compare differences among groups (Gaussian distribution) or Kruskal-Wallis rank sum test (nonnormal distribution). Categorical variables were expressed as frequency (percentage values), and differences among cohorts were determined using the chi-square (χ^2), Fisher's exact test or Kruskal-Wallis rank sum test, as appropriate. A 2-tailed *P* value <0.05 was considered statistically significant.

Results

Out of 2049 operated women in Yantai Yuhuangding Hospital, 964 were immediately referred to IVF due to factors such as male infertility, damaging to bilateral fallopian tubes, or previous unsuccessful attempts at IVF. Among the remaining 1085 women, 13 ceased to plan pregnancy due to personal reasons, 29 women were lost from follow-up, and 30 subjects missed primary items, including HSG, AMH, CA125, TC, TG, INS and Glu. No significant differences were observed between the values before and after removing the missing data (Additional File 1: Supplementary Table 2). Figure 1 displays the flowchart illustrating the process of selecting patients and designing the study.

Using a rate of 50% for the occurrence of the event in the series (spontaneous pregnancy after reproductive surgery) and considering 6 variables selected through multivariable logistic analysis, we conducted a power analysis. This analysis utilized the formula developed by Riley et al. (15), with the aim of achieving a shrinkage of predictor effects of 0.288 (pmsampsize (type = "b", *r* squared = 0.288, parameters = 6, prevalence = 0.50) (15, 16) and obtaining a required sample size of 385 patients and 32.08 events per variable. Finally, a total of 1013 individuals in Yantai Yuhuangding Hospital were enrolled in this study to develop the model, which satisfied the minimum sample size.

Among 1013 enrolled infertile women, the percentage of women who conceived spontaneously is 51.7% (n = 524/1013) in the postoperative 2-year period. The enrolled patients were randomly divided into a training set (70.3%, n = 713) which was used to construct a model, and an internal validation set (29.7%, n = 301). Meanwhile, an additional 195 patients from Linyi People's Hospital were utilized for external validation. The process of patient selection can be seen in Figure 1. No significant difference is observed in the spontaneous pregnancy rate (51.4%, 52.5% and 50.3%, $P<0.05$), clinical baseline characteristics and laboratory data among the three datasets (training, internal and external validation sets), indicating good homogeneity between the three datasets, which was summarized in Table 1.

Independent risk factors

First, 19 variables were analyzed via univariate logistic analysis, and eight features with statistically significant differences ($P<0.2$) were picked out. Next, the aforementioned variables were incorporated into the original multivariate logistic regression model (AIC=614.62), as shown in Table 2. Finally, according to the principle of AIC minimization (AIC=610.43), six independent predictors were selected in the final logistic regression model by the backward stepwise selection. Table 2 displays the precise coefficients for each individual factor.

Regarding LASSO regression, Supplementary Table 3 (Additional File 1) displays the coefficients, while Figure 2A illustrates a profile of the coefficients. Significantly, the optimal tuning parameter for LASSO regression, denoted as "Lambda (λ)", was determined to be 0.036 at the point where the partial likelihood binomial deviance achieved its lowest value (refer to Figure 2A). As shown in Figure 2B, six predictors including dummy variables were independently associated with non-zero coefficients within one standard error of the log λ minimum in the LASSO analysis. These variables were selected for the most regularized and parsimonious model.

Encouragingly, both the number and name of the final independent factors (age, infertility type, duration of infertility, surgical procedures, ovulation monitoring and AMH) were good concordance between

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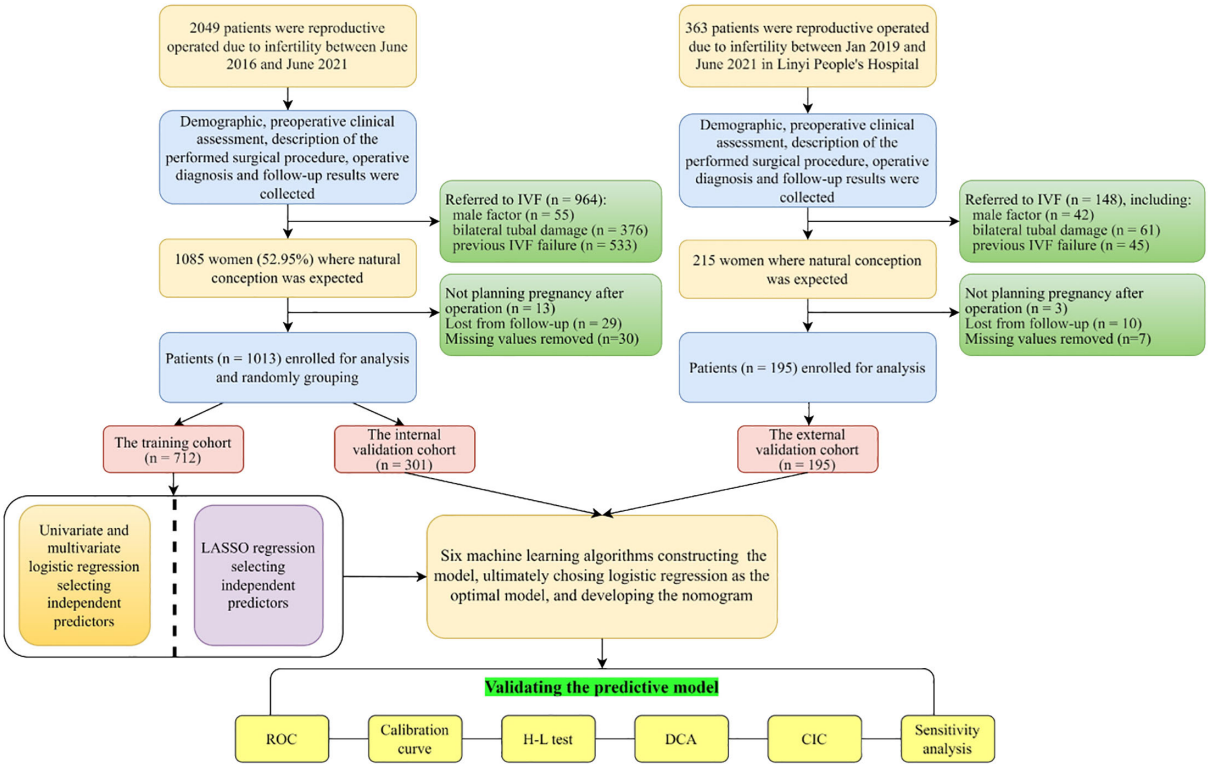

FIGURE 1
Flowchart of the study. IVF, *in vitro* fertilization; LASSO, least absolute shrinkage and selection operator; ROC, receiver operating curve; H-L test, Hosmer-Lemeshow test; DCA, decision curve analysis; CIC, clinical impact curve.

TABLE 1 Baseline characteristics of all patients in the training cohort and validation cohort.

Variables	Total	Training cohort	Internal validation cohort	External validation cohort	*P*-value (overall)
	N=1208	*N=712*	*N=301*	*N=195*	
Pregnancy[a]:					0.886
No	586 (48.5%)	346 (48.6%)	143 (47.5%)	97 (49.7%)	
Yes	622 (51.5%)	366 (51.4%)	158 (52.5%)	98 (50.3%)	
Age (years)[b]	31.2 (3.40)	31.3 (3.41)	31.2 (3.19)	30.9 (3.72)	0.851
BMI (kg/m^2)[a]:					0.075
20~24.9	676 (56.0%)	400 (56.2%)	169 (56.1%)	107 (54.9%)	
<20	243 (20.1%)	136 (19.1%)	68 (22.6%)	39 (20.0%)	
25~29.9	244 (20.2%)	145 (20.4%)	60 (19.9%)	39 (20.0%)	
≥30	45 (3.73%)	31 (4.35%)	4 (1.33%)	10 (5.13%)	
Infertility_type[a]:					0.269
primary	608 (50.3%)	367 (51.5%)	143 (47.5%)	98 (50.3%)	
secondary	600 (49.7%)	345 (48.5%)	158 (52.5%)	97 (49.7%)	
Duration of infertility (years)[b]	2.57 (1.35)	2.62 (1.37)	2.50 (1.33)	2.51 (1.33)	0.181

(Continued)

TABLE 1 Continued

Variables	Total	Training cohort	Internal validation cohort	External validation cohort	*P*-value (overall)
	N=1208	*N=712*	*N=301*	*N=195*	
Previous_pelvic_surgery[a]:					0.868
no	1179 (97.6%)	696 (97.8%)	293 (97.3%)	190 (97.4%)	
yes	29 (2.40%)	16 (2.25%)	8 (2.66%)	5 (2.56%)	
HSG[a]:					0.067
mild	518 (42.9%)	308 (43.3%)	114 (37.9%)	96 (49.2%)	
moderate	351 (29.1%)	197 (27.7%)	105 (34.9%)	49 (25.1%)	
severe	339 (28.1%)	207 (29.1%)	82 (27.2%)	50 (25.6%)	
Surgical_procedures[a]:					0.778
diagnostic	186 (15.4%)	106 (14.9%)	44 (14.6%)	36 (18.5%)	
endometriosis_mild_moderate	235 (19.5%)	134 (18.8%)	62 (20.6%)	39 (20.0%)	
endometriosis_severe	116 (9.60%)	71 (9.97%)	29 (9.63%)	16 (8.21%)	
intramural_fibroids	24 (1.99%)	13 (1.83%)	7 (2.33%)	4 (2.05%)	
tubal_factor_unilateral	226 (18.7%)	144 (20.2%)	48 (15.9%)	34 (17.4%)	
tubal_factor_bilateral	254 (21.0%)	148 (20.8%)	65 (21.6%)	41 (21.0%)	
miscellaneous	167 (13.8%)	96 (13.5%)	46 (15.3%)	25 (12.8%)	
Ovulation_monitoring[a]:					0.398
no	489 (40.5%)	294 (41.3%)	115 (38.2%)	80 (41.0%)	
yes	719 (59.5%)	418 (58.7%)	186 (61.8%)	115 (59.0%)	
AMH[a]:					0.545
normal	1124 (93.0%)	664 (93.3%)	276 (91.7%)	184 (94.4%)	
low	31 (2.57%)	21 (2.95%)	9 (2.99%)	1 (0.51%)	
high	53 (4.39%)	27 (3.79%)	16 (5.32%)	10 (5.13%)	
CA125 (U/mL)[b]	24.3 (8.26)	24.2 (8.21)	24.6 (8.49)	24.2 (8.13)	0.430
TC (mmol/L)[b]	4.57 (0.84)	4.57 (0.85)	4.57 (0.82)	4.58 (0.83)	0.985
TG (mmol/L)[b]	1.19 (0.49)	1.20 (0.50)	1.16 (0.50)	1.21 (0.47)	0.244
HDLC (mmol/L)[b]	1.53 (0.39)	1.53 (0.39)	1.56 (0.39)	1.50 (0.37)	0.152
LDLC (mmol/L)[b]	2.59 (0.63)	2.59 (0.62)	2.58 (0.66)	2.60 (0.60)	0.757
ALT (U/L)[b]	25.6 (11.9)	25.5 (11.0)	26.2 (13.9)	25.2 (11.6)	0.427
AST (U/L)[b]	25.1 (8.45)	25.3 (8.65)	24.9 (8.26)	25.0 (8.05)	0.493
Cr (μmol/L)[b]	57.9 (10.4)	58.2 (10.2)	57.3 (10.5)	57.4 (10.7)	0.208
INS (uU/mL)[b]	13.8 (6.58)	13.8 (6.54)	13.8 (6.88)	13.9 (6.31)	0.963
Glu (mmol/L)[b]	5.04 (0.77)	5.01 (0.76)	5.12 (0.77)	5.03 (0.83)	0.052

[a]Categorical variables were expressed as frequency (percentage values), and differences among cohorts were determined using the chi-square (χ^2), Fisher's exact test or Kruskal-Wallis rank sum test, as appropriate. [b]All values were mean (standard-deviation) and tested by analysis of variance (Gaussian distribution) or Kruskal-Wallis rank sum test (nonnormal distribution). BMI, body mass index; HSG, hysterosalpingography; AMH, anti-Müllerian hormone; CA125, carbohydrate antigen 125; TC, total cholesterol; TG, triglyceride; HDLC, high-density lipoprotein; LDLC, low-density lipoprotein; ALT, alanine aminotransferase; AST, aspartate transaminase; Cr, creatinine; INS, fasting insulin; Glu, fasting glucose.

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TABLE 2 Univariate and multivariate logistic regression analysis to determine independent predictors associated with spontaneous pregnancy according to the backward stepwise selection with the Akaike information criterion (AIC).

Characteristics	Univariate analysis			Multivariate analysis (original) (AIC=614.62)			Multivariate analysis (final) (AIC=610.43)			
	OR	95%CI	*P* value	OR	95%CI	*P* value	β	OR	95%CI	*P* value
Age (years)	1.29	1.22–1.36	<0.001	0.79	0.74–0.85	<0.001	-0.227	0.80	0.75–0.85	<0.001
BMI (kg/m^2)										
20~24.9	reference			reference						
<20	0.94	0.63–1.38	0.737	0.73	0.42–1.27	0.262				
25~29.9	1.16	0.80–1.70	0.435	1.06	0.63–1.78	0.837				
≥30	2.34	1.08–5.11	0.032	0.59	0.21–1.63	0.309				
Infertility_type										
primary	reference			reference			reference			
secondary	0.26	0.19–0.35	<0.001	3.06	2.01–4.66	<0.001	1.107	3.02	1.99–4.60	<0.001
Duration of infertility (years)	2.39	2.05–2.79	<0.001	0.44	0.36–0.54	<0.001	-0.807	0.45	0.37–0.54	<0.001
Previous_pelvic_surgery										
no	reference									
yes	1.37	0.50–3.72	0.537							
HSG										
mild	reference			reference						
moderate	0.73	0.51–1.05	0.088	1.53	0.91–2.58	0.105				
severe	0.85	0.60–1.21	0.359	1.50	0.92–2.45	0.105				
Surgical_procedures										
diagnostic	reference			reference			reference			
EM_mild_moderate	0.19	0.11–0.35	<0.001	7.67	3.58–16.41	<0.001	1.992	7.33	3.45–15.58	<0.001
EM_severe	3.33	1.68–6.62	0.001	0.29	0.12–0.70	0.005	-1.131	0.32	0.14–0.76	0.010
intramural_fibroids	0.4	0.12–1.37	0.143	1.31	0.25–6.85	0.751	0.454	1.58	0.30–8.20	0.590
tubal_factor_unilateral	0.58	0.35–0.97	0.038	2.05	1.05–3.98	0.035	0.700	2.01	1.04–3.89	0.037
tubal_factor_bilateral	0.99	0.6–1.64	0.984	0.96	0.51–1.82	0.906	-0.066	0.94	0.5–1.75	0.836
miscellaneous	2.54	1.40–4.59	0.002	0.22	0.09–0.51	<0.001	-1.440	0.24	0.10–0.54	0.001
Ovulation_monitoring										
no	reference			reference			reference			
yes	0.35	0.26–0.48	<0.001	2.41	1.59–3.65	<0.001	0.876	2.40	1.59–3.63	0.001
AMH										
normal	reference			reference			reference			
low	6.61	1.93–22.63	0.003	0.12	0.02–0.61	0.011	-2.183	0.11	0.02–0.56	0.008
high	0.88	0.41–1.91	0.748	5.18	1.63–16.45	0.005	1.516	4.55	1.46–14.2	0.009
CA125 (U/mL)	1.00	0.98–1.02	0.985							
TC (mmol/L)	0.9	0.75–1.07	0.215							
TG (mmol/L)	1.09	0.81–1.47	0.557							

(Continued)

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TABLE 2 Continued

Characteristics	Univariate analysis			Multivariate analysis (original) (AIC=614.62)			Multivariate analysis (final) (AIC=610.43)			
	OR	95%CI	*P* value	OR	95%CI	*P* value	β	OR	95%CI	*P* value
AMH										
HDL (mmol/L)	1.08	0.74–1.57	0.705							
LDL (mmol/L)	0.85	0.67–1.07	0.167							
ALT (U/L)	1.00	0.98–1.01	0.498							
AST (U/L)	1.00	0.98–1.02	0.846							
Cr (μmol/L)	0.99	0.98–1.01	0.232							
INS (uU/mL)	0.99	0.97–1.01	0.443							
Glu (mmol/L)	0.98	0.8–1.19	0.808							

AIC, Akaike information criterion; OR, odds ratio; CI, confidence interval; BMI, body mass index; HSG=, hysterosalpingography; AMH, anti-Müllerian hormone; CA125, carbohydrate antigen 125; TC, total cholesterol; TG, triglyceride; HDLC, high-density lipoprotein; LDLC, low-density lipoprotein; ALT, alanine aminotransferase; AST, aspartate transaminase; Cr, creatinine; INS, fasting insulin; Glu, fasting glucose.

multivariate logistic analysis and LASSO algorithm, indicating that the above selected factors were appropriate.

Clinical predictive value of the machine learning models

Hyperparameters were further optimized for each model to ensure best performance. In the training set, bootstrapping method with 1000 resamples was used to assess the performance of the models. Initially, as shown in Table 3, random forest exhibited superior performance, with an AUR of 0.902 (95% CI 0.888–0.912), followed by logistic regression, with an AUR of 0.892 (95% CI: 0.870–0.915) in the training set. However, the logistic regression model performed the best among all models in terms of AUC across both internal and external validations. Therefore, from the perspective of the model interpretability and stability, the logistic regression model is chosen as our final model. Consequently, the individualized predictive nomogram (Figure 3A) and an interactive user-friendly online calculator (Figure 3B) were established (https://yyyzhentengliu.shinyapps.io/DynNomforSPRafterRS/). For example, when an infertile woman is aged 31 years old, and the duration of the secondary infertility is 3 years with a normal AMH level, suffering from mild to moderate endometriosis, without ovulation monitoring using transvaginal ultrasounds (≥2 times per menstrual cycle) after surgery, we could impute that her probability of receiving natural conception after surgery during 2-year period is 83.2% (Figures 3A, B).

Model validation of discrimination and calibration

Figures 4A–C demonstrate that the final model had an AUC of 0.892 (95% CI 0.870–0.915) in the training group. In the internal and external validation groups, the AUC was 0.870 (95% CI 0.830–0.910) and 0.880 (95% CI 0.833–0.926) respectively, indicating good predictive ability in discrimination between pregnancy negative and positive cases.

The three calibration curves of this model were fairly similar to the ideal curve (Figures 4D–F), suggesting that the estimated outcomes aligned with the real observations. In addition, Hosmer–Lemeshow test indicated that all *P*-values of the model are greater than 0.05 in the three cohorts (Figures 4D–F), suggesting that there was no statistical fit-departure between the predicted and observed values.

Clinical utility of the predictive model

The DCA revealed that the clinical prediction guided by the nomogram leads to better net benefits and more extensive range of cutoff probabilities in detecting spontaneous pregnancy than either the treat-all scheme or the treat-none scheme in the three datasets (Figures 5A–C, Additional File 1: Supplementary Table 4 displays net benefits for various threshold probabilities).

Concurrently, the CIC demonstrated remarkable predictive accuracy of this nomogram in predicting spontaneous conception, exhibiting greater efficacy in differentiating patients within the high and low probability categories in the training set (Figure 5D) and validation groups (Figures 5E, F).

Sensitivity analyses

AUC values of single independent predictors (female age, infertility type, duration of infertility, surgical procedures, ovulation monitoring and AMH) were significantly lower than that of the predictive nomogram (Figures 6A–C). These trends were also observed in DCA, i.e., our developed nomogram had the highest net benefit within a range of threshold compared with any of the univariate models (Figures 6D–F).

TABLE 3 Performance parameters of the 6 machine learning prediction models in the training, internal and external validation sets.

Predictive models	AUC	Accuracy	Precision	Sensitivity	Specificity
Training set					
Logistic regression	**0.892**	0.784	0.788	0.758	0.806
Decision tree	0.815	0.748	0.757	0.713	0.785
Random forest	0.902	0.792	0.789	0.734	0.809
SVM	0.807	0.727	0.699	0.775	0.679
KNN	0.787	0.711	0.749	0.613	0.803
XGBoost	0.858	0.770	0.773	0.739	0.798
Internal validation					
Logistic regression	**0.870**	0.782	0.786	0.741	0.829
Decision tree	0.812	0.742	0.742	0.711	0.782
Random forest	0.868	0.782	0.784	0.737	0.825
SVM	0.806	0.744	0.701	0.804	0.680
KNN	0.784	0.681	0.691	0.594	0.759
XGBoost	0.857	0.768	0.772	0.738	0.795
External validation					
Logistic regression	**0.880**	0.810	0.849	0.752	0.867
Decision tree	0.802	0.722	0.729	0.702	0.685
Random forest	0.879	0.793	0.787	0.747	0.855
SVM	0.804	0.742	0.698	0.805	0.682
KNN	0.786	0.682	0.694	0.598	0.762
XGBoost	0.861	0.771	0.774	0.739	0.798

AUC, area under the receiver operating characteristic curve; SVM, support vector machine; KNN, k-nearest neighbor; XGBoost, extreme gradient boosting.

Discussion

Benefiting from the technological innovations of recent years and the popularization of standard reproductive surgical procedures, reproductive surgery is widely considered one of the major therapeutic schedules for infertility, even though its significance was once doubted a few years ago (17). Counseling inevitably arises in clinical practice regarding the chance of pregnancy once reproduction function is reconstructed. However, a reliable prediction model has not been reported so far. Our current study developed the first publicly free nomogram that integrates key clinical features (patient age, infertility type, duration of infertility, intraoperative diagnosis, ovulation using ultrasound monitoring and serum AMH level) to impute the likelihood of spontaneous pregnancy following reproductive surgery. Notably, the model demonstrated superior discriminative power, good calibration and clinical utility, which were confirmed by both internal and external validations.

It has been well established that woman's age was strongly associated with conceiving success after reproductive surgery (18) and/or IVF (19). For every extra year of female age during their childbearing years, the pregnancy rate decreases by around 20% (OR = 0.80, P <0.001) according to our findings. In addition, women experiencing secondary infertility have a three-fold higher likelihood of achieving a spontaneous pregnancy (OR = 3.02, P <0.001) in comparison to those with primary infertility. The above findings were a bit higher than those reported by Ban Frangez, H., et al. (3). The effect of age on conception rate after surgery may be related to ovarian function, because age directly influences ovarian reserve, embryos quality and endometrial receptivity. In terms of infertility type, it is likely that the chance of pregnancy in this secondary cohort of women is higher as they have previously proven to be fertile. Additionally, in our study, duration of subfertility is an independent factor to predict natural conception after surgery, which is in accordance with a recent study (4). The possible explanation is that the longer the years of infertility, the longer the underlying pathologies (salpingitis, hydrosalpinx, pelvic adhesions, and endometriosis, etc.) could persist, causing a greater difficulty of the surgical operation, which limits the therapeutic effect. Another possible reason is that women with a longer infertility duration tend to be older.

AMH immunoassays are widely accepted for assessing ovarian reserve and guiding the personalized ovulation induction regimen in IVF (20, 21). Previous studies indicated that there was an

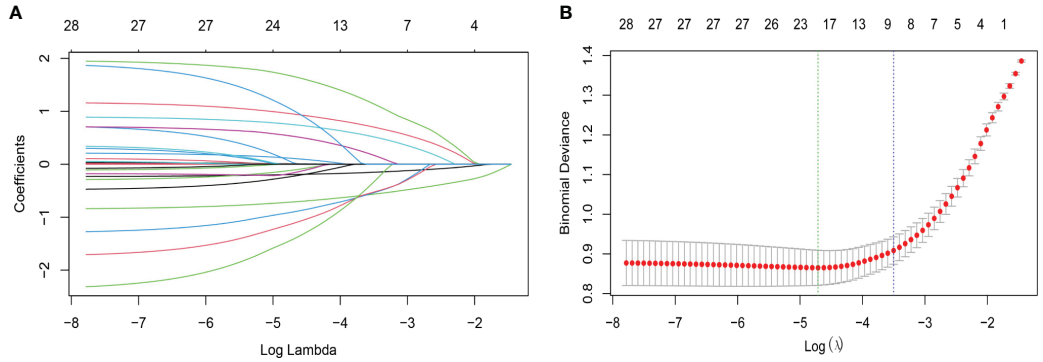

FIGURE 2
Characteristic variable screening based on the LASSO analysis with ten-fold cross-validation. **(A)** Plot of the LASSO coefficient profiles against the log (λ, lambda) sequence. **(B)** Tuning parameter (λ, lambda) selection of deviance in the LASSO regression based on the minimum criteria (left dotted line) and the 1-SE criteria (right dotted line). In the present study, predictor's selection was according to the 1-SE criteria (right dotted line), where 9 nonzero coefficients were selected (6 predictors including dummy variables, more details are in Additional file 1: Supplementary Table 3). LASSO, least absolute shrinkage and selection operator; SE, standard error.

independent correlation between AMH and live birth among women undergoing IVF (22). However, little attention has been paid to the significance of AMH level in predicting natural conception following infertility surgery. In our institution, patients are generally willing to accept the serum AMH detection to assess self-ovarian reserve before surgical treatment. In the present study, it was found that anti-Müllerian hormone (AMH) exhibited an independent predictive value for spontaneous

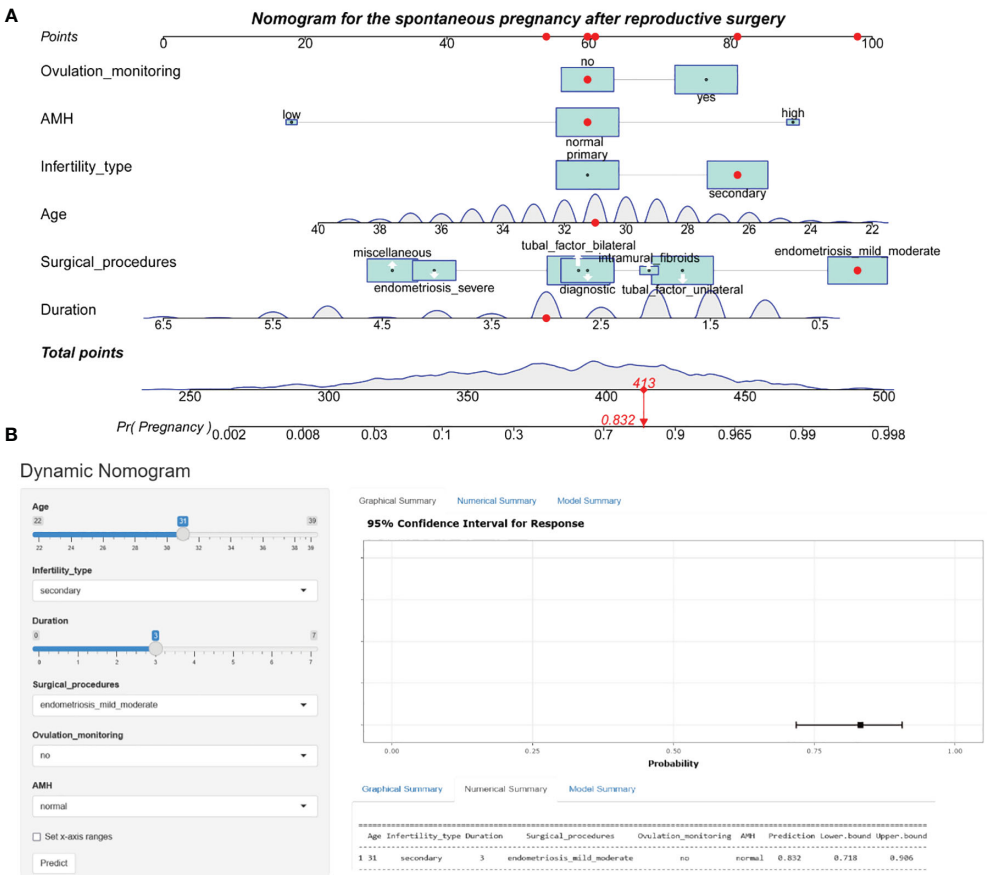

FIGURE 3
Nomogram prediction model for the spontaneous pregnancy after reproductive surgery. **(A)** Established nomogram in the training cohort by incorporating the following six parameters: age, infertility type, duration of infertility, main surgical procedures, ovulation monitoring and AMH. **(B)** Corresponding web-based dynamic nomogram accessible at https://yyyzhentengliu.shinyapps.io/DynNomforSPRafterRS/. AMH, anti-Müllerian hormone.

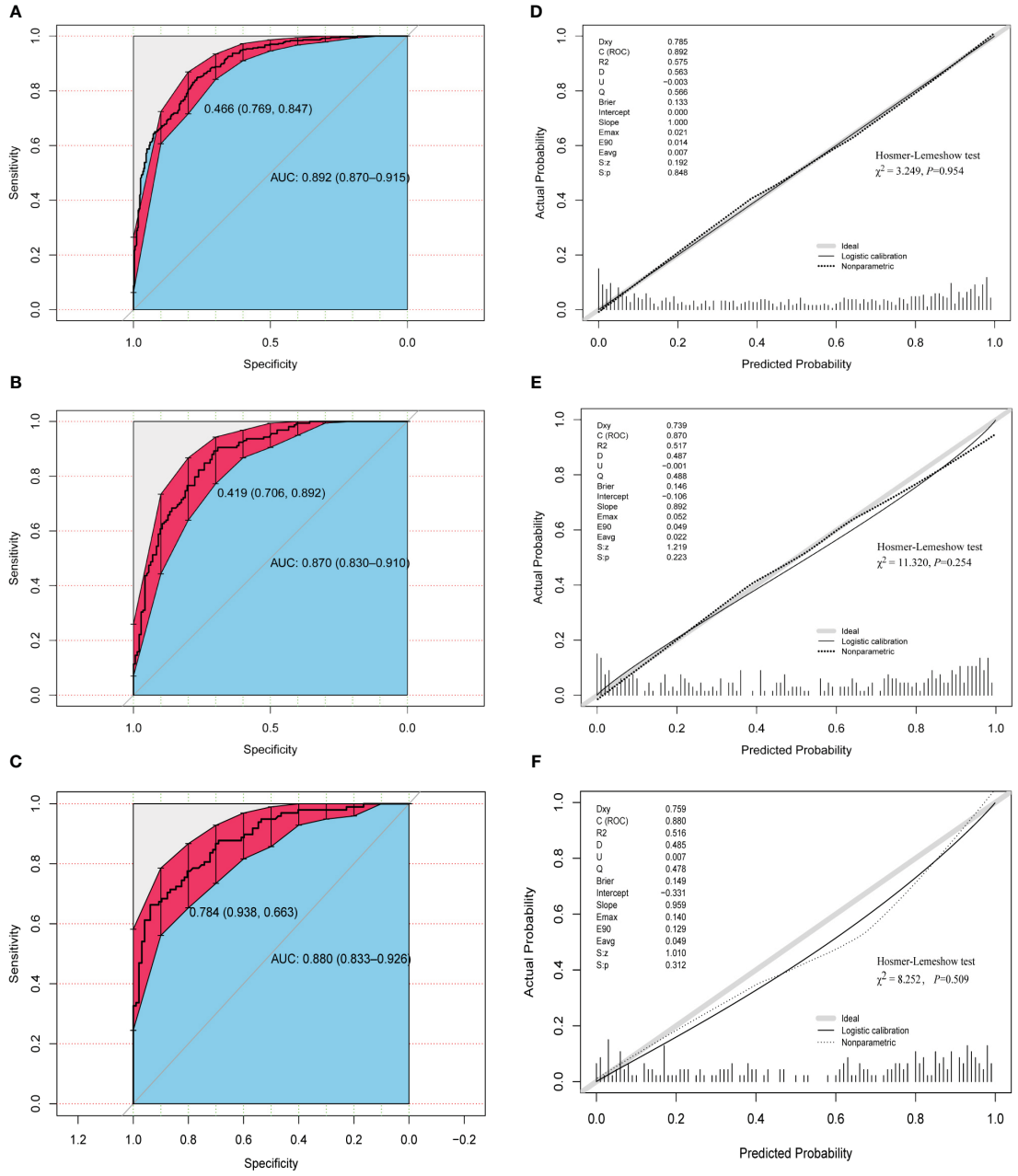

FIGURE 4
Assessment of discrimination and calibration of the model. ROC and AUC using the bootstrap method (resampling = 1000) of the nomogram prediction model in the training cohort **(A)**, internal test cohort **(B)**, and external test cohort **(C)**. The dotted vertical lines represent the 95% confidence interval. The calibration curves and Hosmer-Lemeshow test of the nomogram prediction model for the training cohort **(D)**, internal test cohort **(E)**, and external test cohort **(F)**. ROC, receiver operating characteristic; AUC, area under the ROC curve.

conception following reproductive surgery, leading to its inclusion in the prognostic model. Hence, it is crucial to take into account not only the surgical interventions but also to devote adequate attention to the precise evaluation of ovarian function reserve when predicting the surgical outcomes. Nevertheless, antral follicle count (AFC) and follicle-stimulating hormone level were not tested in most of the patients in the present study, so we could not evaluate the relationship between the two and natural conception.

Another interesting finding in this study was that using transvaginal ultrasound scan to aid in detecting ovulation in our or other clinics significantly improves patients' pregnancy outcomes (OR = 2.40, P = 0.001). Attempts to commence natural gestation as early as possible after surgery, how to accurately judge the day of ovulation is very important. The transvaginal sonogram is widely acknowledged as one of the most convenient and accurate techniques for identifying ovulation. In this study, the decision of detecting ovulation or not is mostly based on patient preference.

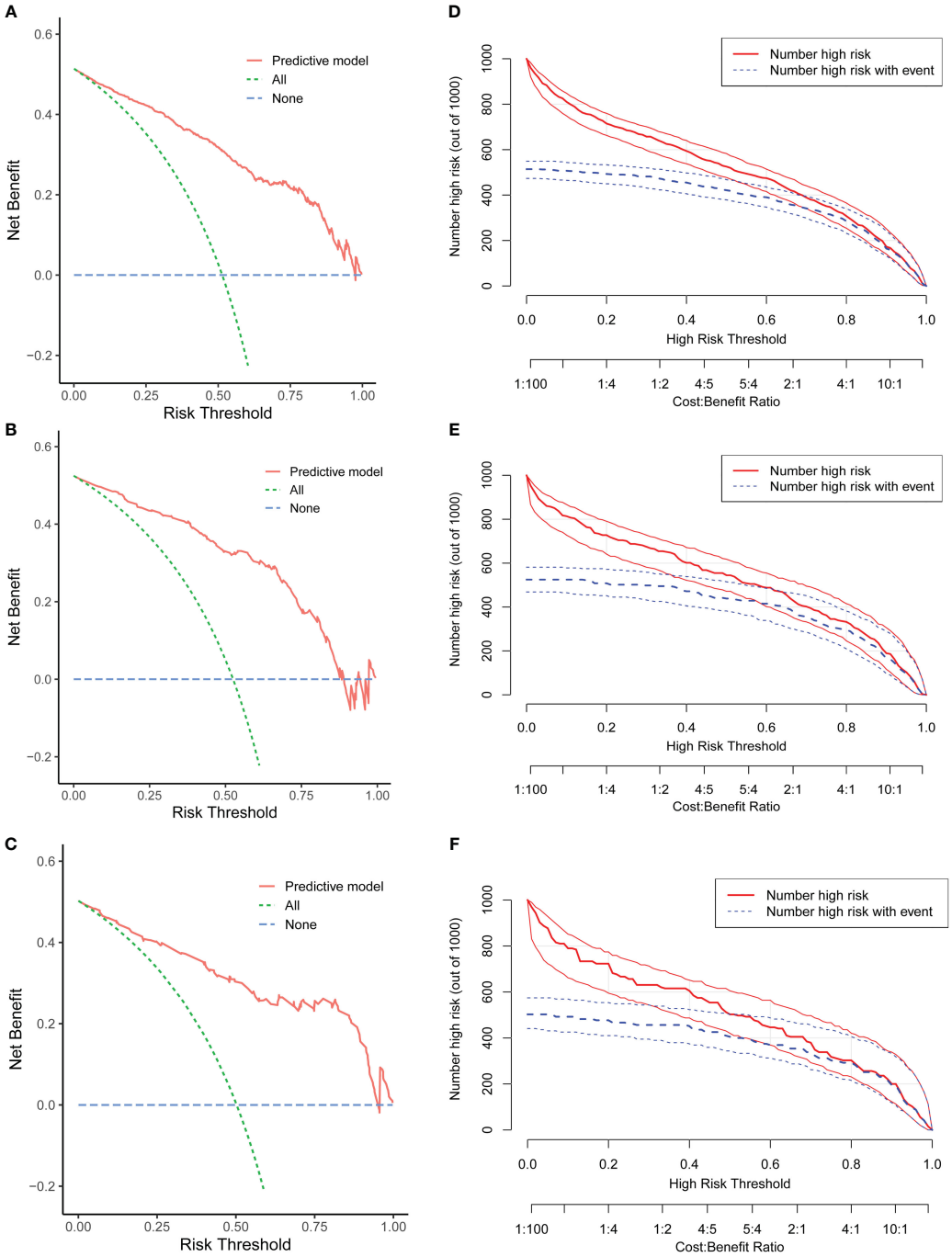

FIGURE 5
Evaluation of the clinical utility of the nomogram. Decision curve analysis (DCA) of the training cohort **(A)**, internal test cohort **(B)**, and external test cohort **(C)**. Clinical impact curve (CIC) of the training cohort **(D)**, internal test cohort **(E)**, and external test cohort **(F)**.

Our data support that patients even with almost regular menstruation should be further assessed for the fertile window in the menstrual cycle after surgery to guide the opportunity of couple's sex life.

Among the three currently most frequently used separate endometriosis classification/scoring systems (i.e. revised American Society for Reproductive Medicine (rASRM), Enzian and Endometriosis Fertility Index (EFI)), the EFI is the only widely recognized to have significant predictive value for natural or IVF conception after surgery for patients affected by endometriosis (23, 24). Nevertheless, several limitations should be noted. On the one hand, the EFI solely relies on the macroscopic assessment of the present condition of the fallopian tubes and ovaries, without considering the biomarker function of ovarian reserve like AMH or AFC. On the other hand, the EFI system does not provide any information to predict pregnancy achievement for non-endometriosis patients. Our model not only has some overlapped features with EFI, such as the fertility history (female age, type and

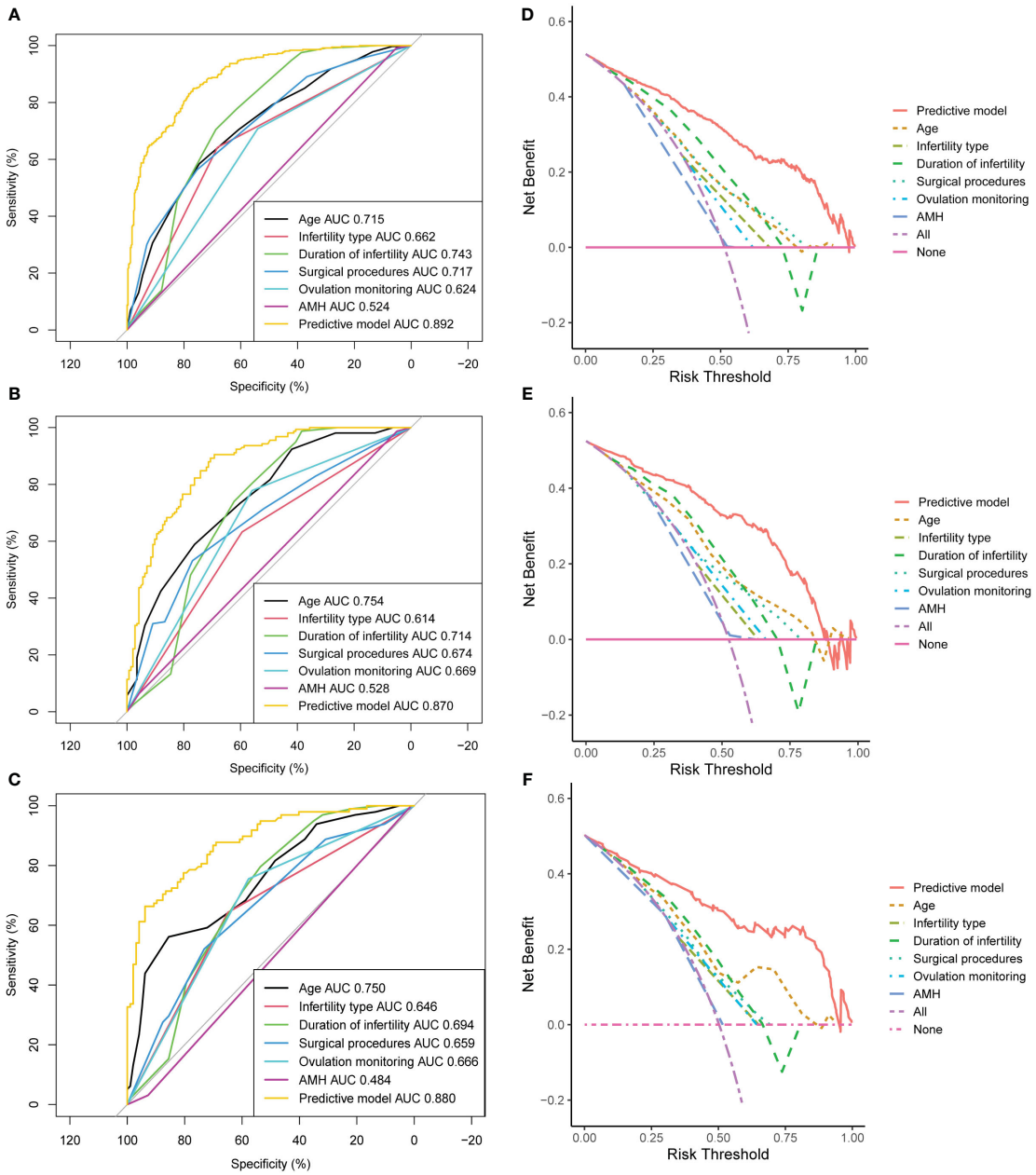

FIGURE 6
Sensitivity analysis of the model. Area under the ROC curve (AUC) of the training cohort **(A)**, internal test cohort **(B)**, and external test cohort **(C)**. Decision curve analysis (DCA) of the training cohort **(D)**, internal test cohort **(E)**, and external test cohort **(F)**. AMH, anti-Müllerian hormone.

years of infertility, rASRM score), but also combines the ovulation monitoring and ovarian reserve information, which would be a useful addition to the EFI to some extent. Moreover, this model basically covers most common etiologies of surgically amenable infertility.

Previous studies demonstrated that the existence of subserous or intraligamentary fibroids and nonmalignant ovarian cysts have no well-defined impact on fertility (3, 25). Due to the limited sample size observed in these diagnoses, we opted to merged the above subgroup with those patients without pronounced pathological changes at laparoscopy to the diagnostic laparoscopy group (Additional File 1: Supplementary Table 1). No significant association was detected between BMI and natural conception after surgical management, which is in line with the previous papers (3, 4). In addition, our multivariate logistic analysis indicates that HSG is not suitable as an independent predictive factor for pregnancy outcome. The reason may be related to the confounding (often low) image quality and the subjectivity of the observer. Therefore, clinical physicians inferring the patient's prognosis should not be formulated based on HSG status alone but should synthetically consider other key factors. Another interesting negative finding was that the probability of natural pregnancy after surgery in women with intramural fibroids

(P=0.158) or bilateral tubal lesions (P=0.836) was comparable to the diagnostic laparoscopy group. When normal anatomy was confirmed at laparoscopy, the patients are termed the unexplained infertility, which has been proven to be more difficult to deal with, even in IVF. Furthermore, for ones suffering from clear driving factors of infertility (mild/moderate endometriosis or unilateral tubal factor), laparoscopy can significantly improve fertility in these patients by correcting anatomical fallopian tubal abnormalities, and destroying concurrent endometriosis lesions, as shown by our results (Table 2). However, severe pathologies (stage III-IV endometriosis or miscellaneous) would inevitably lead to a lower prognosis, even though at least one roughly functionally normal fallopian tube was retained.

In our center, all surgery was carried out by the same professional reproductive surgery team which has been established for about 20 years, and an average of 400 laparoscopic procedures combined with hysteroscopy are performed annually. This can be attributed to the extensive training, meticulous procedures like fimbriae eversion with sutures, delicate tissue handling, preservation of ovarian tissue, minimal electrocoagulation to prevent tissue necrosis and promote optimal healing, precise restoration of normal anatomy, and prevention of adhesions. In this study, the overall rate of spontaneous pregnancy after reproductive surgery is relatively high, achieving 51.7% (524/1013) in our institution and 50.3% (98/195) in the external cohort, which are similar to the previous reports (3, 4, 26). Given that, more and more infertile patients younger than 38 years without absolute indication for IVF are willing to choose to diagnostic or operative laparoscopy combined with hysteroscopy in our hospital. Nonetheless, there is still significant variation regarding the pregnancy result for women desiring to get pregnant following reproductive surgery, which makes it especially challenging to evaluate the prognosis. Therefore, the individualized prediction of the postoperative pregnancy probability has become increasingly important in the era of precision medicine. The nomogram developed in this study represents a pioneering effort to visualize patients' probability of achieving pregnancy in the postoperative 2-year period using machine learning algorithms, and serves as a reference for clinicians and infertile couples to help them with personalized decision-making about the mode of subsequent conception, natural or IVF.

The limitations of our study include its retrospective design, which may introduce some inevitable bias, and the fact that the training and validation cohorts were ethnically homogeneous and limited to East China. Therefore, it is important to validate our data longitudinally in a more ethnically diverse patient population. In addition, unlike IVF's timely feedback outcome (2–3 weeks), pregnancy rates after surgery lack continuous tracing because of the longer expectation period for spontaneous conception. Therefore, the pregnancy outcomes after reproductive surgery were followed only after 2-year at our department, leading to hard to perform survival analysis. In future updates, we will attempt to perform larger, multicenter, prospective studies and analyze long-term follow-up survival data. Third, if women with infertility undergo laparoscopy, it is clinical routine to perform hysteroscopy concurrently to rule out any concurrent endometrial abnormality at our institution. Hence, we were unable to include hysteroscopy as a covariate in the multivariable model. Last, it is the wide heterogeneity of the principle and skill of reproductive surgery in different medical institutions that poses the greatest challenge to the extrapolation capacity of the model.

Conclusions

The first user-friendly web-based nomogram with good predictive ability was proposed in the current study to timely detect the possibility of natural conception after reproductive surgery. The model can be widely applied into the clinical practice and help guide clinicians and infertile couples make sensible decision of choosing the mode of subsequent conception, natural or IVF, to further improve the reproductive health in the population level. However, cross-institutional large-cohort prospective studies are needed to verify our model.

Data availability statement

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

Ethics statement

The studies involving humans were approved by the Ethics Committees of Yantai Yuhuangding Hospital (YT2023-054) and Linyi People's Hospital [LYRMYY (2023-04-036)]. The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because this research was a retrospective cohort study, the data was made anonymous and there was no need for informed consent.

Author contributions

ZL: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Software, Visualization, Writing – original draft, Writing – review & editing. MW: Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Visualization, Writing – original draft, Writing – review & editing. SH: Data curation, Formal analysis, Methodology, Project administration, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing. XW: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Software, Validation, Writing – original draft, Writing – review & editing. XL: Formal analysis, Validation, Visualization, Writing – original draft, Writing – review & editing. XX: Investigation, Methodology, Project administration,

Supervision, Validation, Writing – original draft, Writing – review & editing. HB: Investigation, Methodology, Project administration, Supervision, Validation, 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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1378157/full#supplementary-material

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Does septum resection improve reproductive outcomes for women with a septate uterus? A systematic review and meta-analysis

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Objective: To investigate whether incising the septum facilitates reproductive outcomes for patients with a septate uterus compared to expectant management.

Methods: Research was retrieved from three electronic databases: PubMed, Embase, and the Cochrane Library, with no time or language restrictions. Two authors independently selected the articles and extracted data regarding study characteristics, quality, and results. A random-effects model was employed, and summary risk ratios (RR) with 95% confidence intervals (CI) were calculated.

Results: A total of 468 patients from two randomized controlled trials and one cohort study were included in the systematic review and meta-analysis. Pooled results showed that septum resection did not improve the live birth rate for patients with a septate uterus (RR = 0.84, 95% CI = 0.56 – 1.25, P = 0.39). Additionally, no significant differences were found between the septum resection and expectant management groups in terms of clinical pregnancy (RR = 1.08, 95% CI 0.81 – 1.44, P = 0.60), abortion (RR = 1.99, 95% CI 0.80 – 4.98, P = 0.14), and preterm delivery rates (RR = 0.99, 95% CI 0.42 – 2.31, P = 0.98).

Conclusion: Our data provide clear evidence that septum resection does not improve the reproductive outcomes of patients with a septate uterus. These findings might be useful for revising current clinical guidelines.

KEYWORDS

hysteroscopy, embryo transfer, pregnancy, uterine cavity, assisted reproduction technique

Introduction

The uterine septum is the most common uterine anomaly, accounting for approximately 35% of detected Mullerian abnormalities (1). It is believed to develop from the incomplete resorption of the fused medial walls of the paramesonephric (Mullerian) ducts prior to the 20th embryonic week (2). Thus, a septate uterus exhibits a single fundus and an internal indentation (septum), which originates from the fundal midline and exceeds 50% of the uterine wall thickness, splitting the uterine cavity into two distinct parts (3).

The septate uterus has been associated with declining fertility (4). For example, the incidence of uterine septum is higher in women seeking treatment for subfertility than in the general population, implying an underlying association (5). Additionally, uterine septate has been regarded as a risk factor for miscarriage, as significant risk reduction following surgery has been demonstrated in studies where patients serve as their own internal controls (5). This evidence suggests that removal of the septum via surgery might be a potential approach to improving pregnancy outcomes.

Although the pathophysiology of the uterine septum in reproduction is unclear, it is reasonable to hypothesize that restoring normal anatomy might also improve its function. Initial approaches to incising the septum, such as Bret-Tompkins or Jones metroplasty, required a laparotomy (6, 7). Moreover, the advent of hysteroscopic septum resection, which offers a minimally invasive approach with a shorter recovery time, is now considered first-line therapy (8). Numerous retrospective studies have compared reproductive outcomes for patients with a septate uterus before and after the surgery, reporting superior outcomes in terms of pregnancy rates, preterm birth rates, and live births (9–12). However, this evidence has a high risk of bias due to the study design, with the same group of women serving as both the study and control groups, since before-and-after comparison research tends to favor the intervention (13). Additionally, research with positive results is more frequently published, contributing to publication bias. Thus, there is no solid evidence confirming the benefits of septum resection for patients with a septate uterus.

In the current study, a literature review and meta-analysis were conducted to obtain higher-grade evidence. Both cohort studies and randomized controlled trials were included to evaluate the reproductive outcomes of different treatments (septum resection or expectant management) for women with a septate uterus.

Materials and methods

Literature search

Studies were identified in the following electronic databases: Pubmed, Embase, and Cochrane Library, using the search terms: septal resection OR hysteroscopic metroplasty OR septum resection OR septate uterus OR uterine septum, with adjustments made for each database as necessary. The detailed search strategy is displayed in Supplementary Table 1. There were no restrictions on study design or language. The final research was conducted, including all publications appearing in the databases before 14 August. This systematic review was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) Statement.

Inclusion and exclusion criteria

The inclusion criteria were women with septate uteri and undergoing septum resection or expectant management. Moreover, the included studies had to report at least one of the following reproductive outcomes after treatment: clinical pregnancy, live birth, preterm delivery, term delivery, or abortion. Both randomized controlled trials and cohort studies (retrospective and prospective) published in English were included. Reviews, editorials, letters, case reports, case serials, animal experimental studies, conference abstracts, and articles in other languages were excluded.

Study selection

Titles and abstracts of all identified publications were screened by two of the authors (C.L. and Z.L.). The full texts of the pre-selected articles were reviewed according to the inclusion and exclusion criteria. If consensus could not be reached, disagreements were settled through discussion with a third author (X.G.).

Outcome measurement

All patients in the included studies were diagnosed with a septate uterus via 3D ultrasound, MRI, hysteroscopy, or hysterosalpingography. Following the diagnosis of a uterine septum, patients were expected to conceive naturally or with assisted reproductive technologies, either in the expectant management group or after septum resection. Women in both groups were followed up for 12 months if not pregnant. In addition, patients who conceived continued to be followed up until delivery or abortion. Clinical pregnancy was defined as the presence of a fetal heartbeat at or beyond 6 weeks of pregnancy. The spontaneous demise of a pregnancy, including non-visualized or biochemical pregnancies confirmed by serum or urine b-HCG, was considered abortion. Preterm delivery was defined as birth before a gestational age of complete weeks. The clinical pregnancy, live birth, abortion, and preterm delivery rates were calculated as the number of events that occurred divided by the number of included participants, respectively.

Data extraction and risk of bias assessment

The following data were extracted from all eligible included studies by two of the authors (C.L. and X.G.): authors, year of publication, location of the study groups.

Study design, years of study, age, number of participants, length of follow-up, number of patients assigned to the two groups, and

reproductive outcomes. Any disagreements were resolved by another investigator (Z.L.).

Two investigators (C.L. and Z.L.) independently evaluated the trials for risk of bias. The assessment was based on the criteria outlined in Chapter 8 of the Cochrane Handbook and included random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective reporting, and other biases (14). Each criterion was characterized as low, high, or unclear. Disagreement were resolved through discussion with another investigator (X.G.).

Statistical analysis

All metadata analyses were conducted using Review Manager 5.3 (Cochrane Collaboration, Oxford, UK). Dichotomous variables were analyzed using a risk ratio (RR) with 95% confidence intervals (CIs) employing a random-effects model. Statistical heterogeneity was quantified using the Chi-squared and I^2 statistics. A value of I^2 greater than 50%, or $P < 0.05$, signified significant heterogeneity (15). To assess publication bias, a funnel plot analysis using the Egger test was performed. The results were presented as forest plots. A significance level of $P < 0.05$ was considered statistically significant.

Results

Study selection and characteristics

The detailed selection process for studies is documented in a PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) flow diagram (Figure 1). The literature search yielded a total of 8,565 publications after the removal of duplicates. After reviewing the titles and abstracts, 22 records were assessed for eligibility by full-text screening. Of these, 10 studies were ineligible because they lacked an expectant management group, and 9 were conference abstracts. Finally, three studies met the inclusion criteria and were included in the current meta-analysis (16–18). The risk of bias summary for the included trials is displayed in Figure 2.

The characteristics of the eligible studies are displayed in Table 1. Two of them were randomized controlled trials, and the other was a retrospective cohort study. The investigation periods ranged from 6 years to 18 years, and the length of follow-up ranged from 12 months to 53 months. A total of 468 patients from three studies were enrolled in this meta-analysis. All three studies recruited patients from multiple centers.

Primary outcome

All studies provided data for the primary outcome of live birth. There was heterogeneity for this outcome among studies, as indicated by the I^2 value ($I^2 = 61\%$). The pooled results indicated that incising the uterine septum could improve the live birth rate compared with expectant management (Figure 3, RR = 0.84, 95% CI 0.56 – 1.25, P = 0.39). Based on these data, septum resection was not conclusively suggested for women with a septate uterus and a desire to conceive.

Secondary outcomes

Results for secondary outcomes, including clinical pregnancy, abortion, and preterm delivery, showed no significant heterogeneity. Additionally, there were no significant differences between the two groups regarding the clinical pregnancy rate (Figure 4, RR = 1.08, 95% CI 0.81 – 1.44, P = 0.60, heterogeneity: $I^2 = 0\%$, P = 0.76), abortion rate (Figure 5, RR = 1.99, 95% CI 0.80 – 4.98, P = 0.14; heterogeneity: $I^2 = 0\%$, P = 0.58), and preterm delivery rate (Figure 6, RR = 0.99, 95% CI 0.42 – 2.31, P = 0.98; heterogeneity: $I^2 = 0\%$, P = 0.75).

Publication bias

Funnel plots for publication bias included in different treatment groups are shown in Supplementary Figures 1-4. The results showed no evidence of significant publication bias, as the Egger test was not significant.

Discussion

In this study, we identified two trials and one retrospective study that compared reproductive outcomes between septum resection and expectant management for women with a septate uterus. A total of 468 patients from three studies were included in

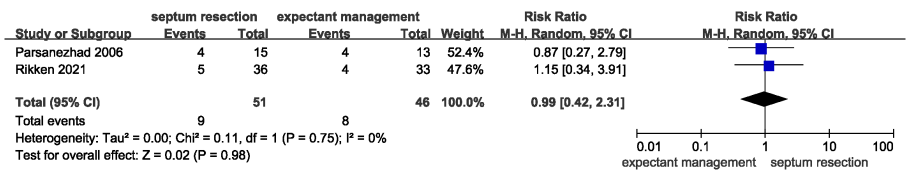

FIGURE 1
Flow diagram of the study identification and selection process for systematic review and meta-analysis.

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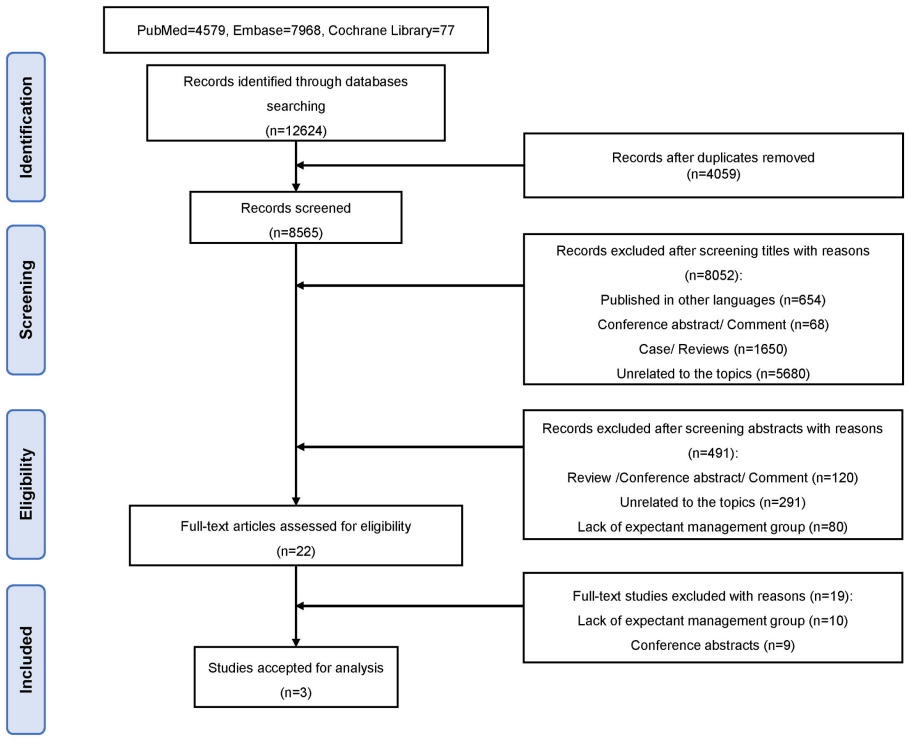

FIGURE 2
Risk of bias summary table.

the meta-analysis. Summary RRs indicated that incising the septum did not increase the chance of live birth and clinical pregnancy rates, nor did it decrease the risk of adverse obstetric outcomes, such as abortion rate and preterm birth rate. Based on these results, patients with a septate uterus may not gain any improvements in reproductive outcomes from septum resection, questioning the rationale behind the surgery.

Currently, incising the septum via hysteroscopy has been recognized as an effective approach to improving reproduction, as suggested by multiple studies (5, 19). However, the results of this meta-analysis indicated that no differences were found between septum resection and expectant management, which was in line with a previous retrospective cohort study (17). However, such findings seem to contradict the results of prior observational research with a before/after study design, which reported significant improvements in live birth and clinical pregnancy rates after surgery (20). We speculate on two possibilities for this divergence. First, the study design of such observational research

TABLE 1 Characteristics of the included studies.

Author, Year	Location	Study design	Years of study	Age	Patients (N)	Length of follow-up	Septum resection (N)	Expectant management (N)	Reproductive outcomes
Parsanezhad, 2006 (16)	Iran and Germany	randomized controlled trial	1999–2005	18–35	132	12 months	15	13	Pregnancy, abortion, preterm delivery, live birth, and cesarean rates
Rikken, 2020 (17)	Netherlands, USA, and UK	Retrospective cohort	2000–2018	/*	257	Up to 53 months	151	106	Conception, live birth, ongoing pregnancy, abortion, and preterm birth rates
Rikken, 2021 (18)	Netherlands, UK, USA, and Iran	randomized controlled trial	2010–2018	29–33	79	12 months	36	33	Live birth, ongoing pregnancy, clinical pregnancy, abortion, and preterm birth rates

*the age of the included participants was displayed as the mean (SD) in the original study. To be specific, the average age of the participants was 31.7 years (4.18) in the septum resection group and 30.8 years (5.09) in the expectant management group, respectively.

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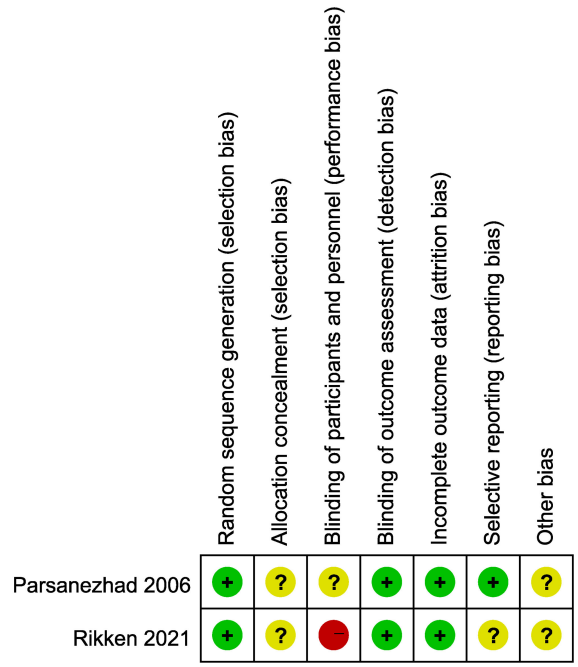

FIGURE 3
Forest plots of the live birth rate in patients with a septate uterus receiving septum resection and expectant management.

was "before/after," which always favors the tested intervention. Second, the conclusions of these studies could be limited by their retrospective nature. These non-randomized comparative studies did not accurately account for confounders, and some were also at high risk of selection bias. For example, there was an unequal distribution of patients in a previous study, with 109 in the surgery group and 15 in the control group (21).

In fact, incising the uterine septum without improvements in fecundity is not surprising. When women experiencing infertility present to a reproductive center without identifiable risks, such as a uterine septum, there may be pressure from both the provider and patient to pursue immediate resection based on the stereotype that restoring normal anatomy also restores normal function (1, 3). The conventional view holds that the main composition of the uterine septum was fibromuscular tissue, with more connective tissues and fewer muscular fibers (22, 23). However, this assumption contradicts histological findings that the muscle bundles accounted for over 50% of the septum (24). Besides, the linear arrangement of smooth muscle and vessels in the core of the septa is similar to that of the normal myometrium (25). Thus, metroplasty corrects uterine anatomy while also injuring the inner face of the myometrium and the endometrium, which may take considerable time for recovery (26). In this study, the follow-up period from surgery to pregnancy was only 12 months, which might be too short for functional recovery of the uterus. Therefore, there were no

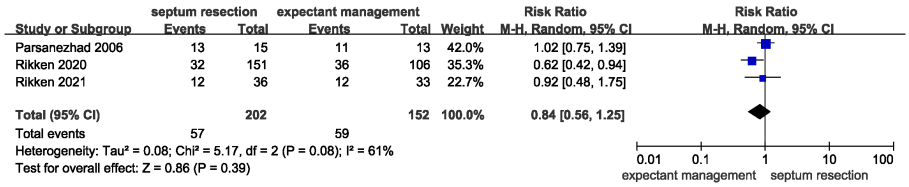

FIGURE 4
Forest plots of the clinical pregnancy rate in patients with a septate uterus receiving septum resection and expectant management.

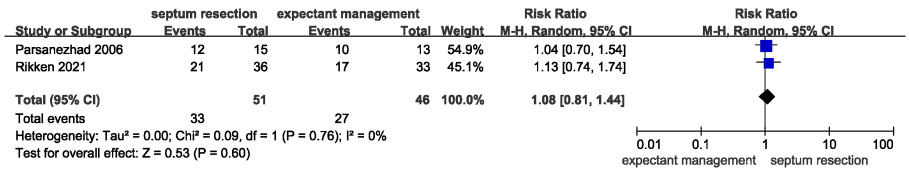

FIGURE 5
Forest plots of the abortion rate in patients with a septate uterus receiving septum resection and expectant management.

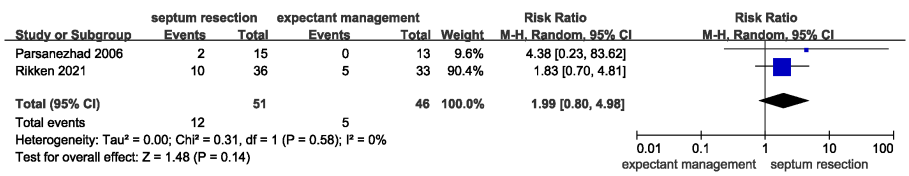

FIGURE 6
Forest plots of the preterm delivery rate in patients with a septate uterus receiving septum resection and expectant management.

significant differences in clinical pregnancy and live birth rates between the two treatments, which is reasonable.

To date, studies comparing the prognosis of uterine septum resection or expectant management are limited. This review, to our best knowledge, is the first meta-analysis to address these issues. Moreover, this study provided high-quality evidence and raised questions about routine hysteroscopic septum resection for women with a septate uterus. However, this meta-analysis was limited by the lack of trials on the topic. After literature selection, only two trials were included in the meta-analysis, reducing its potential impact. Besides, owing to the small sample size, the included studies could not evaluate the differential effects of septum resection in women with pregnancies compared to those presenting with subfertility. Therefore, large-scale studies with subgroup analysis for patients with different conditions are required to confirm the effectiveness of uterine septum resection on reproduction.

Data availability statement

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

Author contributions

CL: Conceptualization, Writing – original draft. ZL: Investigation, Writing – original draft. XG: Investigation, Writing – original draft. YC: 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.2024.1361358/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Funnel plots for publication bias evaluation for the live birth rate for women who underwent septum resection versus expectant management.

SUPPLEMENTARY FIGURE 2
Funnel plots for publication bias evaluation for the clinical pregnancy rate for women who underwent septum resection versus expectant management.

SUPPLEMENTARY FIGURE 3
Funnel plots for publication bias evaluation for the abortion rate for women who underwent septum resection versus expectant management.

SUPPLEMENTARY FIGURE 4
Funnel plots for publication bias evaluation for the preterm birth rate for women who underwent septum resection versus expectant management.

SUPPLEMENTARY TABLE 1
The detailed search algorithm used in electronic databases.

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Survey of *in vitro* fertilization add-ons in Japan (Izanami project)

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Objective: To identify any correlations between evidence levels, adoption rates, and additional costs of *in vitro* fertilization (IVF) add-ons.

Design: Online survey.

Subjects: The survey was conducted in 621 assisted reproductive technology-registered facilities that are members of the Japanese Society of Obstetrics and Gynecology from December 22, 2021, to February 13, 2022.

Exposure: The survey included details regarding the specific add-on modalities employed and their associated costs; inquiries pertained to the fertility healthcare infrastructure in Japan before the implementation of the National Health Insurance scheme.

Main outcome measures: The correlation between the adoption rate and cost of IVF add-ons and their evidence levels were analyzed. The evidence level of the add-on treatments was classified into Green, Amber, and Red categories based on the United Kingdom's Human Fertilisation and Embryology Authority and Cochrane systematic reviews.

Results: A total of 438 eligible responses were analyzed, with clinics constituting 70.9% of the respondents' facilities. A total of 18 add-ons were assessed, and 96.5% (423/438) of facilities used at least one add-on. A positive correlation of the adoption rate and an inverse correlation of the cost with the evidence level of the IVF add-on treatment were observed (not significant). Outpatient clinics, defined as medical facilities with no beds, had a significantly higher adoption rate (Amber, 65.7%; Red, 52.0%) of add-ons than other facilities, regardless of the evidence rating, although the costs were similar across all site attributes.

Conclusion: Accumulating evidence on the efficacy and safety of add-ons will lead to the development of medical care with a high-cost benefit, as an increase in the adoption rate and a decrease in cost are expected when limiting to medical care with a high level of evidence.

KEYWORDS

adoption, cost, *in vitro* fertilization, add-ons, Japan

1 Introduction

Routine cycles of proven fertility treatment are effective without the addition of unproven treatment add-ons. Assisted reproductive technology (ART) is considered the most effective method for treating infertility, although it has been reported that only approximately 60%–70% of couples who undergo ART are able to achieve live births (1), indicating the existence of limitations in the therapeutic approach. A study conducted by the United Kingdom's Human Fertilisation and Embryology Authority (HFEA) revealed that 74% of patients undergoing medical treatment for infertility utilized "*in vitro* fertilization (IVF) add-on" treatments, with the number of patients using them increasing yearly (2). An agreement between the HFEA and other professional and patient groups (Consensus Statement, October 19, 2023) states that treatments without strong evidence of safety and/or efficacy should only be offered in research settings. According to a survey conducted in Australia, the most frequently used "add-on" treatments are preimplantation genetic testing for aneuploidy (PGT-A) (27%), time-lapse technology (TLT) (23%), hyaluronic acid-containing culture media (22%), and assisted hatching (AHA) (8%) (3).

Despite the lack of scientific evidence, "IVF add-on" treatments are widely used and have become an international issue, owing to the high financial burden placed on patients (4). In Japan, some add-ons, such as artificial oocyte activation (AOA), AHA, and Hyaluronate, have been made eligible for national health insurance coverage from 2022 onward. When introducing "add-on" treatments, medical professionals are required to explain their effectiveness and safety and obtain informed consent. However, it is suspected that many patients undergo add-on treatments without sufficient explanation, which could increase the likelihood of patient regret. In a survey conducted by Lensen et al., the percentage of regret increased when the patient's role in the decision to use the selected add-on was <50% (5). Furthermore, when medical professionals fail to provide sufficient information regarding the evidence level and details of the treatment, patients may rely on misinformation from external sources and experience regret when the therapeutic approach fails to yield positive results.

To alleviate the burden on patients, it is essential to clarify the clinical status of add-on treatments. The Japanese government began providing national insurance coverage for ART in 2022. However, little information is available regarding the adoption rates and additional costs of IVF add-ons at IVF centers. Therefore, to gain insight into the medical system and reality of add-on treatments before insurance coverage, we conducted a survey to establish evidence for the introduction of new therapeutic approaches and to determine the essential medical systems required for this purpose.

2 Materials and methods

2.1 Methods and timelines of the survey

We conducted an empirical survey, named the IZANAMI survey project (toward the Introduction of new technologies for handling Zygotes Survey on treatment ADD-ONS and Assisted Reproductive MedIcine in Japan), targeting 616 ART facilities, after modifying for closures/integrations (five facilities), cessation of handling of IVF (three facilities), and additional/newly established facilities (three facilities), based on 621 ART facilities registered with the Japan Society of Obstetrics and Gynaecology. We conducted the survey using a Google Form (December 22, 2021, to February 13, 2022) (6). Of the 478 responses obtained, 41 duplicate responses were removed; therefore, 437 responses were included (response rate, 70.9%). Based on the responses obtained from the target facilities, we analyzed the implementation status and cost of IVF add-on treatments in Japan.

2.2 Adoption rate of IVF add-on treatment

In Japan, medical institutions are classified based on the number of beds they have. According to the Japanese Medical Care Act, a clinic is defined as a medical facility with 19 or fewer beds, distinguishing it from a hospital, which must have 20 or more beds. A unique feature of the Japanese healthcare system is the prevalence of clinics without any inpatient beds, which focus entirely on outpatient care. These no-bed clinics play a significant role in providing accessible medical services, particularly in urban areas where space is limited and outpatient care is in high demand. Accordingly, each facility was defined and consistently referred to as

"outpatient clinics (no bed)," "inpatient clinics (19 or fewer beds)," "hospital (more than 20 beds)," or "university hospital.".

Outpatient clinics are considered dominant because they handle the largest number of oocyte retrieval cycles and account for approximately half of all the facilities in Japan. Accordingly, we examined variations in add-ons using outpatient clinics as controls. Add-on treatments with adoption rates that are more than twice as high between facility types compared with outpatient clinics were defined as "variations" and analyzed accordingly.

2.3 Evidence level rating of IVF add-ons

In addition to add-ons listed in the HFEA, we included those identified by a Japanese Ministry of Health, Labour and Welfare survey (7). We independently classified the add-on treatments into Green, Amber, and Red categories, based on the level of evidence of clinical effectiveness currently available in the HFEA (8) and Cochrane systematic reviews. No add-ons were classified as the Green category (where there is more than one high quality randomized controlled trial [RCT] which shows that the procedure is effective at improving live birth rate for most fertility patients). The add-ons included in the Amber category (where there is conflicting evidence from RCTs to show that an add-on is effective at improving live birth rate for most fertility patients) were AOA, hyaluronic acid-containing culture media, and TLT. The add-ons included in the Red category (no RCT studies have shown an effect on improving the chances of having a child for most infertile patients) were AHA, endometrial receptivity analysis (ERA), interferon-γ-producing helper-T cell (Th1)/IL-4-producing helper-T cell (Th2) ratio test, intracytoplasmic morphologically selected sperm injection (IMSI) (9), and PGT-A. The evidence level ratings of add-ons, including AHA (10), hyaluronic acid-containing culture media (11), endometrial injection of embryo culture supernatant (12), immunosuppressant agent treatment (13), PGT-A (14), and granulocyte colony-stimulating factor infusion (15) were verified based on Cochrane systematic reviews. Add-ons not addressed by either HFEA or Cochrane (testing and treatment of chronic endometritis, including ERA, endometrial microbiome metagenomic analysis [EMMA], two-step embryo transfer, platelet-rich plasma [PRP] intrauterine injection, and *in vitro* maturation [IVM]) were also classified as Red. The Cochrane review (16) updated the recommendations for endometrial scratch and included the Lensen 2019 trial, which found no significant difference between endometrial scratch (n = 690, live birth rate 26.1% [180/690]) and controls (n = 674, live birth rate 26.1% [176/674]) (odds ratio, 1.00 [95% confidence interval, 0.78-1.27]) (5). Accordingly, in the current study, we decided to classify endometrial scratch as Red instead of Amber.

2.4 Statistical analyses

We performed statistical analysis by conducting a t-test using the JMP software program (JMP®, Version 15; SAS Institute Inc., Cary, NC, USA) to examine the adoption rate and cost among facilities in relation to IVF add-ons. A total of 208 outpatient clinics, accounting for 47% of all facilities, were used as controls. Facilities that utilized an add-on but did not provide information on costs were excluded from the analysis. The following notations were adopted:

1. The adoption rate of each add-on medical treatment was indicated in the order of the number of facilities implementing it/total number of facilities × 100 (%).
2. The cost for each add-on medical treatment was indicated as the median cost for each add-on (interquartile range).

2.5 Ethics

This study was a survey of medical facilities and was not suited to the "Ethical Guidelines for Medical and Health Research Involving Human Subjects." Therefore, the requirement for ethics approval was waived by the local ethics committee.

3 Results

3.1 Facility types

Facilities were categorized as outpatient clinics, inpatient clinics, hospitals, and university hospitals. Outpatient clinics accounted for the largest proportion, followed by hospital-based clinics and university hospitals. Clinics accounted for 69.5% of the total number of facilities (Figure 1A). The numbers of ART cycles (Figure 1B) and oocyte retrieval cycles (Figure 1C) were significantly higher in outpatient clinics than in other types of facilities.

3.2 Adoption rate of IVF add-on treatment

Add-on treatments with high adoption rates of >50% across all facility types were AHA, chronic endometritis testing and treatment, and AOA, whereas those with low adoption rates of <30% across all facility types were PGT-A, IVM, granulocyte colony-stimulating factor infusion, and IMSI (Table 1).

The add-on treatments with "variations" in adoption rates were ERA, EMMA, Th1/Th2 cell ratio test, endometrial injection of embryo culture supernatant, immunosuppressive agents, and PRP therapy (Table 1). These were commonly associated with high adoption rates in outpatient clinics and low adoption rates in university hospitals.

3.3 Cost of IVF add-on treatment

We examined the extent to which an add-on treatment was provided according to facility type in Japan. Medical care that requires human and medical resources may incur high costs.

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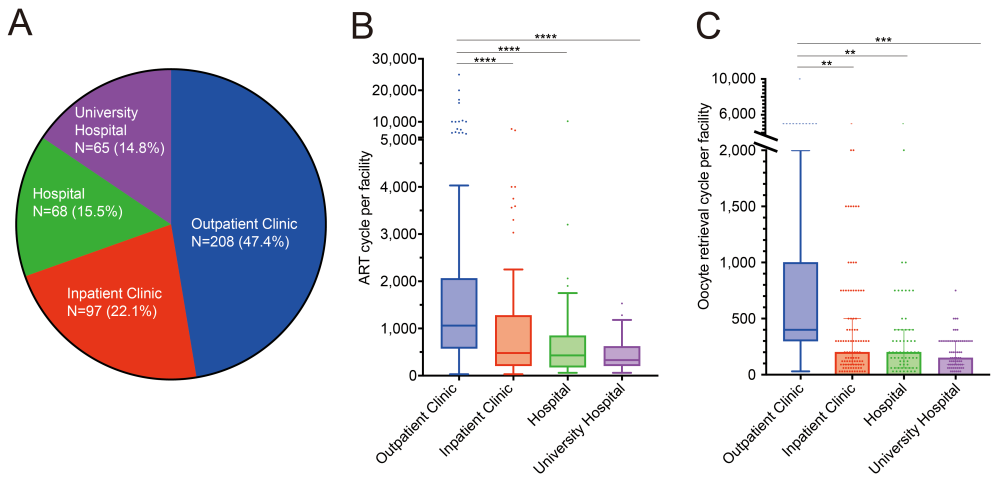

FIGURE 1
Facilities treating assisted reproductive technology in Japan. **(A)** Proportion of *in vitro* fertilization facilities in Japan. **(B, C)** Number of assisted reproductive technology cycles: total number of oocyte retrievals and embryo transfers per facility **(B)** and oocyte retrieval cycles **(C)**. ****p<0.0001, ***p<0.001, **p<0.01.

TABLE 1 Adoption rate of *in vitro* fertilization add-on treatments according to facility.

	Rating in the current study	Total	Outpatient Clinic	Inpatient Clinic	Hospital	University Hospital	
		(n=438)	(n=208)	(n=97)	(n=68)	(n=65)	*p* value
Artificial Oocyte Activation (AOA)	Amber	295 (67.3%)	167 (80.3%)	51 (52.6%)	38 (55.9%)	39 (60.0%)	p<0.00001
Timelapse incubator (TLT)	Amber	211 (48.2%)	122 (58.6%)	42 (43.2%)	26 (38.2%)	27 (41.5%)	p<0.005
Hyaluronate	Amber	206 (47.0%)	121 (58.2%)	34 (35.1%)	26 (38.2%)	25 (38.5%)	p<0.0005
Assisted-hatching (AHA)	Red	372 (85.1%)	196 (94.2%)	76 (78.4%)	51 (75.0%)	49 (75.4%)	p<0.00001
Chronic endometritis examination	Red	338 (77.2%)	176 (84.6%)	57 (58.8%)	52 (76.5%)	53 (81.5%)	p<0.00001
Chronic endometritis treatment	Red	337 (76.9%)	179 (86.1%)	57 (58.8%)	49 (72.1%)	52 (80.0%)	p<0.00001
Endometrial receptivity array (ERA)	Red	281 (64.2%)	165 (79.3%)	53 (54.6%)	39 (57.4%)	24 (36.9%)	p<0.00001
Endometrial microbiome metagenomic analysis (EMMA)	Red	258 (58.9%)	148 (71.2%)	46 (47.4%)	40 (58.8%)	24 (36.9%)	p<0.00001
Two-step embryo transfer	Red	214 (48.9%)	120 (57.7%)	45.4 (44)	25 (36.8%)	25 (38.5%)	p<0.005
Helper-T (Th)1/Th2 cell ratio test	Red	194 (44.3%)	117 (56.3%)	40 (41.2%)	22 (32.4%)	15 (23.1%)	p<0.00001
Endometrial injection of embryo culture supernatant	Red	173 (39.5%)	105 (50.5%)	33 (34%)	21 (30.9%)	14 (21.5%)	p<0.00001
Immunosuppressant agent treatment	Red	142 (32.4%)	93 (44.7%)	30 (30.9%)	15 (22.1%)	4 (6.2%)	p<0.00001
Endometrial scratching	Red	116 (26.5%)	70 (33.6%)	17 (17.5%)	12 (17.6%)	17 (26.2%)	p<0.01
Platelet-Rich Plasma (PRP) intrauterine infusion	Red	97 (22.1%)	65 (31.2%)	19 (19.6%)	11 (16.2%)	2 (3.1%)	p<0.00001
Preimplantation genetic testing for aneuploidy (PGT-A)	Red	93 (21.2%)	54 (26.0%)	15 (15.5%)	8 (11.8%)	16 (24.6%)	p<0.05
***In vitro* maturation (IVM)**	Red	76 (17.4%)	45 (21.6%)	14 (14.4%)	5 (7.4%)	12 (18.5%)	p<0.05
G-CSF administration	Red	71 (16.2%)	46 (22.1%)	14 (14.4%)	8 (11.8%)	3 (4.6%)	p<0.005
Intracytoplasmic morphologically selected sperm injection (IMSI)	Red	68 (15.5%)	44 (21.2%)	12 (12.4%)	6 (8.8%)	6 (9.2%)	p<0.05

Values are presented as % (n/total), unless otherwise indicated. Statistical significance is set at p<0.05.

Nevertheless, several add-on treatments were provided at no extra cost, regardless of facility type, including chronic endometritis treatment, TLT, IVM, and IMSI. One facility, classified as a outpatient clinic, was found to charge 400,000 yen (2697.5 USD, as of September 23, 2023) for IVM. These results highlight the unique characteristics of the IVF add-on supplement system in Japan (Table 2).

Medical care should be provided at the same price regardless of the facility. Nevertheless, IVF add-ons, including AHA, AOA, two-step embryo transfer, and endometrial injection of embryo culture supernatant, were found to have significant differences in cost according to facility type. All these add-on treatments were significantly more expensive in outpatient clinics than in university hospitals (Table 2).

3.4 Outpatient clinics are actively incorporating IVF add-on treatments

It seems evidence-based medicine is emphasized more by university facilities emphasize than by general clinical facilities. Therefore, we examined whether there was a correlation between the evidence level and adoption rate and cost of add-ons among the four attributes. Contrary to expectations, the adoption rate of add-on treatments classified as Amber was significantly lower in university hospitals than in outpatient clinics ($p<0.01$) (Figure 2A). Furthermore, the add-ons rated as Red had significantly higher adoption rates at outpatient clinics than at any other facility ($p<0.0001$, each) (Figure 2B). In contrast, there was no significant difference in cost between facility types for both Amber and Red categories (Figures 2C, D).

TABLE 2 Cost of *in vitro* fertilization add-on treatments according to facility.

	Rating in the current paper	Total	Outpatient Clinic	Inpatient Clinic	Hospital	University Hospital	
		(n=438)	(n=208)	(n=97)	(n=68)	(n=65)	*p* value
Artificial Oocyte Activation (AOA)	Amber	12,000 (0-20,000)	16,500 (0-22,000)	15,000 (0-20,000)	0 (0-10,000)	0 (0-19,000)	$p<0.01$
Timelapse incubator (TLT)	Amber	0 (0-20,000)	0 (0-10,000)	0 (0-0)	0 (0-0)	0 (0-0)	$p=0.65$
Hyaluronate	Amber	0 (0-11,000)	4,180 (0-15,000)	0 (0-6,700)	0 (0-10,000)	0 (0-11,250)	$p=0.14$
Assisted-hatching (AHA)	Red	20,000 (10,000-25,000)	20,000 (15,000-27,875)	15,750 (5,000-22,550)	20,000 (6,250-26,500)	19,000 (0-22,000)	$p<0.05$
Chronic endometritis examination	Red	10,000 (4,000-15,500)	11,000 (1,875-18,000)	4,000 (0-10,750)	5,000 (0-21,000)	0 (0-10,000)	$p=0.19$
Chronic endometritis treatment	Red	0 (0-30,00)	0 (0-3,000)	0 (0-2,575)	0 (0-3,000)	0 (0-2,000)	$p=0.66$
Endometrial receptivity array (ERA)	Red	120,000 (100,000-136,375)	125,000 (110,000-140,000)	110,000 (100,000-138,000)	120,000 (95,750-132,750)	110,330 (100,000-120,000)	$p=0.13$
Endometrial microbiome metagenomic analysis (EMMA)	Red	50,000 (40,000-66,000)	50,000 (40,000-66,000)	50,000 (40,000-61,000)	50,000 (35,000-65,000)	50,000 (40,000-65,515)	$p=0.70$
Two-step embryo transfer	Red	30,000 (10,000-50,000)	30,000 (20,000-54,930)	30,000 (10,000-46,500)	22,500 (7,500-37,250)	10,000 (0-31,000)	$p<0.05$
Helper-T (Th)1/Th2 cell ratio test	Red	15,000 (10,000-20,000)	15,000 (10,000-21,587)	15,150 (5,500-12,000)	10,000 (5,750-20,000)	13,750 (10,250-19,000)	$p=0.41$
Endometrial injection of embryo culture supernatant	Red	20,000 (10,000-27,500)	20,000 (11,085-30,000)	15,000 (10,000-20,000)	20,000 (13,000-25,000)	0 (0-12,500)	$p<0.01$
Immunosuppressant agent treatment	Red	1,500 (0-10,000)	1,000 (0-10,000)	2,500 (0-12,000)	5,000 (2,000-10,000)	3,000 (1,500-6,500)	$p=0.55$
Endometrial scratching	Red	2,500 (0-5,000)	2,500 (0-7,300)	3,650 (2,000-5,125)	700 (0-4,000)	0 (0-6,250)	$p=0.28$
Platelet-Rich Plasma (PRP) intrauterine infusion	Red	165,000 (142,250-200,000)	178,300 (150,000-200,000)	150,000 (95,000-182,500)	162,500 (142,500-200,000)	200,000 (200,000-200,000)	$p=0.09$

(Continued)

TABLE 2 Continued

	Rating in the current paper	Total	Outpatient Clinic	Inpatient Clinic	Hospital	University Hospital	
		(n=438)	(n=208)	(n=97)	(n=68)	(n=65)	*p* value
Preimplantation genetic testing for aneuploidy (PGT-A)	Red	80,000 (65,750-90,000)	86,000 (70,000-90,000)	70,000 (55,000-95,000)	75,000 (60,202-82,000)	67,500 (59,300-82,500)	*p*=0.29
***In vitro* maturation (IVM)**	Red	0 (0-22,000)	0 (0-0)	0 (0-22,000)	0 (0-55,000)	0 (0-52,500)	*p*=0.74
G-CSF administration	Red	20,000 (15,000-30,000)	23,600 (16,125-30,000)	15,500 (12,250-21,500)	25,000 (17,500-30,000)	20,000 (15,000-20,000)	*p*=0.68
Intracytoplasmic morphologically selected sperm injection (IMSI)	Red	0 (0-0)	0 (0-5,000)	0 (0-0)	0 (0-0)	0 (0-0)	*p*=0.77

Values are presented as median (IQR) unless otherwise indicated. Statistical significance is set at p<0.05.

3.5 Evidence levels, adoption rates, and cost of IVF add-ons

We hypothesized that as the evidence levels of IVF add-ons increase, adoption rates increase and costs decrease. Adoption rates and costs were analyzed to determine whether they correlated with the evidence levels of each individual IVF add-on treatment. The median adoption rate of Amber (48.2%) was higher than that of Red (32.4%) (Figure 3A). The wide variability of Red compared with Amber suggests that there are large differences in the adoption rates of add-on treatments according to facility type. The median cost of Red (20,000 yen, equivalent to 134.8 USD at the exchange rate as of September 23, 2023) was higher than that of Amber (0 yen, 0 USD) (Figure 3B). Furthermore, the wide range of minimum to maximum values for Red indicates that there are large differences in costs according to the type of add-on treatment. Although there was a trend towards a positive correlation between the level of evidence for IVF add-ons and adoption rates and an inverse correlation with costs, it was not statistically significant.

3.6 Cost did not correlate with the adoption rate

Based on our results, the adoption rate and cost were suggested to be inversely correlated. Accordingly, a linear regression analysis was performed. Although there was an overall trend towards an inverse correlation, there was no significant difference (Supplementary Figure 1A). The same trend was observed when the analysis was conducted separately according to the facility type (Supplementary Figures 1B-E). This trend was similar when linear regression analysis was performed by categorizing the add-on introduction rate as the adoption rate (0%–33%, 34%–66%, 67%–99%) (Supplementary Figure 2).

4 Discussion

The current survey showed that outpatient clinics had the highest number of oocyte retrievals per facility per year, indicating their central role in reproductive medicine in Japan. These facilities have a greater number of embryologists and nurses but fewer obstetricians and gynecologists than others. Outpatient clinics actively adopted significantly more IVF add-ons than other ART centers, regardless of the evidence level. There was a positive relationship between the level of evidence and the adoption rate of IVF add-ons and a negative relationship between the level of evidence and the cost of add-ons.

Add-ons have a history of being originally developed to improve pregnancy outcomes. Although add-ons are important for the advancement of reproductive medicine, their implementation in clinical practice has faced criticism from various media outlets, owing to the perceived lack of sufficient evidence (4). To assess the effectiveness of add-ons in Japan, several studies have examined the efficacy of add-on treatments within the framework of advanced research coinciding with the initiation of insurance coverage (17). In addition to explaining to patients that add-ons are distinct from essential medical care, this may provide crucial evidence for determining whether add-ons constitute a more essential medical treatment by establishing a stronger evidential basis for their efficacy. However, because the medical facilities responsible for clinical research may derive benefits from add-ons, caution should be exercised when interpreting the results, considering potential conflicts of interest.

In addition to the effectiveness and safety of each medical treatment, it is important to improve cost-effectiveness and patient satisfaction. The current study showed that the accumulation of evidence on the effectiveness and safety of add-on treatments may lead to an increasing adoption rate, which would produce scale merit and reduce costs. Additionally, as represented by next-generation sequencing, technological advances may lead to decreased costs (18). High-quality medical care is expected to be more cost-effective, and a positive cycle of increasing adoption rates and accumulating evidence is expected to emerge.

In contrast, there were no significant differences between evidence levels, adoption rates, and costs. There are three reasons for this: (i) The uniqueness of the clinics: Outpatient clinics adopted a significantly higher number of add-ons than other facility types, regardless of the evidence level. They may anticipate gain from the publicity effect of introducing “novel” and “costly” tests and treatments (19). Clinics have more freedom than university

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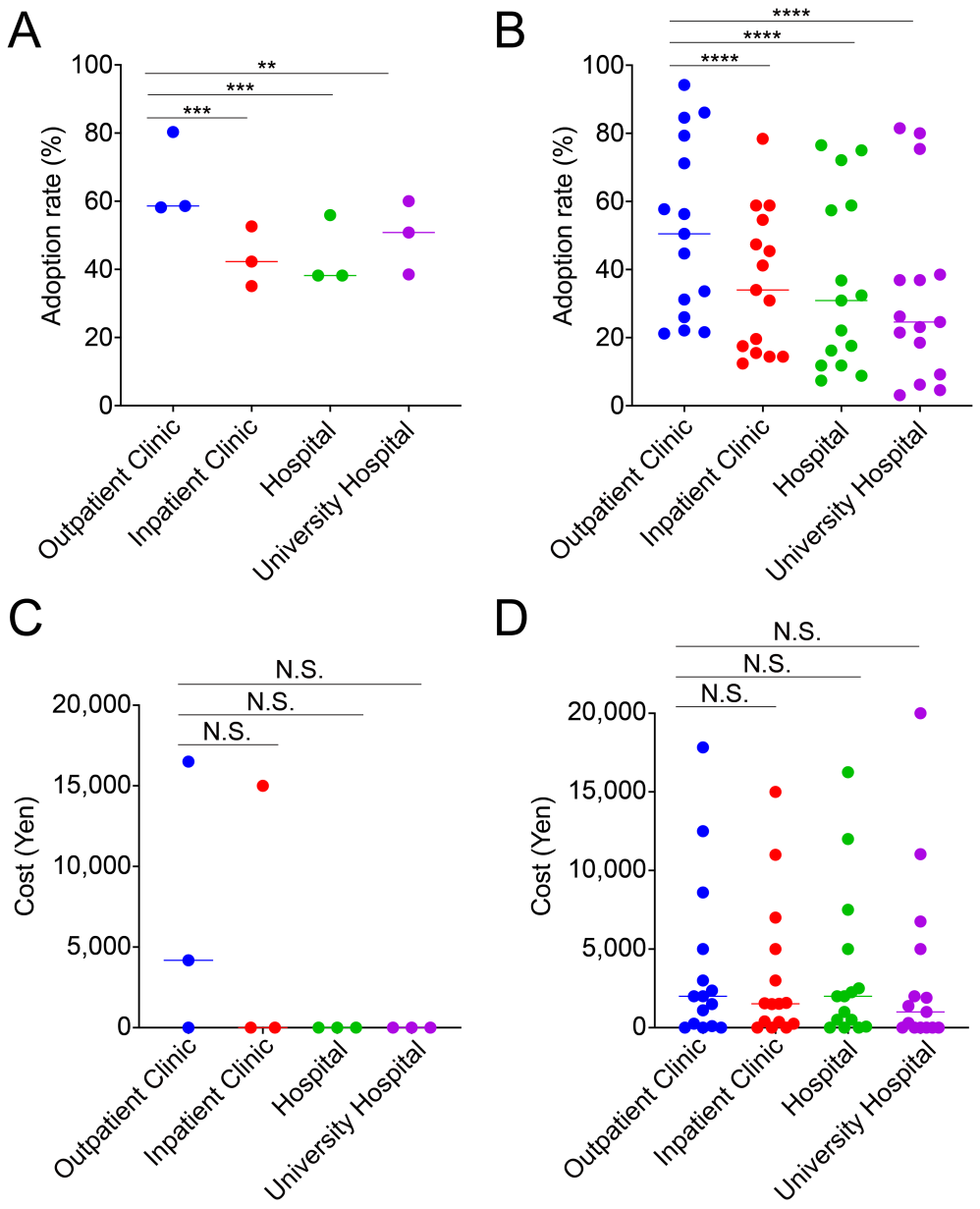

FIGURE 2
Outpatient clinics adopt significantly more add-on treatments than other facilities, but the median costs are comparable. The adoption rate of *in vitro* fertilization add-on treatments is rated as Amber **(A)** and Red **(B)**, and the cost is rated as Amber **(C)** and Red **(D)**, based on facility type. ****p<0.0001, ***p<0.001, **p<0.01, N.S., not significant.

hospitals to offer commercially available treatments, even those that have not been adequately proven to be safe or effective. (ii) Cost-free add-on treatments, e.g., TLT: The median cost of TLT in the current study was 0 yen (0 USD), regardless of facility type, even though TLT is considered expensive (20), with an initial implementation cost of hundreds and thousands of USD (21). According to a French survey of embryologists, the reasons for not implementing TLT were high initial implementation costs (50%) and a lack of data to support its clinical usefulness (37.5%) (22), as proven by a recent RCT trial (23). One reason for the discrepancy between the actual situation in the United States, France, and Japan is that the standardization of TLT, which facilitates the expansion of embryo culture capacity (24), may lead to the loss of the option of culture methods with a conventional incubator, limiting patients' ability to bear additional costs. There is a concern that cost-free add-on treatments could be attributed to financial constraints that prevent payment of salaries to healthcare providers, which could result in a shortage of staff able to provide adequate support in the decision-making process. Because of the free pricing available under free treatment, facilities that do not incur add-on costs may add these costs to the normal ART costs. (iii) Limited evidence of only three treatments classified as Amber: Add-ons that allow medical facilities to control costs only to a limited extent are likely to have a significant impact on outcomes. In cases where no significant differences between different facilities were found, such as ERA, EMMA, analysis of infectious chronic endometritis, PRP, and PGT-A, costs are determined by the outsourcing company. In

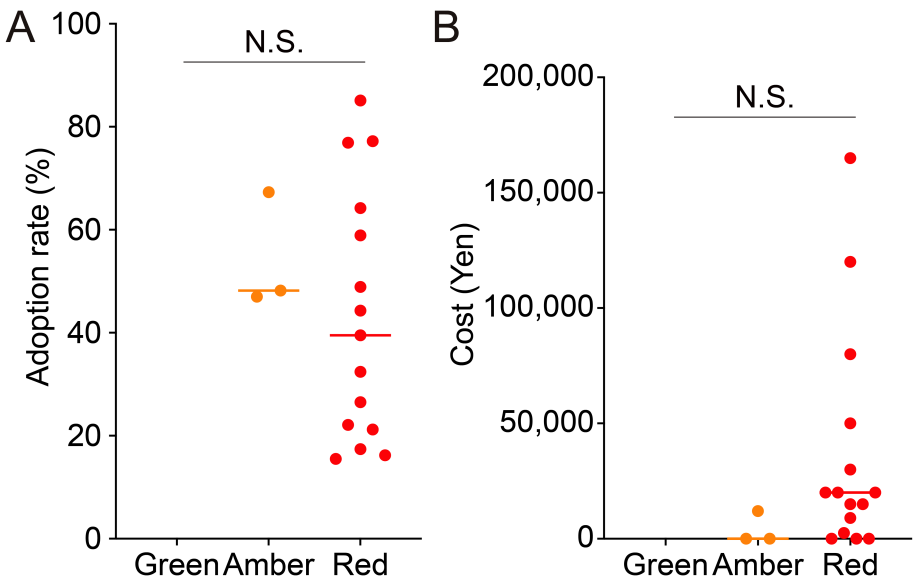

FIGURE 3
Correlation of evidence level with adoption rate and median cost of *in vitro* fertilization add-on treatments. The figure shows the adoption rate **(A)** and cost **(B)** of *in vitro* fertilization add-on treatments according to the evidence level. N.S., not significant.

particular, the environment surrounding PGT-A has undergone significant changes over the past 25 years. The substantial expenses associated with the equipment required for PGT-A and the potential for cost reduction through batch processing of samples have led companies to offer genetic services to multiple IVF clinics (25). Therefore, the absence of variation in the additional cost of PGT-A based on facility type is because of the commonality of the contractors involved. In contrast, AHA, AOA, and two-step embryo transfer showed significant differences in overall costs, but their pricing was left to the discretion of the facility.

One of the strengths of the current study is that it relies on a cost survey of healthcare providers, which may represent actual costs more accurately than previous studies on patients (3). In addition, the response rate to the survey was >70%, which is the highest response rate ever recorded in Japan (51%–63% in a survey conducted by the Ministry of Health, Labour and Welfare in 2020). This high response rate appropriately represents the actual status of ART in Japan before insurance coverage.

4.1 Limitation

As this survey was conducted in medical facilities, the extent to which patients choose add-ons and the total cost paid for reproductive health-related services were unclear. In addition, a few add-ons were classified as Amber, which limits the statistical analysis.

4.2 Conclusion

This survey of ART facilities in Japan showed that IVF add-ons are widespread and that the use of some add-ons creates a significant financial burden. Although numerous add-ons are available for IVF, their efficacy in improving pregnancy outcomes has not been scientifically demonstrated. Add-ons that have strong scientific evidence regarding safety and efficacy are widely adopted, regardless of facility type, whereas costly add-ons have low adoption rates because of the emphasis placed by healthcare professionals on scientific evidence. In Japan, some add-ons have been made eligible for national health insurance coverage from 2022 onwards; however, concurrent collaborative research by the Japan Society of Obstetrics and Gynaecology and the Japan Society for Reproductive Medicine as well as performance evaluations based on advanced medical care are being conducted to assess the safety and efficacy of add-ons. Based on these results, a review of insurance medical care is planned for fiscal year 2024. This survey played an important role in the formation of consensus. Additionally, it is expected to be a decision-making tool for clinicians and patients who suffer from infertility and consider add-ons as medical treatments.

Data availability statement

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

Author contributions

NS: Data curation, Formal analysis, Investigation, Software, Writing – original draft, Writing – review & editing. MY: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration,

Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. SH: Data curation, Investigation, Writing – original draft, Writing – review & editing. HS: Writing – original draft, Writing – review & editing. SJ: Data curation, Investigation, Supervision, Writing – original draft, Writing – review & editing. KK: Writing – original draft, Writing – review & editing. MH: Supervision, Writing – original draft, Writing – review & editing. YO: Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1404601/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Correlation of adoption rate with the median cost of *in vitro* fertilization add-on treatments. Linear regression of all facility types **(A)**, outpatient clinics **(B)**, inpatient clinics **(C)**, hospitals **(D)**, and university hospitals **(E)**.

SUPPLEMENTARY FIGURE 2
Correlations of adoption rate with the median costs of *in vitro* fertilization add-on treatments, categorized into 0%–33%, 34%–66%, and 67%–99% adoption rate. The adoption rate of *in vitro* fertilization add-on treatments (>34%) for all categories **(A)**, 0%–33% vs. 34%–66% **(B)**, 0%–33% vs. 67%–99% **(C)**, 34%–66% vs. 67%–99% **(D)**. There is no relationship between cost and the adoption rate, which is divided into three categories.

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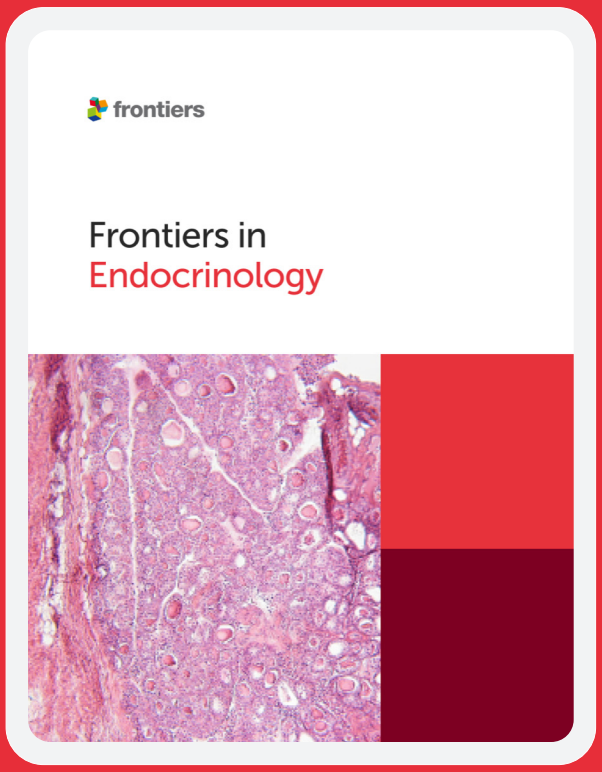

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