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Recurrent pregnancy loss and endocrine dysfunction

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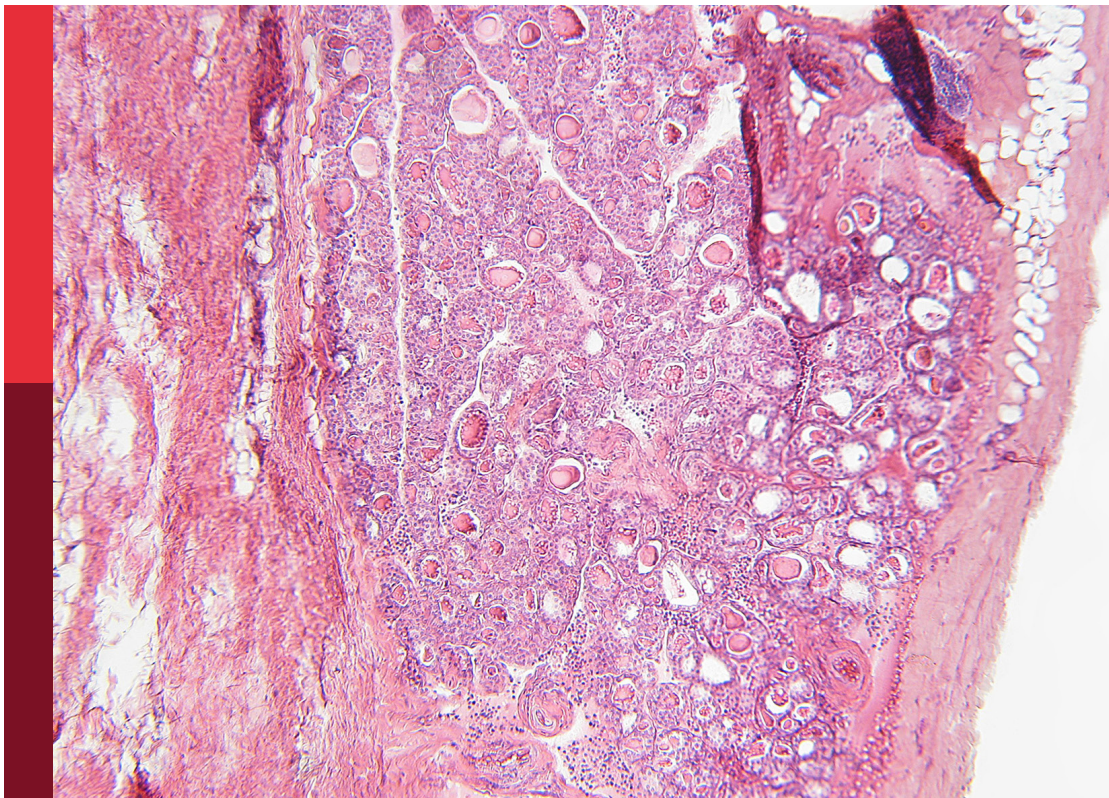

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Recurrent pregnancy loss and endocrine dysfunction

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Editorial: Recurrent pregnancy loss and endocrine dysfunction

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KEYWORDS

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Editorial on the Research Topic
Recurrent pregnancy loss and endocrine dysfunction

Recurrent pregnancy loss (RPL) is a distressing condition affecting a significant number of women globally, with complex etiologies that often remain elusive (1). It is estimated that approximately 8% to 12% of all cases of RPL are caused by endocrine diseases, which include thyroid dysfunction, luteal phase deficiency, hyperprolactinemia, diabetes mellitus, insulin resistance, polycystic ovarian syndrome (PCOS), and so on (2). The Research Topic "Recurrent Pregnancy Loss and Endocrine Dysfunction", aims to deepen our understanding of how endocrine issues contribute to RPL and to identify potential therapeutic targets.

The manuscripts accepted for this Research Topic present a diverse range of studies, each providing valuable insights into different aspects of endocrine-related RPL.

The cross-sectional study by Zhang et al. highlights the high prevalence of endocrine dysfunctions, such as thyroid dysfunction, hyperprolactinemia, obesity, PCOS, and glucose abnormalities in women with RPL. They emphasize the importance of comprehensive endocrine evaluations of endocrine dysfunction in recurrent pregnancy loss, proposing that obesity may be a key endocrine factor among patients with two or more pregnancy losses, and suggesting screening of patients for endocrine-related etiology after two miscarriages.

Several studies focus on specific endocrine disorders and their impact on RPL.

Huang et al. investigates the effect of thyroid-stimulating hormone (TSH) levels post-controlled ovarian hyperstimulation on IVF/ICSI outcomes. It finds that while TSH levels do not significantly affect pregnancy rates, lower TSH levels are associated with higher preterm delivery rates.

In terms of treatment strategies, sequential embryo transfer could improve the clinical outcomes of patients with recurrent implantation failure (Gao et al.). Fang et al. found that the number of previous embryo implantation failures is an independent factor affecting implantation rate, clinical pregnancy rate, spontaneous abortion rate and live birth rate of patients underwent IVF/ICSI- ET.

Adenomyosis can induce heavy menstrual bleeding, chronic pelvic pain, infertility and RPL. Ge et al. explore the effects of ovarian stimulation protocols on assisted reproductive technology outcomes in women with adenomyosis, they recommended that an ultra-long or long protocol might be beneficial for fresh embryo transfer.

The link between lupus and RPL is explored in another study, which finds that women with lupus have a higher risk of pregnancy loss due to the autoimmune nature of the disease, adding to the complexity of managing RPL in these patients (Valeff et al.).

Ren et al. found that exposure to environmental pollutants is also a risk factor for RPL. Insulin resistance, vitamin D insufficiency, and abnormal thyroid and sex hormone concentrations might contribute to heavy metal-related abortion.

Low follistatin levels have also been identified as a potential risk factor for RPL. This study indicates that follistatin may play a protective role in maintaining pregnancy, highlighting another endocrine factor that could be crucial in managing RPL (Gong et al.).

Li et al. provide a metabolic perspective by analyzing follicular fluid in patients with diminished ovarian reserve (DOR), showing unique metabolic characteristics that could be associated with RPL.

Conclusion

In conclusion, these studies collectively enhance our understanding of the multifaceted relationship between endocrine dysfunction and RPL. They underscore the importance of comprehensive and individualized endocrine evaluations in women with RPL to improve clinical outcomes. The knowledge gained from this compilation of studies aims to reduce the burden of RPL on affected women and their families, ultimately leading to better clinical practices and improved reproductive health outcomes.

We express our gratitude to all contributors for their invaluable research and to the reviewers for their critical evaluations. We hope this Research Topic will stimulate further research and enhance clinical practices in managing endocrine-related recurrent pregnancy loss.

Author contributions

HZ: Writing – review & editing. LD: Writing – original draft.

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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Metabonomic analysis of follicular fluid in patients with diminished ovarian reserve

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Background: Ovarian reserve is an important factor determining female reproductive potential. The number and quality of oocytes in patients with diminished ovarian reserve (DOR) are reduced, and even if *in vitro* fertilization-embryo transfer (IVF-ET) is used to assist their pregnancy, the clinical pregnancy rate and live birth rate are still low. Infertility caused by reduced ovarian reserve is still one of the most difficult clinical problems in the field of reproduction. Follicular fluid is the microenvironment for oocyte survival, and the metabolic characteristics of follicular fluid can be obtained by metabolomics technology. By analyzing the metabolic status of follicular fluid, we hope to find the metabolic factors that affect the quality of oocytes and find new diagnostic markers to provide clues for early detection and intervention of patients with DOR.

Methods: In this research, 26 infertile women with DOR and 28 volunteers with normal ovarian reserve receiving IVF/ET were recruited, and their follicular fluid samples were collected for a nontargeted metabonomic study. The orthogonal partial least squares discriminant analysis model was used to understand the separation trend of the two groups, KEGG was used to analyze the possible metabolic pathways involved in differential metabolites, and the random forest algorithm was used to establish the diagnostic model.

Results: 12 upregulated and 32 downregulated differential metabolites were detected by metabolic analysis, mainly including amino acids, indoles, nucleosides, organic acids, steroids, phospholipids, fatty acyls, and organic oxygen compounds. Through KEGG analysis, these metabolites were mainly involved in aminoacyl-tRNA biosynthesis, tryptophan metabolism, pantothenate and CoA biosynthesis, and purine metabolism. The AUC value of the diagnostic model based on the top 10 metabolites was 0.9936.

Conclusion: The follicular fluid of patients with DOR shows unique metabolic characteristics. These data can provide us with rich biochemical information and a research basis for exploring the pathogenesis of DOR and predicting ovarian reserve function.

KEYWORDS

diminished ovarian reserve, follicular fluid, metabonomics, oocytes, embryos, amino acids, steroids

1 Introduction

The ovarian reserve reflects the sum of follicles at different stages of development in the female ovary and the ability of these follicles to grow, develop and fertilize. With increasing age, follicles decrease gradually in number and function (1). Diminished ovarian reserve (DOR) refers to a decrease in the number and quality of oocytes, DOR is also currently diagnosed mainly by a decrease in basal sinus follicle count and antimilorphan hormone as well as elevated serum basal follicle stimulating hormone levels. DOR that occurs after the age of 40 is usually physiological, while women's early experience of DOR leads to an early decline in their reproductive function. The most common clinical manifestations of DOR are menstrual disorder, endocrine disorder, poor response to ovarian stimulation and infertility. If not treated in time, it is likely to progress to premature ovarian failure within a few years (2). The etiology of DOR is complex and mainly includes age, genetics, immunity, psychology, environment and other factors. In most patients, the main reasons are still difficult to determine. Recent studies have shown that even if IVF-ET is selected as a treatment means, the pregnancy rate of DOR patients is still reduced due to their low response to ovulation inducing drugs, and their live birth rate is significantly lower than that of women with normal ovarian reserve (3). In addition, patients with DOR have an increased incidence of hypertension during pregnancy (4) and an increased risk of recurrent abortion and aneuploid blastocysts after IVF (5, 6). Due to the unclear etiology and limited therapeutic effect of DOR, it is still one of the great challenges in the clinical treatment of infertility.

Follicular fluid (FF) is formed by the secretion of granulosa cells, membrane cells and oocytes and the diffusion of plasma components from capillaries to the follicular cavity. Its main components include hormones, growth factors, cytokines, proteins, steroids, amino acids and polysaccharides. As the microenvironment for the growth and development of follicles and oocytes, FF is the medium for the exchange of substances and energy between oocytes and the extracellular environment, and alterations in components in FF can reflect the metabolic level and developmental potential of oocytes (7). Studies also show that changes in follicular fluid metabolites, such as progesterone (8), phosphatidylcholine (9), and high-density lipoproteins (10), have an impact on fertilization and early embryonic development. Therefore, studying changes in metabolites in follicular fluid can reveal the factors that affect the development of oocytes and pregnancy outcome in DOR patients.

Metabonomics is a new omic technology developed after genomics and proteomics, and it can simultaneously qualitatively and quantitatively analyze all low molecular weight metabolites in the samples to be detected. Metabonomics reflects events downstream of gene expression and provides valuable information about cell metabolism. Common methods include nuclear magnetic resonance (NMR), gas chromatography–mass spectrometry (GC–MS), liquid chromatography–mass spectrometry (LC–MS), and capillary electrophoresis mass spectrometry (CE–MS) (11). In recent years, the rapid development of metabonomics has also promoted the study of follicular fluid, which is used to assess the metabolic status of oocytes, study the pathogenesis of diseases and identify potential biomarkers (12). The researchers found that the FF showed different metabolic characteristics at different stages of follicular development (13), and the characteristics of FF metabolism in women of different ages were also different (14). In addition, changes in the metabolic map were also found in FF under the conditions of endometriosis (EMS), polycystic ovary syndrome (PCOS) and other diseases (15, 16). Several targeted metabonomic studies have revealed abnormalities in the metabolism of amino acids and oxidized lipids in the FF of DOR patients, and these differential metabolites are significantly related to the number of oocytes retrieved and embryo quality during IVF-ET, which indicates that the decline in oocyte quality in DOR patients may be involved in the impairment of energy utilization and the increase in oxidative stress. Differential metabolites may be used as biomarkers to predict ovarian reserve and embryonic development (17–19). At present, there are few studies on the nontargeted metabonomics of FF from patients with DOR. In this study, the researchers used nontargeted metabonomics technology (high-performance liquid chromatography–mass spectrometry) to expand the detection range of metabolites in the FF of DOR patients and screen potential biomarkers, which supplemented new data for studying the pathogenesis of DOR and the impact of metabolic changes on IVF-ET outcomes of DOR patients.

2 Materials and methods

2.1 Sample collection and preparation

This study is a prospective clinical experiment that has been approved by the Ethics Committee of Wuhan University People's Hospital (WDRY2018-K009), and all subjects signed an informed consent form. The subjects were all IVF or intracytoplasmic sperm injection (ICSI)–assisted pregnancy patients at Wuhan University People's Hospital from December 2020 to January 2022, and they were divided into an experimental group (DOR group, n=26) and a control group (CON, n=28) according to whether the ovarian reserve was normal.

The inclusion criteria of the DOR group were as follows: 1: 20-40 years old; 2. body mass index (BMI) ≤ 24 kg/m2; and 3. patients meeting the clinical diagnostic criteria of DOR (20): 1) AMH ≤ 1. 1 ng/mL; 2) basic follicle stimulating hormone (bFSH) ≥ 10 IU/L, with or without FSH/luteinizing hormone (LH) ≥ 3.2; and 3) number of follicles in the unilateral basal sinus (bAFC) ≤ 7. Any two of the above three items can be diagnosed as DOR. In the control group, patients with normal ovarian reserve were treated with IVF/ICSI only because of male infertility or oviduct factors (oviduct adhesion, excision, ligation, etc.). The inclusion criteria of the control group were as follows: 1, 20-35 years old; 2. BMI ≤ 24kg/m2; 3, AMH ≥ 2 ng/mL, with or without bAFC ≥ 7; 4. bFSH < 10 IU/L, and estradiol (E2) < 80 pg/mL.

All subjects in this experiment should meet the following exclusion criteria: 1. History of ovarian surgery: cyst stripping, oophorectomy, etc.; 2. EMS, PCOS, ovarian cysts and other diseases that may affect the ovarian reserve function; 3. Received hormone therapy three months before the visit; 4. Any contraindication for ovulation induction therapy; 5. Other systemic abnormalities included hereditary diseases such as chromosome abnormalities, endocrine diseases such as hyperthyroidism and diabetes, infectious diseases such as hepatitis B, HIV and syphilis, and autoimmune diseases such as systemic lupus erythematosus.

2.2 Oocyte acquisition and follicular fluid collection

Subjects received an individualized ovarian stimulation protocol according to their ovarian reserve, age, weight and previous ovulation induction. In our reproductive center, the gonadotropin-releasing hormone antagonist protocol (GnRH-ant protocol) and ovarian stimulation protocol under progesterone (PPOS protocol) are used to help patients with DOR. The GnRH-ant protocol: patients begin daily gonadotropin (Gn) on days 2-3 of the menstrual cycle, and follicular development and serum hormone levels are monitored at the same time. When one follicle diameter reaches 12 mm or serum E2 > 300 pg/ml, patients should take GnRH ant 0.25/d; until three follicles have a diameter greater than 18 mm, and 60% of the follicles have a diameter of 16 mm or E2 has no significant increase or decline, they should be injected with HCG6000-10000 IU that night, and after 34-38 hours, they are punctured for oocytes under ultrasonic monitoring. The PPOS protocol is as follows: patients begin daily progesterone (8-10 mg) on days 2-5 of the menstrual cycle, and at the same time, they are injected with FSH or human menopausal gonadotropin (HMG) 150-300 IU/day. The amount of Gn was adjusted according to follicular growth. When one follicle diameter is greater than 17 mm, patients are injected with HCG 6000-10000 IU or GnRH-a 0.2 mg combined with HCG 2000 IU to induce ovulation. Oocytes were retrieved 34-38 h after ovulation induction. Patients who still have more than 2 small follicles (diameter ≤ 8 mm) after oocyte retrieval in the follicular phase, they should continue to take oral Gn, FSH or HMG (150-300 IU) in the luteal phase and adjust the Gn dose 2-3 days later according to follicular development. When at least 2-3 follicles had a diameter greater than 18 mm, and the serum E2 level reached an average of approximately 200 pg/ml for each dominant follicle, HCG (10000 IU) was injected. Oocytes were retrieved 34-38 h after injection.

Only follicular fluid from follicles larger than 18 mm in diameter was collected. The patient's oocytes were retrieved and placed in the incubator for 4-6 hours, and the follicular fluid was collected in Eppendorf tubes after merging. The collected follicular fluid was centrifuged at 4°C and 15000 rpm for 10 minutes, and the supernatant was collected and frozen at -80°C for the next step of detection.

2.3 Embryo quality assessment

The embryo quality was recorded on the third day after fertilization. The evaluation criteria for embryos are as follows: Grade I: the shape of blastomere is regular, the size is uniform, and there is no obvious DNA fragment; Grade II: the size of blastomere is slightly uneven, the shape is slightly irregular, and the fragments are not more than 20%; Grade III: blastomeres vary in size, fragments between 20% to 50%; Grade IV: blastomeres of different sizes, fragments ≥ 50%. High-quality embryos at cleavage stage are defined asfollows: Day3 embryos are Class I~II, and the number of blastomeres is 6~9.

2.4 Mass spectrometry detection: sample preparation and spectrum collection

In this study, we used a timsTOF Pro mass spectrometer (Bruker Daltronics, Germany) in combination with an Ultimate 3000 liquid system (Thermo Scientific) for LC–MS analysis. MS data were obtained using a timsTOF Pro mass spectrometer with a TOP 3 data-dependent acquisition (DDA) method. The scanning range of the timsTOF Pro mass spectrometer was 20-1300 Da, and the collision energy of MS/MS was 20 eV. The ESI source conditions were set as follows: capillary voltage=3.6 kV; dry gas flow=10.0 L/min; Spray gas = 2.2 bar; drying temperature=220 °C. Before injecting each mode, we performed external calibration with sodium formate and injected 15 QC samples before FF samples to balance the instrument. During data collection, one QC sample was inserted for every 10 FF samples to monitor the consistency and stability of the whole operation process. The Ultimate 3000 liquid system was mainly used for chromatographic separation. To improve the quality and quantity of identification of hydrophilic and hydrophobic metabolites, two chromatographic separation methods, HILIC and RPLC, were both used for nontargeted metabonomic analysis. In HILIC mode, we used a Waters ACQUITY UPLC BEH amide column (2.1 mm * 100 mm, 1.7 μm), the column temperature was 40 °C, and the injection volume was 5 μL. The mobile phase consisted of A (H2O containing 10 mM ammonium acetate and 0.1% acetic acid) and B (containing 95% ACN and 10 mM ammonium acetate and 0.1% acetic acid), with a constant flow rate of 0.5 mL/min. The chromatographic gradient program was as follows: 0 ~ 1 min, 100% B; 1 ~ 11 min, 60% B; 11~11.5 min, 40% B; 11.5 ~ 12 min, 40% B; 12 ~ 12.1 min, 100% B; 12.1 ~ 18 min, 100% B. In RPLC mode, we used Waters ACQUITY UPLC BEH C18 column (2.1mm * 100mm, 1.7 μm), the column temperature was maintained at 50°C, and the injection volume was 5μ. The mobile phase consisted of A (H2O containing 0.1% formic acid) and B (containing 0.1% formic acid and ACN), with a constant flow rate of 0.4 mL/min. The chromatographic gradient program was as follows: 0 ~ 2 min, 2% B; 2 ~ 17 min, 100% B; 17 ~ 20 min, 100% B; 20 ~ 20.1 min, 2% B; 20.1 ~ 23 min, 2%.

2.5 Data processing and statistical analysis

MetaboScape4.0 software was used to process the original data with peak filtering (greater than 5000), calibration, feature matching and standardization to conduct comprehensive molecular feature extraction. After determining the mass to charge ratio and peak intensity of metabolites, we searched the Human metabolome Database (HMDB) and the database established by local standards to retrieve and identify the detected metabolites. Subsequently, the data of all metabolites were normalized on Metaboanalyst 5.0 (www.metaboanalyst.ca). We excluded features with CV values greater than 0.25, filled in the missing value (5.5%) with 1/5 of the positive minimum value, and normalized the data according to the sum. Benjamin Hochberg correction was applied to keep the risk of type I errors below 5%. Using a nonparametric test, we set the fold change (FC)>1.2 and false discovery rate (FDR)<0.05 to screen potential differential metabolites related to DOR. Unsupervised pattern recognition principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were also analyzed on SIMCA software after autoscaling. By reducing the dimensions of the obtained multidimensional data to form a matrix, OPLS-DA could show the separation trend between the two groups more clearly. In addition, the permutation test was used to verify the imitative effect and prediction ability of the OPLS-DA model. Metabolites with first principal component projection (VIP) > 1.0 and P value < 0.05 were considered to be significantly different between the two groups. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was conducted to identify pathways that might be involved. Finally, a random forest algorithm was used to establish the diagnostic model.

The baseline characteristics of the subjects were analyzed, and the data are expressed as the mean ± standard deviation (SD). SPSS 25.0 software was used to analyze the normality of the data. Variables with normal or near normal distributions were analyzed by Student's t test or the chi-square test, and data with nonnormal distribution were analyzed by the Kruskal −Wallis test. The Spearman correlation coefficient between differential metabolites and the ovarian function index was calculated using the psych package in R. Statistical significance was defined as $P < 0.05$.

3 Results

3.1 Clinical characteristics of patients with DOR

The patients' clinical characteristics are showed in Table 1. The average age, BMI, years of infertility, fasting blood glucose (FBG) level, serum hormone level and IVF-ET outcome were compared between the DOR and CON groups. Age, bAFC, AMH, and bFSH were significantly different between the two groups ($P < 0.001$). BMI ($P=0.016$, $P<0.05$) and years of infertility ($P=0.021$, $P<0.05$) had a slight increase in the DOR group, while bLH, bE2, P and FBG had no differences ($P>0.05$). Analysis of the results of oocytes obtained and fertilization revealed that the number of oocytes retrieved, MII oocytes, 2PN fertilization and high-quality embryos on the third day in DOR patients were significantly lower than those in CON patients ($P<0.001$). This shows that the ovarian reserve of patients in DOR group is indeed reduced and our inclusion criteria are reliable. DOR is an age-related infertility, ovarian reserve and

TABLE 1 The demographic and clinical characteristics of patients with DOR and CON.

Item	DOR group (n=26)	CON group (n=28)	P value
Age (year)	34.04 ± 3.8	30.29 ± 2.62	< 0.001 ***
BMI (kg/m^2)	21.57 ± 1.43	20.48 ± 1.74	0.016 *
bFSH (mIU/ml)	11.11 ± 4.71	6.60 ± 1.75	< 0.001 ***
bLH (mIU/ml)	4.22 ± 4.36	3.65 ± 1.71	0.52
bE2 (pg/ml)	47.55 ± 30.86	36.02 ± 17.33	0.094
P (ng/ml)	1.49 ± 2.77	0.78 ± 1.03	0.21
bAFC (n)	3.5 ± 1.61	10.18 ± 3.35	< 0.001 ***
AMH (ng/mL)	0.72 ± 0.36	4.70 ± 2.49	< 0.001 ***
FBG (mmol/L)	4.78 ± 0.45	5.02 ± 0.31	0.474
Duration of infertility (year)	3.28 ± 2.77	2.78 ± 2.31	0.021 *
Oocytes retrieved (n)	4.31 ± 2.49	15.07 ± 6.43	< 0.001 ***
MII oocytes (n)	3.12 ± 1.77	10.21 ± 4.83	< 0.001 ***
2PN Fertilizations (n)	2.38 ± 1.68	7.14 ± 4.09	< 0.001 ***
Day3 High-quality embryos (n)	1.04 ± 1.08	4.43 ± 3.56	< 0.001 ***

DOR, Diminished ovarian reserve; NOR, Normal ovarian reserve; BMI, Body mass index; bFSH, basic follicle-stimulating hormone; bLH, basic luteinizing hormone; bE2, basic estrogen; P, progesterone; bAFC, basic antral follicle count; AMH, anti-Mullerian hormone; FBG, fasting blood glucose *P ≤ 0.05 **P ≤ 0.005 ***P ≤ 0.001.

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oocyte quality decline with advancing age, the difference in age between the DOR group and the control group in the results may be explained by the fact that age is to some extent an etiological or synergistic factor in DOR. To make the study more rigorous, future studies should expand the sample size and distinguish between different age groups before comparing metabolic levels

3.2 Multivariate analysis of metabolites

In this experiment, we detected metabolites in follicular fluid under HILIC positive and negative ion modes and RPLC positive and negative ion modes. A total of 2897 variable features were detected under HILIC mode, and 3419 features were detected under RPLC mode; a total of 994 metabolites were identified. The Wayne diagram in Supplementary Figure 1 (Figure S1) shows that the metabolites detected in the four modes are very different, which proves that detection in different modes can obtain more information about metabolites.

To compare the overall difference in the metabolic spectrum between DOR patients and the control group, PCA and OPLS-DA discriminant models were established based on the metabolite data obtained under four modes. The OPLS-DA discriminant model reveals that the DOR group and control group can be well separated, as shown in Figures 1A, C, E, G, the DOR group is all clustered on the right side, the control group is all clustered on the left side, and the values of R2Y and Q2Y are close to 1, which indicates that the difference between the two groups is significant

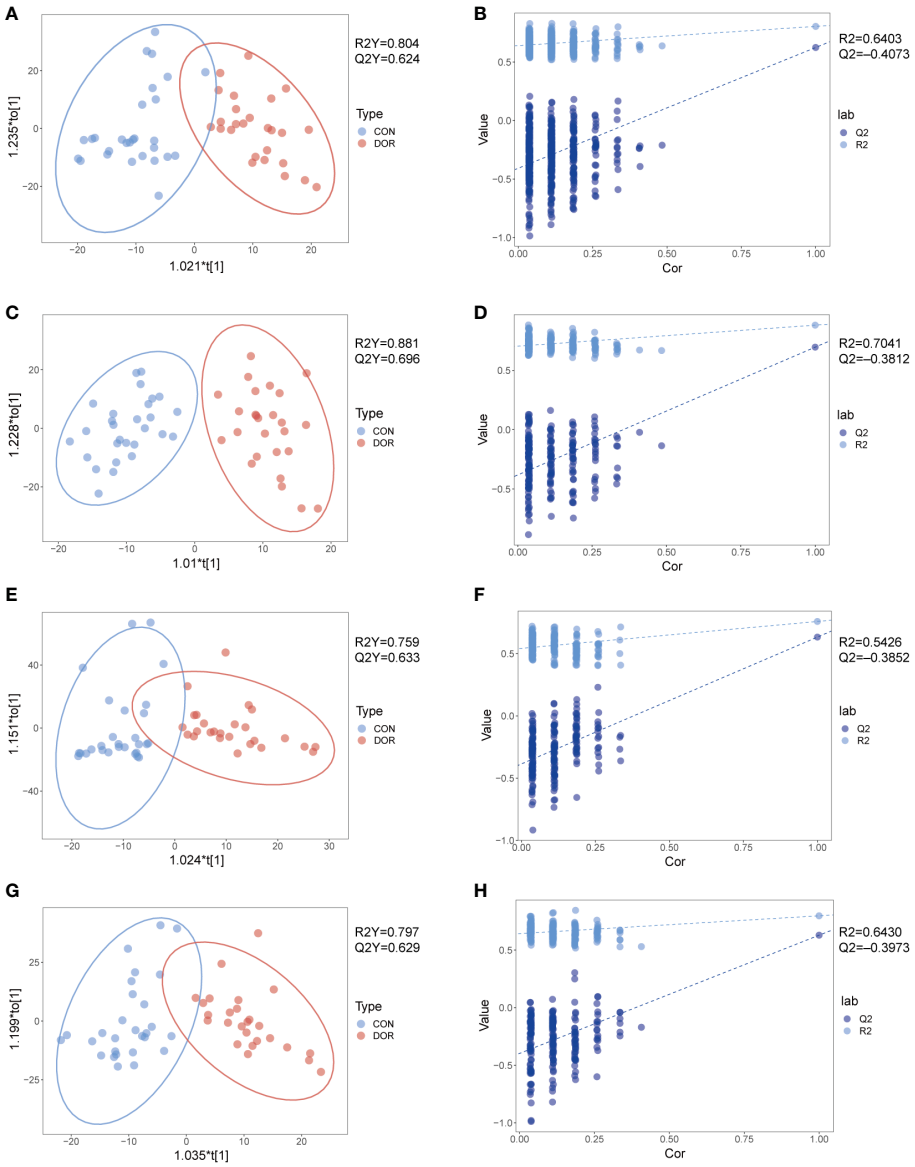

FIGURE 1
Metabolomic analysis of follicular fluid from patients with diminished ovarian reserved (n = 26, green dots) and healthy controls (n = 28, red dots) under HILIC and RPLC mode. OPLS-DA score plots for positive ionization mode **(A, E)** and negative ionization mode **(C, G)**, the OPLS-DA model's permutation test for positive ionization mode **(B, F)** and negative ionization mode **(D, H)**. **(A-D)**: HILIC mode; e-h: RPLC mode.

and the prediction ability of the OPLS-DA model is good. As shown in Figures 1B, D, F, H, the permutation test shows the value of Q2 and R2 under the four modes, further verifying that the OPLS-DA model has a good imitative effect and prediction ability. In addition, the PCA models also show that the DOR group and control group are distributed in different regions (Figure S2), but the two groups are not completely separated in the PCA model, which indicates that the samples have individual differences. In Figure S2, the quality control samples (QC) are gathered together, which shows that the stability and repeatability of the detection system is satisfactory, and the OPLS-DA is reliable.

3.3 Identification of differential metabolites and analysis of related pathways

Univariate analysis was carried out on all features detected in follicular fluid, and a volcano plot is shown in Figures 2A–D, downregulated features in DOR samples were clustered on the left, and upregulated features were clustered on the right. A total of 12 upregulated and 32 downregulated metabolites were identified from all differential features. Differential metabolites mainly include amino acids, indoles, nucleosides, organic acids, steroids, phospholipids, fatty acyls and organic oxygen compounds. The VIP value of these differential metabolites in OPLS-DA was greater than 1, and $P < 0.05$ in univariate analysis (Supplementary Tables 1, 2), which also indicated that they were related to ovarian reserve. In addition, the heatmap also intuitively shows the change in metabolites in DOR patients (Figure 2E).

According to KEGG analysis, the differential metabolites are mainly involved in aminoacyl-tRNA biosynthesis, tryptophan metabolism, pantothenate and CoA biosynthesis, and purine metabolism (marked in Figure 3). These metabolic pathways play an important role in maintaining the normal function of cells and tissues, and the results add new data for exploring the pathogenesis and clinical treatment of DOR.

3.4 Correlation analysis between differential metabolites and clinical indicators

In this experiment, Spearman correlation analysis was used to study the relationship between differential metabolites and clinical detection indicators in FF. Ten metabolites with correlation coefficients of $R>0.6$ and $P<0.05$ are shown in Figure 4A. We found that pregnanediol-3-glucuronide, N-acetyl-D-tryptophan, L-aspartic acid and indole-3-carboxaldehydewere significantly positively correlated with AMH, bAFC, number of oocytes retrieved, MII oocytes, and 2PN fertilizations, while almost all metabolites did not correlate with BMI, bLH, bP, bE2, and duration of infertility ($R < 0.3$, not shown in the figure). In addition, for further study on how the metabolic profile changed in the FF of DOR patients, the correlation between metabolites is shown in Figure 4B.

3.5 Establishment of a diagnostic model based on 10 metabolites

To intervene earlier and more individually in the development of DOR, we used a random forest algorithm to distinguish the DOR groups and the CON groups and finally selected the top 10 metabolites (Figure 5A) to be included in the diagnostic model. Using a receiver operating characteristic (ROC) curve to evaluate the diagnostic performance of this model, as shown in Figure 5B, the AUC reached a very high value of 0.9936. When all the metabolites were chosen for diagnostic models, the AUC value (AUC=0.9936) was similar to that of the top 10 metabolites. Although the top 20 metabolites had the highest AUC values (AUC=0.9952) when included in the diagnostic model, it was not much higher than 0.9936, so we finally determined the top 10 metabolites for the sake of efficient clinical diagnosis. In addition, unsupervised cluster analysis showed that the top 10 metabolites could distinguish all DOR patients from normal subjects (Figure 5C). In summary, these 10 metabolites can be used as promising tools to predict ovarian function.

4 Discussion

In this study, we used nontargeted metabonomics to detect the follicular fluid of 54 subjects, and found that there were 12 upregulated and 32 downregulated metabolites in the FF of DOR patients compared with normal women. These metabolites were mainly involved in aminoacyl-tRNA biosynthesis, tryptophan metabolism, pantothenate and CoA biosynthesis, purine metabolism, which are closely related to the number of oocytes and embryo quality. In the following, we discuss in detail the possible mechanisms of differential metabolites.

As an essential amino acid, tryptophan has three metabolic pathways *in vivo*: 1) tryptophan is hydroxylated and decarboxylated to form serotonin and melatonin; 2) tryptophan generates nicotinic acid, nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) through karinurine metabolism; and 3) tryptophan can generate indole, indole pyruvic acid, indole lactic acid, etc., through deamination and decarboxylation. Tryptophan and its metabolites participate in many physiological processes, such as maintaining cell growth and regulating immune function. In recent years, research on tryptophan metabolism in the reproductive field has made some progress. A large number of studies have shown that serotonin, as one of the main metabolites of tryptophan, plays an important role in regulating placental function and fetal development (21). Serotonin can affect the development of oocytes by regulating progesterone secretion by granulosa cells. Animal experiments have also shown that a decrease in serotonin in mouse blood leads to damage to early embryo development (22). Serotonin is the main source of melatonin. The latest evidence showed that human ovarian granulosa cells cultured *in vitro* can express melatonin, and melatonin reduces oxidative stress by improving the mitochondrial function of oocytes (23). In addition, adding an appropriate amount of melatonin during embryo culture improves

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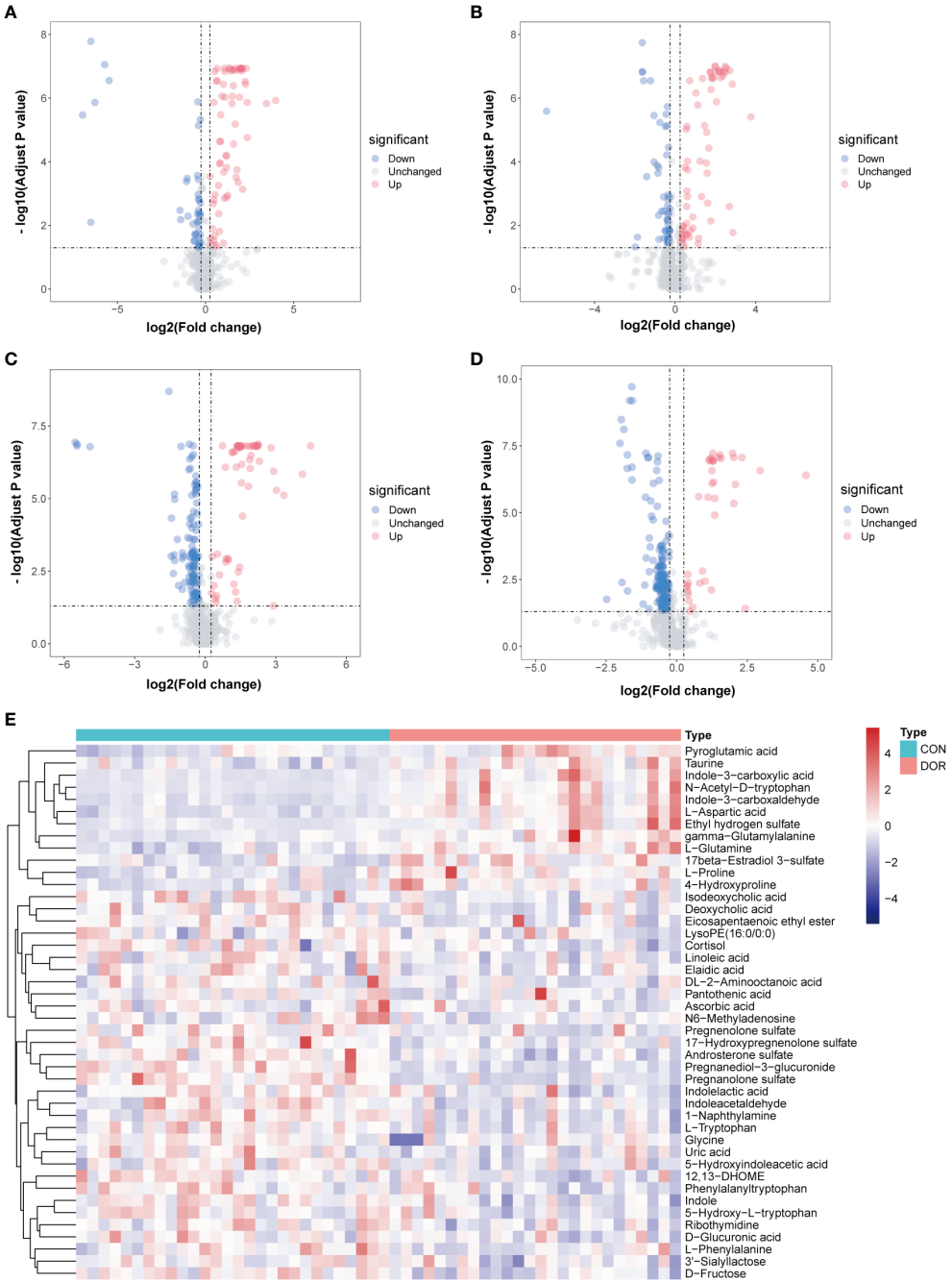

FIGURE 2
Volcanic maps under different detection modes show the distribution of different features. The blue dot represents the up regulated features while the red dot represents the down regulated. **(A)**: positive ionization mode in HILIC; **(B)**: negative ionization mode in HILIC; **(C)**: positive ionization mode in RPLC; **(D)**: negative ionization mode in RPLC; **(E)**: Heatmap based on 44 metabolites, unsupervised cluster analysis showed that differential metabolites could distinguish DOR patients from healthy subjects.

the rate of high-quality embryos in patients with repeated low-quality embryos on the third day (24), and the quality of embryos and clinical results of patients with repeated cycles after the failure of IVF/ICSI can also be improved (25). Only a small amount of tryptophan is metabolized through the indoleacetic acid pathway, and the role of metabolites generated from this pathway in oocyte and embryo development is still unclear. In this experiment, we detected that tryptophan and its decomposition products 5-hydroxy-L-tryptophan, 5-hydroxyindoleacetic acid (the metabolic end product of serotonin), indole, indoleacetaldehyde, and indoleacetic acid in the FF of DOR patients decreased, and were positively correlated with the number of oocytes, fertilized embryos, and high-quality embryos. The results of this study indicate that the decrease in tryptophan and its decomposition products, especially in the ovary, will affect the quantity and quality of oocytes, and may be detrimental to the early development of embryos. In addition,

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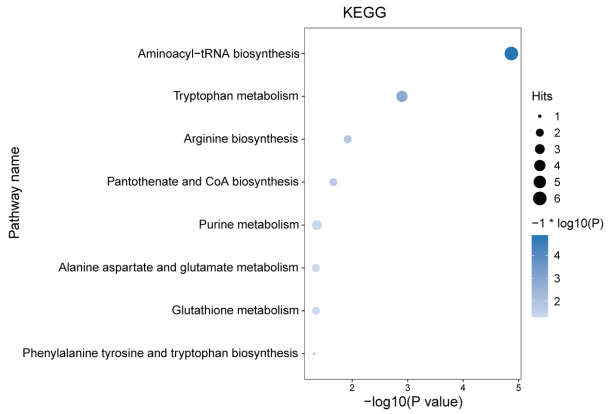

FIGURE 3
KEGG's metabolic pathway for differential metabolites. As the bubble color deepens, the P value decreases, and the larger the bubble is, the greater the impact of this pathway on DOR.

karinurine metabolism plays a dominant role in tryptophan catabolism, and changes in the concentration of karinurine in the placenta are closely related to the occurrence of several pregnancy complications (26). Recent studies have also shown that the tryptophan-karinurine pathway is abnormally activated in PCOS patients (27). However, in the FF of patients with DOR, we did not detect differential metabolites related to this pathway. Does this indicate that the effect of tryptophan on the development of oocytes and early embryos in DOR patients is through the serotonin and indoleacetic acid metabolic pathways, but not through the karinurine pathway? This need to be further explored.

We also found that other amino acids in FF changed: glycine, phenylalanine and DL-2-aminooctanoic acid were downregulated, and aspartic acid, proline, L-glutamine and pyroglutamate were upregulated. Glycine is a component of the endogenous antioxidant glutathione (GSH), and the reduction may mean that the oocytes are suffering from enormous oxidative stress; therefore, glycine is consumed to synthesize glutathione and resist oxidative stress. Studies have shown that glycine treatment of pig oocytes cultured *in vitro* promoted oocyte maturation by reducing the level of intracellular reactive oxygen species (ROS) and increasing mitochondrial function (28). In addition, glycine also has an effect on mammalian oocyte maturation and early embryo implantation through a volume regulation mechanism (29). L-proline is a key regulator of embryogenesis, placental development and fetal growth. It increased the viability of porcine trophoblastic ectodermal cells by activating the TORC1 signaling

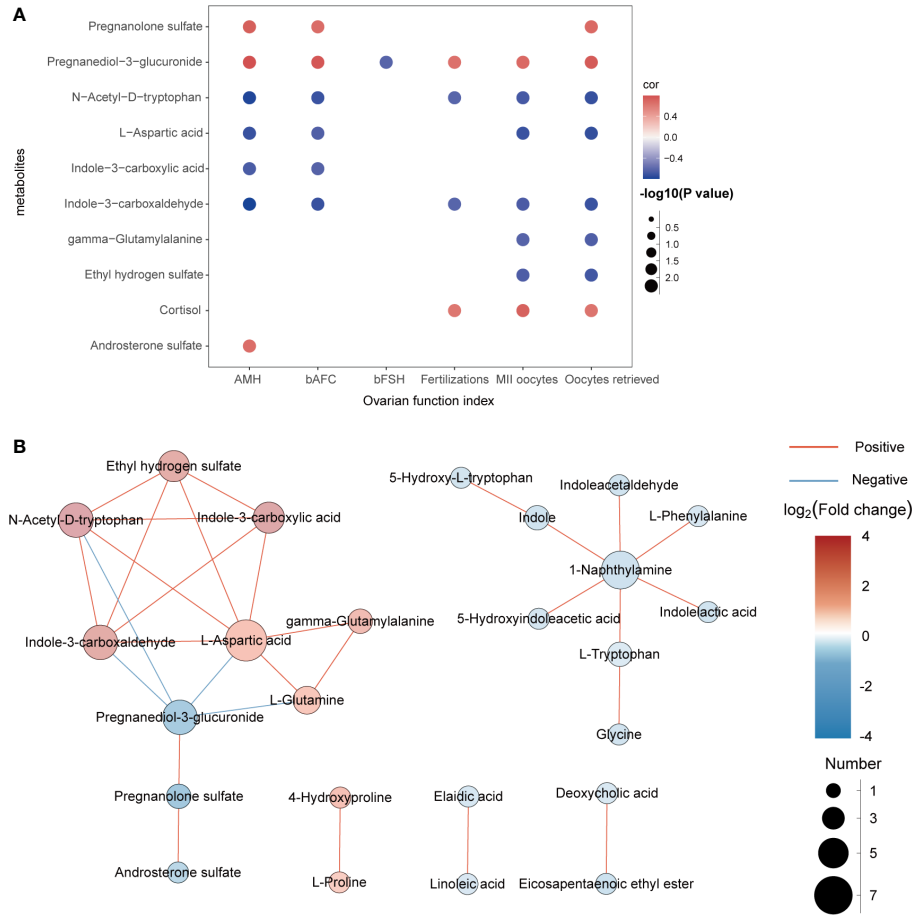

FIGURE 4
(A): Correlation analysis between differential metabolites and clinical indicators. Red represents positive correlation, while blue represents negative correlation. The darker the color is, the stronger the correlation is. **(B)**: Network diagram of the interactions between metabolites.

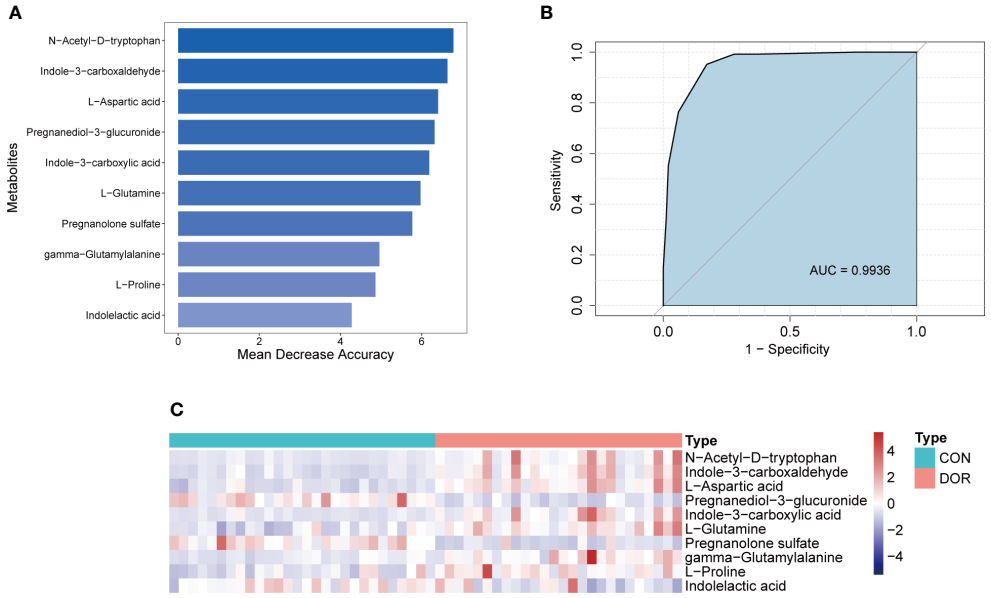

FIGURE 5
Establishment and test of diagnostic model based on 10 metabolites. **(A)**. Top 10 metabolites selected according to random forest algorithm; **(B)**. ROC curve and AUC value of the combined diagnostic model of the top 10 metabolites; **(C)**. Unsupervised cluster analysis of the top 10 metabolites.

pathway (30). Maternal L-proline supplementation could also improve mouse placental development and fetal survival (31). L-glutamine is another important energy substrate that plays a role in the development of preimplantation embryos (32, 33). However, how these two amino acids affect oocyte development is still unclear. We detected changes in amino acids and their derivatives in the FF of DOR patients, and it has been shown that amino acids promote oocyte maturation and early embryonic development by reducing oxidative stress and enhancing mitochondrial function. However, whether amino acids participate in glucose metabolism to regulate cell energy utilization and what kind of synergistic or antagonistic effects they have in DOR patients need further study.

Steroids are another group of metabolites that are significantly altered in the FF of DOR patients. We found that androsterone sulfate, pregnenolone sulfate, pregnanolone sulfate, and 17-hydroxypregnenolone sulfate were decreased; taurine, glucuronic acid and pregnenol-3-glucuronic acid which are closely related to the excretion of gonadal hormones were also downregulated; while 17beta-Estradiol 3-sulfate (E2-3S) was upregulated. Pregnenolone sulfate, 17-hydroxypregnenolone sulfate and androsterone sulfate are the starting points for the synthesis of progesterone, estrogen and cortisol (34). Progesterone is mainly converted into pregnenol-3-glucuronic acid, and estrogen is mainly excreted in the form of sulfate and glucuronic acid. Our results seemed to indicate that compared with normal women, estrogen and progesterone in the FF of DOR patients are decreased. Yang et al. found that genes regulating cholesterol synthesis and transport, such as SCAP, FDFT1, CYP51A1, SRB1 and STARD1 were significantly downregulated in granulosa cells of DOR patients, and serum estradiol and progesterone were significantly lower in DOR groups (35), which complemented our findings. Dehydroepiandrosterone (DHEA) is mainly derived from 17-hydroxypregnenolone sulfate, and study have proved that DHEA treatment improved the clinical outcome of DOR patients receiving IVF-ET (36). DHEA improves the quality of oocytes by increasing the oxidative phosphorylation of mitochondria and reducing the apoptosis of cumulus cells (37, 38). During oocyte activation, DHEA at the normal threshold could significantly improve the fertilization rate and live birth rate (39). Our study found that the downregulation of 17-hydroxypregnenolone sulfate was related to the number of oocytes retrieved, MII oocytes and high-quality embryos, which also implied that 17-hydroxypregnenolone sulfate and DHEA played an important role in the growth and development of oocytes and embryos. At present, the research on metabolites in the steroid group is not perfect, and the metabolic map obtained may not be complete, so more experiments are needed to further understand how the steroid group affects oocyte and embryo development.

The energy required for oocyte growth and meiosis mainly depends on the oxidative phosphorylation of mitochondria. However, the ability of oocytes to ingest and consume glucose is limited, the pyruvate needed for oxidative phosphorylation is mostly supplied by the glycolysis pathway of cumulus cells and granulosa cells (40), and increasing glycolysis in granulosa cells also promotes the activation of primordial follicles (41). In our study, D-fructose, pantothenic acid and 3-sialyllactose were downregulated, which seemed to point to the decreased energy metabolism of cumulus cells and oocytes in DOR patients. Pantothenic acid is necessary for coenzyme A (CoA), which is a cofactor of many enzymes. CoA participates not only in the metabolism of sugar, fat and protein but also in the antioxidation of the body. This study also detected that ascorbic acid was reduced in FF, and the bioactive form of ascorbic acid is vitamin C, which is considered an antioxidant. The results indicated that the follicular

microenvironment in patients with DOR seemed to be damaged by oxidative stress, thus adversely affecting the growth and maturation of oocytes.

Some fatty acids, such as linoleic acid, eicosapentaenoic ethyl ester, elaidic acid and 12,13-DHOME, were also downregulated, and they were significantly positively correlated with the number of oocytes retrieved, fertilized and high-quality embryos. It has been proved that defects in the synthesis of polyunsaturated fatty acids such as linoleic acid and linolenic acid lead to follicular arrest, oocyte atresia and infertility in female mice (42). Fatty acids are important energy source, mainly providing ATP for oocytes through β-Oxidation, and inhibition of β-Oxidation lead to meiosis arrest of oocytes and development failure after fertilization (43, 44). The decrease in fatty acids leads to DNA damage in granulosa cells and then accelerates cell apoptosis, which further reduces the quality of oocytes and embryos (45, 46). We know that the imbalance of oxidation/antioxidation is an important reason for DOR (47). There is evidence that arachidonic acid metabolism disorder is closely related to the reduction of ovarian reserve (18),and fatty acids and their derivatives participate in the regulation of cellular oxidative stress as antioxidants (48), which may be one of their roles in DOR.

In the FF of DOR patients, we also detected abnormal nucleotide metabolism, including decreased ribothymidine, N6-methyladenosine (m6A) and uric acid (UA). UA is the final product of human purine metabolism, and its physiological level can act as an antioxidant to reduce the damage of oxygen radicals and nitrite to cells (49). PCOS is closely involved in inflammation and oxidative stress, and some patients have obviously increased serum uric acid, which is related to the severity of the disease (50). Although no research has shown the role of UA in DOR, it can also be reasonably speculated that low UA levels in FF may cause increased oxidative stress and mitochondrial dysfunction and thus affect the growth and maturation of oocytes. N6-methyladenosine is one of the most common and abundant RNA modifications; it affects the stability, splicing and/or translation of modified RNA and therefore plays an important role in posttranscriptional regulation (51). A recent study found that in granulosa cells of aging ovaries, there was more m6A involved in the modification of mRNA, which damaged the normal process of mRNA degradation and accelerated the aging of granulosa cells (52). Mu. et al. also confirmed that m6A is indispensable in all stages of mouse follicular development, and oocytes lacking m6A cannot complete meiosis and form oosperms (53). With an increasing number of studies on m6A in granulosa cells and oocytes, m6A may become a new target for studying the pathogenesis and treatment of DOR.

5 Innovation and limitations

In the metabonomic study on the follicular fluid of patients with DOR, we used nontargeted metabonomic technology for the first time, obtained a more informative metabolic profile and established a diagnostic model based on metabolites. Our results provide data support for the study of the pathogenesis of DOR and the search for new diagnostic markers. However, our study still has some limitations. First, the sample size of the experimental groups is less than 30, which is only one small sample study. After expanding the sample, more metabolites with apparent differences may be obtained. Second, after obtaining the differential metabolites, we did not conduct cell experiments or animal experiments for validation or further study. Third, in subsequent research, targeted metabonomic studies could be carried out according to the metabolites obtained, which may be helpful to improve the metabolic pathway of DOR. Finally, our research is limited to metabonomics, and combining our results with genomics, transcriptomics and proteomics will hopefully promote research on the mechanism of ovarian reserve reduction.

Data availability statement

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

Ethics statement

This study is a prospective clinical experiment that has been approved by the Ethics Committee of Wuhan University People's Hospital (WDRY2018-K009), and all subjects signed an informed consent form. The patients/participants provided their written informed consent to participate in this study.

Author contributions

JL, YW and TY conceived the original ideas. JL and YW collected samples and clinical data. JL, ZZ, YW and PZ conducted the metabolomics analysis. ZZ performed the statistical analysis. JL interpreted the data. JL and ZZ cowrote the manuscript. TY, SMC and QW supervised and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Although the author Yan Zhang (YZ) does not appear in the author list of this study, she also provided some suggestions for this study and is gratefully acknowledged here.

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.2023.1132621/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Number of metabolites detected in different modes. HP: HILIC positive ionization mode; HN: HILIC negative ionization mode; RP: RPLC positive ionization mode; RN:RPLC negative ionization mode.

SUPPLEMENTARY FIGURE 2
PCA analysis and evaluation of quality control samples in different modes. a-b: HILIC (+) mode; c-d: HILIC (-); e-f: RPLC (+); g-h: RPLC (-).

SUPPLEMENTARY FIGURE 3
Correlation between differential metabolites.

SUPPLEMENTARY TABLE 1
Down-regulated metabolites in FF samples from DOR groups

SUPPLEMENTARY TABLE 2
Up-regulated metabolites in FF samples from DOR groups

SUPPLEMENTARY TABLE 3
Diagnostic models based on top5, top10, top 20 and total differential metabolites

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Effects of ovarian stimulation protocols on outcomes of assisted reproductive technology in adenomyosis women: a retrospective cohort study

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Objective: To evaluate the effects of different ovarian stimulation protocols on in vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI) outcomes in infertile women with adenomyosis.

Methods: We carried out a retrospective cohort study among infertile women with adenomyosis receiving IVF/ICSI treatment, including 257 fresh embryo transfer (ET) cycles and 305 frozen embryo transfer (FET) cycles. In fresh ET cycles, ultra-long, long, short, and antagonist protocols were adopted. In FET cycles, patients received long-acting GnRH agonist (GnRHa) pretreatment or not. The primary outcome was clinical pregnancy rate (CPR), and the secondary outcomes included implantation rate (IR), miscarriage rate (MR), and live birth rate (LBR).

Results: In fresh ET cycles, compared with ultra-long and long protocols, IR (49.7%, 52.1% versus 28.2%, P=0.001) and CPR (64.3%, 57.4% versus 35.6%, P=0.004) significantly decreased in the short protocol. Similarly, compared with ultra-long and long protocols, a decreased inclination of IR (49.7%, 52.1% versus 33.3%) and CPR (57.4%, 64.3% versus 38.2%) existed in the antagonist protocol, although no statistical significance was detected because of strict P adjustment of Bonferroni method (P_{adj}=0.008). Compared with long protocol, LBR in short protocol decreased obviously (48.2% versus 20.3%, P<0.001). In FET cycles, no matter which origin of embryos, there were no statistical differences in IR, CPR, and LBR. For women ≥35 years receiving fresh ET, CPR was higher in ultra-long and long protocols (52.1%, 50.0% versus 20.0%, 27.5%, P=0.031) compared to antagonist and short protocols. For women ≥35 years receiving

FET, compared with ultra-long and antagonist protocols, cycles with embryos originating from long and short protocols had higher proportions of long-acting GnRHa pretreatment (30.4%,30.00 versus 63.9%, 51.4%, P=0.009). IR (61.1%, 48.6% versus 32.6%, 25.0%, P=0.020) and CPR (58.3%, 48.6% versus 30.4%, 25.0%, P=0.024) in long and short protocols were higher than rates of ultra-long and antagonist protocols, but no statistical differences were supported because of strict Bonferroni method (P_{adj}=0.008).

Conclusion: In infertile women with adenomyosis, if a fresh embryo was planned for transfer, an ultra-long or long protocol might be beneficial. If antagonist and short protocols were used, whole embryos frozen followed by FET was recommended. In FET cycles, embryos derived from different protocols had no impact on pregnancy outcomes.

KEYWORDS

adenomyosis, infertility, *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), pregnancy outcomes

Introduction

As a continuing conundrum, adenomyosis has besieged clinicians for more than one hundred years, which is manifested in the displacement of endometrial glands and stroma in the myometrium (1). Adenomyosis can induce a series of clinical problems, such as heavy menstrual bleeding, chronic pelvic pain, and infertility (2–4). As time passes, the lesions gradually exacerbate, and eventually result in infertility or other severe impacts. A cross-sectional study showed that the incidence of adenomyosis was 20%-29.7% in the infertile population (5). In infertile women receiving assisted reproductive technology (ART), the proportion could rise to 30%-40% (6).

The negative effect of adenomyosis on ART outcomes was accumulated, and persistent endeavors were made to improve the pregnancy outcomes (6–8). So far, the usage of ultra-long protocol was more extensive because of possible improvement in clinical pregnancy rate (CPR) or live birth rate (LBR) (9–12). A widely accepted mechanism was that downregulation induced by long-acting GnRHa could counter hyperestrogenism and progesterone resistance of adeomyosis (13–15). However, the ultra-long protocol had an obvious defect, that was, deep inhibition of ovarian function, which usually resulted in the increase of the duration and dosage of gonadotropin (10). More seriously, for adenomyosis patients with poorer ovarian reserve, the inhibition of long-acting GnRHa could induce poor ovarian response, manifesting in decreased oocyte retrieval and negative pregnancy outcomes. Besides ultra-long protocol, conventional protocols, such as long, antagonist, and short protocols, all could be adopted, however, systematic evaluation of these protocols was absent. In frozen embryo transfer (FET) cycles, if embryos originated from different protocols, did any differences in pregnancy outcomes exist? There were no answers.

So, we designed this study and tried to systematically evaluate the pregnancy outcomes of different protocols in fresh embryo transfer (ET) cycles. Additionally, we also aimed to elucidate whether embryo deriving from different protocols could affect the outcomes of FET cycles.

Materials and methods

Study design and population

Patients with adenomyosis who underwent *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) at the Center for Reproductive Medicine, Shandong University from January 2016 to December 2020 were included in the retrospective cohort study. The follow-up time was up to April 2023. The study has been reviewed and approved by Ethics Committee at the Center for Reproductive Medicine, Shandong University (No. 2021-133). The inclusion criteria were as follows: (i) age below 42 years at the start of the IVF/ICSI cycles; (ii) diagnosis of adenomyosis based on a consensus opinion from the Morphological Uterus Sonographic Assessment (MUSA) group with subjective enlargement of the uterine corpus and heterogeneous myometrium, accompanying with or without asymmetrical thickening, cysts, hyperechoic islands, fan-shaped shadowing, echogenic subendometrial lines and buds, translesional vascularity, irregular junctional zone and interrupted junctional zone or not (16, 17); (iii) no history of uterine malformation, untreated hydrosalpinx or intrauterine lesions. All scans were performed by experienced imaging doctors, who had

over 8 years of experience in gynecological practice. The baseline data of scans were recorded on the electronic medical system.

Controlled ovarian stimulation protocols during IVF/ICSI

Pretreatment

Long acting GnRHa downregulation was an important pretreatment method with duration varing from 1 to 6 cycles according to the response of patients to GnRHa, which was explained in detail in the ultra-long protocol. Surgery and anti-inflammatory drugs were not used as pretreatment.

Ultra-long protocol

to receive the first injection of long-acting GnRHa (Triptorelin Acetate®; Ipsen, France; Leuprorelin Acetate®; Lizhu, China) with a dose of 3.75 mg on day 2 or 3 of the menstrual cycle. The anteroposterior diameter of the uterus was measured 28 days after each injection. If the diameter was more than 70 mm, another injection was repeated until the sixth injection. Twenty-eight days after the last dose, the effect of downregulation was evaluated by transvaginal ultrasonography (TVS) and serum hormone examination. Eligible downregulation was defined as endometrium thickness ≤5mm, serum estradiol ≤ 50pg/mL, LH ≤ 5IU/L, and diameters of follicles <8mm in bilateral ovary without functional cysts.

Long protocol

A daily dose of triptorelin acetate (0.05–0.1mg) of GnRHa (triptorelin acetate®; Ipsen, France) for 14 days. If downregulation criteria were achieved, ovarian stimulation started.

Short protocol

A daily dose (0.05–0.1 mg) of GnRHa (triptorelin acetate®; Ipsen, France) was given on days 2-4 of the menstrual cycle until HCG trigger. After 1-2 days of GnRHa, gonadotropin was used for ovarian stimulation and lasted for about 8-12 days.

Antagonist protocol

A daily dose (0.25mg) of GnRH-ant (Orgalutran®, MSD, Netherlands) was used on the fifth or sixth day of gonadotropin until the trigger day.

Ovarian stimulation

Gonadotropin (Gonal F®, Merck Serono, Switzerland; Lishenbao®, Lizhu, China) at 150–300 IU daily was administered for COS according to age, body mass index (BMI), and ovarian reserve. The adjustment of gonadotropin and addition of recombinant LH (Luveris®, Merck Serono, Germany) were decided by follicular development. Routinely, 8000-10000 IU of urinary human chorionic gonadotropin (HCG) (hCG®; Livzon, China) was used intramuscularly for triggering when at least two follicles measured ≥18mm. If a high risk of ovarian hyperstimulation syndrome (OHSS) existed, 2000-4000 IU of HCG combined with 0.2mg GnRHa was administrated. Oocyte retrieval was performed 34–36h later. The choice of IVF or ICSI depended on sperm quality. Cancellation of fresh ET and whole embryo frozen was carried out in the conditions of high risk of OHSS, hydrosalpinx, or unsynchronized endometrium status. All embryos were cultured for 3 or 5 days with two high-quality cleavage-stage embryos on day 3 or one blastocyst on day 5 transferring into the uterus under the guidance of abdominal ultrasound. The high-quality embryos were defined as 2PN-derived embryos with 7-10 cells and scores ≥3 on day 3 or ≥4BC on day 5 (18). Oral dydrogesterone tablet (Duphaston®, Abbott, Netherlands) 10 mg twice daily and vaginal progesterone gel (Crinone gel®, Merck Serono, Switzerland) 90 mg once daily or oral dydrogesterone tablet (Duphaston®, Abbott, Netherlands) 20 mg twice daily and vaginal progesterone soft capsules (Utrogestan®, Besins, Belgium) 200 mg once daily were administered as luteal phase support.

Protocols of endometrial preparation in FET cycles

GnRHa pretreatment before FET: GnRHa pretreatment was performed in patients with severer adenomyosis before FET for ≥1 month with 3.75 mg GnRHa per month. The uterine anteroposterior diameter was measured 28 days after each injection. If it was more than 70 mm, injection of the same dose of GnRHa was repeated until the sixth injection. Hormone replacement cycles would be administered 4 weeks after the last dose of GnRHa.

Oral estradiol valerate (Progynova®; Bayer, Germany) was administrated in a dose-escalating method, 4mg/day for the first 5 days and subsequently 6mg/day for the second 5 days. According to the assessment of endometrial thickness and serum hormone levels of E_2, 8mg/day for another 3-4 days was continued or not. When endometrial thickness ≥7 mm, progesterone addition was started. One frozen-thawed blastocyst was transferred into the uterus 5 days after progesterone addition. Other protocols for endometrial preparation in FET cycles had been described in previous studies (19).

Observational parameters and outcome variables

Baseline parameters included age, BMI, duration of infertility, primary infertility, antral follicle count (AFC), basal follicle-stimulating hormone (FSH), anti-Müllerian hormone (AMH), the mean diameter of the initial uterus, history of dysmenorrhea, and IVF-related parameters. The mean diameter of the uterus was calculated as the average of long and wide diameter in the longitudinal section measured by TVS on days 2-6 of the menstrual cycle. According to the verbal multidimensional scoring system (20), dysmenorrhea was classified into none, mild,

moderate, and severe. The primary pregnancy outcome was CPR, and the secondary pregnancy outcomes included implantation rate (IR), biochemical pregnancy rate (BPR), ectopic pregnancy rate (EPR), miscarriage rate (MR), early MR, late MR, and LBR, cumulative live birth rate (CLBR). Serum β-hCG level was examined 14 days after ET. Biochemical pregnancy as defined as elevated serum β-hCG level ≥10mIU/mL. Clinical pregnancy was defined as the presence of intra-uterine pregnancy or visible extra-uterine pregnancy. Ectopic pregnancy was defined as the presence of a gestational sac or mass outside the uterine cavity. Miscarriage was defined as pregnancy loss before 28 gestational weeks. Clinical pregnancy loss ≤12 gestational weeks was an early miscarriage, vice versa, pregnancy loss >12 gestational weeks was a late miscarriage. IR was defined as the ratio of the total number of gestational sacs confirmed by TVS to the total number of transferred embryos. CLBR was defined as the number of cycles in which patients had the first live birth/number of all transfer cycles per oocyte retrieval, including all fresh and frozen ET.

Statistical analysis

All statistical analysis was conducted by SPSS 25.0 (SPSS, Chicago, USA). The normality of continuous variables was assessed by the Shapiro-Wilk test. If conforming to normality distribution, mean ± SD and analysis of variance (ANOVA) were adopted for variables description and intra-group comparisons. Vice versa, if the non-normality distribution was ascertained, the median (25th-75th percentile) and Kruskal-Wallis test were used for variables description and intragroup comparisons. Frequencies (percentages) were utilized to describe categorical variables. The comparisons of categorical variables were evaluated by χ (2) test or Fisher's exact test. Among the pairwise comparisons of four subgroups, the Bonferroni method was used to adjust p-values. In order to account for the differences among subgroups, parameters, such as age, BMI, duration of infertility, types of infertility, AFC, AMH, FSH, initial uterine diameter, and dysmenorrhea history, were included in the logistic regression model. P<0.05 was considered as statistical significance.

Results

A total of 562 cycles were included with 257 cycles of fresh ET and 305 cycles of FET. In fresh ET cycles, cycles of ultra-long, long, antagonist, and short protocols were 108, 56, 34, and 59, respectively. In FET cycles, cycles of FET with embryos originating from ultra-long, long, antagonist, and short protocols were 98, 101, 54, and 52.

Pregnancy outcomes of adenomyosis in fresh ET cycles

In fresh ET cycles, except for BMI, there were significant differences in age, duration of infertility, primary infertility, AFC, FSH, AMH, mean initial diameter of the uterus, and dysmenorrhea in intragroup comparisons. Compared with ultra-long, long, and antagonist protocols, the short protocol had older age (34.00, 32.50, 33.50 versus 37.00, P<0.001), poorer AMH (1.65, 2.75, 1.32 versus 1.30, P<0.001), and lower AFC (11.00, 13.00, 10.00versus 9.0, P<0.001). The proportion of severe dysmenorrhea in ultra-long protocol was highest compared with long, antagonist, and short protocols (37.96% versus 17.86%, 11.76%, 13.56%, P<0.001). The data was shown in Table 1.

COS-related parameters and pregnancy outcomes were shown in Table 2. Compared with the long, antagonist, and short protocols, the duration of gonadotropin (9.00, 9.00, 9.00 versus 11.00, P<0.001) and the dosage of gonadotropin (1800.00, 2175.00, 1975.00 versus 2775.00, P<0.001) increased clearly in the ultra-long protocol. Compared with ultra-long, long and antagonist protocols, endometrial thickness on HCG trigger day was thinnest (1.1, 1.1, 1.0versus 0.9, P=0.002), and the number of retrieved oocytes was lowest (7.00, 10.00, 6.00 versus 4.00, P<0.001) in the short protocol. However, no statistical significance existed among intragroup comparisons of ultra-long, long, and antagonist protocols. For high-quality embryos on day 3, long protocol had more good embryos compared with ultra-long, antagonist, and short protocols (4.00 versus 2.00, 2.00, 2.00, P<0.001).

The parameters of pregnancy outcomes included IR, BPR, EPR, CPR, MR, early MR and late MR, LBR, and CLBR. Compared with ultra-long and long protocols, IR (49.7%, 52.1% versus 28.2%, P=0.001) and CPR (64.3%, 57.4% versus 35.6%, P=0.004) in short protocol significantly decreased. Compared with ultra-long and long protocols, the decrease of IR (49.7%, 52.1% versus 33.3%) and CPR (57.4%, 64.3% versus 38.2%) also existed in antagonist protocol, although no statistical significance was detected because of strict P adjustment of Bonferroni method (P_{adj}=0.008). Compared with the long protocol, LBR in the short protocol decreased obviously (48.2% versus 20.3%, P<0.001) and LBR in the antagonist protocol was also pessimistic (48.2% versus 26.5%), although no statistical significance. There were no significant differences in intragroup comparisons of BPR, MR, early MR, and late MR.

Considering the differences in baseline parameters among the four protocols, we carried out CPR and LBR-associated multinomial logistics regression analysis, which was shown in Supplementary Tables 1, 2. When CPR was set as the outcome variable, we observed that long protocol was a protective factor compared with short protocol (OR 2.414, 95% CI 1.011-1.144, P=0.047). AFC was also a protective factor (OR 1.073, 95% CI 1.007-1.144, P=0.030), and the main risk factor was age (OR 0.87, 95% CI 0.809-0.936, P<0.001). When LBR was set as the outcome variable, ultra-long (OR 2.47, 95% CI 1.005-6.07, P=0.049) and long protocol (OR 2.786, 95% CI 1.055-7.353, P=0.039) were both protective factors compared with short protocol. Secondary infertility (OR 2.088, 95% CI 1.018-4.283, P=0.045) and AFC (OR 1.069, 95% CI 1.001-1.142, P=0.047) were also protective factors. Age (OR 0.809, 95% CI 0.743-0.882, P<0.001) was still a risk factor.

Pregnancy outcomes of adenomyosis in FET cycles

In FET cycles, baseline data of frozen embryo transferred was shown in Table 3. Compared with ultra-long, long, and antagonist

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TABLE 1 Baseline characteristics of different COS protocols in fresh ET cycles.

	Ultra-long protocol	Long protocol	Antagonist protocol	Short protocol	P value
No. of cycles	108	56	34	59	
Age, years	34.00 (31.00,37.00)[a]	32.50 (30.00,36.00)[b]	33.50 (32.00,41.00)	37.00 (33.00,40.00)[ab]	0.001
BMI, kg/m2	24.40 (22.09,26.84)	23.37 (21.37,26.63)	25.70 (23.02,27.03)	23.95 (21.76,26.26)	0.249
Duration of infertility, years	3.00 (2.00,5.00)	3.00 (2.00,5.00)	3.50 (2.50,5.50)[d]	2.00 (1.00,3.75)[d]	0.007
Primary infertility, n(%)	40 (37.04)[a]	23 (41.07)[b]	9 (26.47)	6 (10.17)[ab]	<0.001
AFC	11.00 (7.00,14.00)[ac]	13.00 (10.00,16.00)[bce]	10.00 (6.00,14.00)[e]	9.00 (5.00,11.00)[ab]	<0.001
Basal FSH, IU/L	6.45 (5.30,7.78)[a]	6.50 (5.88,7.23)	6.76 (5.60,8.97)	7.60 (6.32,8.87)[a]	0.015
AMH, ng/ml	1.65 (1.06,3.03)[ac]	2.75 (1.66,3.90)[bc]	1.32 (0.92,3.37)	1.30 (0.77,1.90)[ab]	<0.001
Mean initial diameter of uterus, cm	6.22 (5.64,7.30)[ac]	5.43 (4.65,6.35)[c]	6.05 (4.81,6.85)	5.45 (4.72,6.03)[a]	<0.001
History of dysmenorrhea					<0.001
None, n(%)	11 (10.19)	19 (33.93)	16 (47.06)	22 (37.29)	
Mild, n(%)	30 (27.78)	19 (33.93)	8 (23.53)	18 (30.51)	
Moderate, n(%)	26 (24.07)	8 (14.29)	6 (17.65)	11 (18.64)	
Severe, n(%)	41 (37.96)[af]	10 (17.86)	4 (11.76)[f]	8 (13.56)[a]	

Data were presented as median (25th-75th percentile) for non-normality distribution variables and frequencies (percentages) for categorical variables.
COS, controlled ovarian stimulation; BMI, body mass index; AFC, antral follicle count; FSH, follicle stimulating hormone; AMH, anti-müllerian hormone; ET, embryo transfer.
[a]ultra-long vs short; [b]long vs short; [c]ultra-long vs long; [d]antagonist vs short; [e]long vs antagonist; [f]ultra-long vs antagonist.

TABLE 2 Outcomes of ultra-long, long, antagonist, and short protocols in fresh ET cycles.

	Ultra-long protocol	Long protocol	Antagonist protocol	Short protocol	P_{adjust}
No. of cycles	108	56	34	59	
Total dosage of Gn, IU	2775.00 (2250.00,3993.75)[acf]	1800.00 (1500.00,2325.00)[c]	2175.00 (1656.25,2728.12)[f]	1975.00 (1425.00,2700.00)[a]	<0.001
Duration of Gn stimulation, days	11.00 (10.00,12.25)[acf]	9.00 (9.00,11.00)[c]	9.00 (8.00,10.00)[f]	9.00 (7.50,10.00)[a]	<0.001
LH on HCG trigger day, IU/L	1.04 (0.56,1.59)[acf]	2.30 (1.43,3.05)[bc]	2.51 (1.80,5.54)[df]	5.24 (3.80,7.04)[abd]	<0.001
E_2 on HCG trigger day, pg/ml	2061.50 (1407.75,2884.25)	2650.50 (1708.00,3468.25)[be]	1653.50 (1081.00,2172.25)[e]	1619.00 (1294.50,2575.50)[b]	<0.001
P on HCG trigger day, ng/ml	0.66 (0.46,0.84)	0.58 (0.44,0.94)	0.46 (0.35,0.80)	0.65 (0.39,0.81)	0.549
Endometrial thickness on HCG trigger day, cm	1.10 (0.90,1.20)[a]	1.10 (0.90,1.25)[b]	1.00 (0.80,1.10)	0.90 (0.80,1.10)[ab]	0.002
No. of oocytes retrieved	7.00 (4.75,12.00)[a]	10.00 (6.00,12.00)[b]	6.00 (3.00,9.75)	4.00 (3.00,8.00)[ab]	<0.001
No. of 2PN zygotes retrieved	5.00 (3.00,7.25)[a]	6.00 (4.00,9.00)[b]	4.00 (2.25,6.50)	3.00 (2.00,5.00)[ab]	<0.001
No. of high-quality embryos retrieved on Day 3	2.00 (1.00,4.00)[c]	4.00 (2.00,6.00)[bc]	2.00 (2.00,4.00)	2.00 (1.00,3.00)[b]	<0.001
Pregnancy outcomes, %(n/N)					
IR	49.7 (85/171)[a]	52.1 (49/94)[b]	33.3 (18/54)	28.2 (24/85)[ab]	0.001
BPR	10.2 (11/108)	10.7 (6/56)	11.8 (4/34)	8.5 (5/59)	0.960
EPR	0.01 (1/108)	–	–	–	–
CPR	57.4 (62/108)[a]	64.3 (36/56)[b]	38.2 (13/34)	35.6 (21/59)[ab]	0.004
MR	30.6 (19/62)	25.0 (9/36)	30.8 (4/13)	42.9 (9/21)	0.575

(Continued)

TABLE 2 Continued

	Ultra-long protocol	Long protocol	Antagonist protocol	Short protocol	P_{adjust}
Early MR	16.1 (10/62)	22.2 (8/36)	23.1 (3/13)	38.1 (8/21)	0.230[g]
Late MR	14.5 (9/62)	2.8 (1/36)	7.7 (1/13)	4.8 (1/21)	0.241[g]
LBR	39.8 (43/108)	48.2 (27/56)[b]	26.5 (9/34)	20.3 (12/59)[b]	0.007

Data were presented as median (25th-75th percentile) for non-normality distribution variables. Gn, gonadotropin; LH, luteinizing hormone; E2, estradiol; P, progesterone; HCG, human chorionic gonadotropin IR, implantation rate; BPR, biochemical pregnancy rate; EPR, ectopic pregnancy rate; CPR, clinical pregnancy rate; MR: miscarriage rate; LBR, live birth rate.
[a]ultra-long vs short; [b]long vs short; [c]ultra-long vs long; [d]antagonist vs short; [e]long vs antagonist; [f]ultra-long vs antagonist; [g]Fisher's exact test.

TABLE 3 Baseline characteristics of frozen embryo originating COS cycles.

	Ultra-long protocol	Long protocol	Antagonist protocol	Short group	P value
No. of cycles	98	101	54	52	
Age, years	33.00 (30.00,37.00)[a]	31.00 (30.00,35.00)[b]	33.00 (30.25,36.00)[d]	37.00 (34.00,40.00)[abd]	<0.001
BMI, kg/m2	23.14 (20.70,26.33)[a]	23.28 (21.13,27.18)	24.69 (23.01,26.17)	25.33 (23.06,26.70)[a]	0.016
Duration of infertility, years	3.00 (2.00,4.50)	3.50 (1.50,4.00)	3.50 (2.50,5.50)	2.50 (2.00,4.00)	0.084
Primary infertility, n(%)	51 (52.04)[a]	50 (49.5)[b]	25 (46.3)[d]	7 (13.46)[abd]	<0.001
AFC	13.50 (10.00,19.75)[a]	15.00 (11.00,20.00)[b]	14.00 (9.00,28.5)[d]	9.00 (5.75,12.00)[abd]	<0.001
Basal FSH, IU/L	6.08 (5.06,7.57)	6.16 (5.61,7.03)[b]	6.31 (5.92,7.71)	6.87 (5.57,8.35)[b]	0.013
AMH, ng/ml	2.84 (1.57,5.90)[a]	4.17 (2.78,5.54)[b]	4.09 (1.73,6.90)[d]	1.35 (0.84,2.17)[abd]	<0.001
Mean diameter of initial uterus, cm	6.25 (5.50,7.30)[c]	5.70 (4.70,6.75)[c]	5.80 (4.76,7.05)	5.80 (4.94,6.61)	0.010
History of dysmenorrhea					<0.001
None, n(%)	15 (15.31)	21 (20.79)	15 (27.78)	15 (28.85)	
Mild, n(%)	21 (21.43)	43 (42.57)	13 (24.07)	17 (32.69)	
Moderate, n(%)	26 (26.53)	13 (12.87)	22 (40.74)	9 (17.31)	
Severe, n(%)	36 (36.73)[f]	24 (23.76)	4 (7.41)[f]	11 (21.15)	
long-acting GnRHa pretreatment before FET					0.004
yes	62(63.26%)[c]	39(38.61%)[c]	27(50.00%)	22(42.31%)	
no	36(36.74%)[c]	62(61.39%)[c]	27(50.00%)	30(57.7%)	

Data were presented as median (25th-75th percentile) for non-normality distribution variables and frequencies (percentages) for categorical variables.
BMI, body mass index; AFC, antral follicle count; FSH, follicle-stimulating hormone; AMH, anti-müllerian hormone; COS, controlled ovarian stimulation; FET, frozen embryo transplant.
[a]ultra-long vs short; [b]long vs short; [c]ultra-long vs long; [d]antagonist vs short; [e]long vs antagonist; [f]ultra-long vs antagonist.

protocols, the short protocol had older age (33.00, 31.00, 33.00 versus 37.00, P<0.001) and lower AMH (2.84, 4.17, and 4.09 versus 1.35, P<0.001). Age, BMI, duration of infertility, primary infertility, AFC, FSH, and AMH had no statistical difference among ultra-long, long and antagonist protocols. The related parameters of frozen-embryo-originating COS protocols and pregnancy outcomes were shown in Table 4. Compared with the long, antagonist, and short protocols, the dosage of gonadotropin (2025.00, 1875.00, and 2025.00 versus 2512.50, P<0.001) and the stimulation duration of gonadotropin (10.00, 9.00, 9.00 versus 11.00, P<0.001) significantly increased in the ultra-long protocol. Ultra-long and long protocols had higher E2 levels on HCG trigger day compared with the short protocol (3117.00, 3000.00 versus 2131.50, P<0.001). The number of retrieved oocytes (6.5 versus 12.00, 12.00, 11.00, P<0.001) and 2PN zygotes (4.00 versus 7.00, 8.00, 6.50, P<0.001) in short protocol were significantly lower than those in ultra-long, long and antagonist protocols. Absolutely different from fresh ET cycles, no statistical differences were detected among ultra-long, long, antagonist and short protocols on IR (45.5%, 47.2%, 41.8%, 50.0%, P=0.851), BPR (12.2%, 6.9%, 5.6%,13.5%, P=0.313), CPR (43.9%, 49.5%, 40.7%, 50.0%, P=0.658), MR (41.9%, 32.0%, 45.5%, 38.5%, P=0.791), early MR (27.9%, 32.0%, 36.4%, 30.8%, P=0.917), late MR (14.0, -, 9.1%, 7.7%, P=0.12), and LBR (25.5%, 33.7%, 22.2%, 30.8%, P=0.403). The CLBR in the long protocol was significantly higher than the corresponding rate in ultra-long, antagonist, and short protocols (68.2% versus 46.2%, 36.2%, 35.9%, p<0.001). We also noticed that in the ultra-long protocol, even if women had severer dysmenorrhea and larger uterus, the

TABLE 4 Outcomes of FET and characteristics of frozen embryo originating COS cycles.

	Ultra-long protocol	Long protocol	Antagonist protocol	Short protocol	P_{adjust}
No. of cycles	98	101	54	52	
Total dosage of Gn, IU	2512.50 (2025.00,3862.50)[acf]	2025.00 (1500.00,2775.00)[c]	1875.00 (1418.75,2531.25)[f]	2025.00 (1556.25,2625.00)[a]	<0.001
Duration of Gn stimulation, days	11.00 (10.00,12.75)[acf]	10.00 (9.00,12.00)[c]	9.00 (9.00,11.00)[f]	9.00 (8.00,10.25)[a]	<0.001
LH on HCG trigger day, IU/L	1.00 (0.57,1.40)[acf]	1.74 (1.15,2.61)[bce]	3.28 (1.98,4.92)[ef]	4.79 (3.40,6.50)[ab]	<0.001
E_2 on HCG trigger day, pg/ml	3117.00 (2445.00,4806.75)[a]	3000.00 (2373.00,4300.00)[b]	2158.00 (1741.00,4566.25)	2131.50 (1597.75,3408.25)[ab]	<0.001
P on HCG trigger day, ng/ml	0.75 (0.53,0.99)	0.74 (0.50,1.08)	0.83 (0.43,1.18)	0.71 (0.43,0.91)	0.542
Endometrial thickness on HCG trigger day, cm	0.90 (0.80,1.00)	0.90 (0.80,1.00)	0.90 (0.80,1.00)	0.88 (0.80,1.00)	0.676
No. of oocytes retrieved	12.00 (9.00,17.75)[a]	12.00 (10.00,16.00)[b]	11.00 (5.25,15.00)[d]	6.50 (4.00,8.25)[abd]	<0.001
No. of 2PN zygotes retrieved	7.00 (5.00,12.00)[a]	8.00 (6.00,10.00)[b]	6.50 (4.00,10.00)[d]	4.00 (3.00,6.00)[abd]	<0.001
No. of high-quality embryos retrieved on Day 3	4.00 (2.00,6.00)	5.00 (3.00,8.00)[b]	3.00 (1.25,6.00)	3.00 (2.00,4.00)[b]	<0.001
Pregnancy outcomes, %(n/N)					
IR	45.5 (45/99)	47.2 (51/108)	41.8 (23/55)	50.0 (26/52)	0.851
BPR	56.1(55/98)	56.4 (57/101)	46.3 (25/54)	63.5 (33/52)	0.313
EPR	–	–	–	0.02 (1/52)	–
CPR	43.9 (43/98)	49.5 (50/101)	40.7 (22/54)	50.0 (26/52)	0.658
MR	41.9 (18/43)	32.0 (16/50)	45.5 (10/22)	38.5 (10/26)	0.791
Early MR	27.9 (12/43)	32.0 (16/50)	36.4 (8/22)	30.8 (8/26)	0.917
Late MR	14.0 (6/43)	–	9.1 (2/22)	7.7 (2/26)	0.120[g]
LBR	25.5 (25/98)	33.7 (34/101)	22.2 (12/54)	30.8 (16/52)	0.403
CLBR	46.2(67/145)[c]	68.2(60/88)[bce]	36.2(21/58)[e]	35.9(21/58)[b]	<0.001
$CLBR^{hypo}$	59.8(116/194)[c]	76.5(91/119)[bce]	50.7(38/75)[e]	49(48/98)[b]	<0.001

Data were presented as median (25th-75th percentile) for non-normality distribution variables. Gn, gonadotropin; LH, luteinizing hormone; E2, estradiol; P, progesterone; HCG, human chorionic gonadotropin IR, implantation rate; BPR, biochemical pregnancy rate; EPR, ectopic pregnancy rate; CPR, clinical pregnancy rate; MR, miscarriage rate; LBR, live birth rate; CLBR, cumulative live birth rate, was calculated based on current finished embryo transfer cycles. $CLBR^{hypo}$ was calculated by hypothesizing that all surplus embryos were transferred and live birth was achieved. [a]ultra-long vs short; [b]long vs short; [c]ultra-long vs long; [d]antagonist vs short; [e]long vs antagonist; [f]ultra-long vs antagonist; [g]Fisher's exact test.

CLBR was higher than those in antagonist and short protocol (46.2% versus 36.2%, 35.9%), although no statistical differences existed because of strict P adjustment of Bonferroni method (P_{adj}=0.008). So far, 117 blastocytes were still frozen, and we calculated the highest rate of CLBR by hypothesizing that live birth can be achieved by surplus embryo transfer. The real CLBR will fluctuate between the current data and the hypothesized data.

Pregnancy outcomes of adenomyosis with age ≥35 years in fresh ET and FET cycles

Considering the possible selection bias of different protocols, we carried out an analysis among women≥35 years. In fresh ET cycles, cycles of ultra-long, long, antagonist, and short protocols were 48, 20, 15, and 40, respectively. There were no significant differences in BMI, basal FSH, AMH, and mean diameter of the initial uterus (Supplementary Table 3).

Antagonist protocol had significantly older age compared with ultra-long, long, and short protocols (42.00 versus 37.00, 38.00, and 39.00, P=0.002). The proportion of no dysmenorrhea in short protocol was significantly higher than in ultra-long protocols (45.0% versus 10.40%, P=0.010). Compared with antagonist and short protocols, ultra-long and long protocols had more number of oocytes (3.00, 4.00 versus 6.00, 8.00, P=0.02, P=0.011) and 2PN zygotes (3.00, 2.00 versus 4.50, 5.00, P=0.012). However, there was no statistical difference in high-quality embryos on day 3. As to the pregnancy outcomes, ultra-long and long protocols had higher CPR (52.1%, 50.00% versus 20.0%, 27.5%, P=0.031). Although there were no significant differences on IR (P=0.183), BPR (P=0.87), MR (P=0.417), and LBR (P=0.071), LBR in ultra-long and long protocols was higher than the corresponding rates in antagonist and short protocols (27.1%, 30.0% versus 6.7%, 10.0%). The data was shown in Supplementary Table 4).

In FET cycles, the FET cycles with embryos originating from ultra-long, long, antagonist, and short protocols were 46, 36, 20, and

37, respectively. The data was shown in Supplementary Tables 5, 6. No significant differences existed in age between the four groups (P=0.331). Except for BMI, there were no statistical differences in the duration of infertility, the proportion of primary infertility, basal FSH, AMH, initial mean diameter of the uterus, the duration and dosage of gonadotropin, oocyte retrieved, number of 2PN zygotes and the final number of high-quality embryos retrieved on Day 3. The proportion of long-acting GnRHa pretreatment before FET in the long and short protocols groups was higher than in the ultra-long and antagonist groups (63.9%, 51.4% versus 30.4%, 30.00%, P=0.009). IR (61.1%, 48.6% versus 32.6%, 25.0%, P=0.020) and CPR (58.3%, 48.6% versus 30.4%, 25.0%, P=0.024) in long and short protocols were higher than rates of ultra-long and antagonist protocols, but no statistical differences were supported because of strict Bonferroni method (Padj=0.008). No significant differences in BPR (P=0.338), MR (P=0.634), and LBR (P=0.078) were detected. The CLBR in long protocol was significantly higher than those in ultra-long, antagonist, and short protocols (68.8% versus 34.4%, 16%, 26.4%, P<0.001). In the ultra-long protocol, an even higher proportion of dysmenorrhea and larger uterus existed, and the CLBR was better compared with the antagonist and short protocols (34.4% versus 16%,26.4%), although no statistical differences existed because of strict P adjustment of Bonferroni method (P_{adj}=0.008).

We carried out multinomial logistic regression analysis and CPR was set as the outcome variable. In fresh ET cycles, older age was an important risk factor (OR 0.787, 95% CI 0.658-0.941, P=0.008) and no protective factors were observed (Supplementary Table 7). In FET cycles, AFC (OR 0.903, 95% CI 0.821-0.994, P=0.037) and secondary infertility (OR 0.200, 95% CI 0.058-0.690, P=0.011) were observed as protective factors in FET cycles, which were shown in Supplementary Table 8.

Discussion

Ultra-long protocol could mediate hypo-estrogen status caused by pituitary downregulation and produce positive effects on pregnancy outcomes (21). The long protocol also could mediate pituitary downregulation with weaker function. Because of endogenous inhibition of GnRHa, the duration and dosage of gonadotropin significantly increased in the ultra-long protocol. There were no statistical differences in the number of retrieved oocytes and 2PN zygotes in the intragroup comparison of ultra-long and long protocols. In our study, the ultra-long protocol had a higher proportion of severe adenomyosis, however, after long-acting GnRHa pretreatment, IR, CPR, MR, and LBR in ultra-long and long protocols were similar. Hou X's study analyzed 362 ultra-long protocol cycles and 127 long protocol cycles in fresh ET cycles among adenomyosis patients, and the dosage and duration of gonadotropin in ultra-long protocol also significantly increased. Compared with the long protocol, CPR and LBR in the ultra-long protocol were higher (10). Lan J's study ascertained that the ultra-long protocol improved pregnancy outcomes in women with adenomyosis, especially in women with diffuse adenomyosis (12). All in all, the ultra-long protocol had its advantages for the improvement of pregnancy outcomes for severer adenomyosis, although time and economic cost was increased.

Antagonists could produce a rapid suppression of pituitary function, which could decrease the dosage of gonadotropin and the risk of ovarian hyperstimulation (22). Compared with ultra-long and long protocols, AFC in antagonist protocol was lower, however, the number of retrieved oocytes and 2PN zygotes had no statistical significance. Compare with ultra-long and long protocols, IR, CPR, and LBR in antagonist protocol were poorer, however, strict P adjustment of the Bonferroni method (P_{adj}=0.008) in pairwise under multiple comparisons made the statistical difference difficult to achieve. The BPR, MR, early MR, and late MR were similar among ultra-long, long, and antagonist protocols. Thalluri V. reported CPR in antagonist protocol was 23.6% in patients with adenomyosis, which was similar to our data and significantly lower than the reported CPR in ultra-long or long protocols (9–11, 23). Kolanska K compared pregnancy outcomes of GnRH-agonist versus GnRH-antagonist protocols in women with endometriosis-associated infertility and inferred GnRH-antagonist associated dysfunction of endometrial receptivity might result in decreased CPR and LBR (24).

The advantages of a short protocol could promote the secretion of FSH by flare-up effect, strengthen recruitment of early follicles and improve the response of the ovaries. Compared with ultra-long and long protocols, CPR and LBR in the short protocol were significantly decreased. Vice versa, compared with antagonist protocol, there were no statistical differences in IR, CPR, MR, and LBR. Sheng compared pregnancy outcomes of ultra-long, long, and short protocols, and CPR in the short protocol was 22.4% (25), which was similar to our data. Khan KN et al. reported short and antagonist protocols could not induce hypo-estrogenism and recover potential endometrial normalization of adenomyosis (14, 26), so when they were used, fresh ET should be prudent because of the obviously negative pregnancy outcomes.

In FET cycles, except for larger uterine diameter and a higher proportion of severe dysmenorrhea in ultra-long protocols, other baseline data of embryos originating COS cycles were similar in ultra-long, long and antagonist protocols. However, baseline differences between short and other protocols still had statistical differences, such as age, AFC, and AMH. What we should pay attention to is that no matter which was the origin of the embryo, IR, CPR, MR, and LBR had no statistical difference in FET cycles. So far, there were no other similar comparisons. As to CLBR, because patients still had frozen embryos, we calculated fluctuation ranges by hypothesizing successful live birth with embryo transfer in the future. Compared with other protocols, current data showed that long protocol had its advantages on CLBR. The baseline parameters between long and antagonist were matched, however, the CLBR was poor in antagonist protocol, therefore, antagonist protocol should be cautiously adapted. For severer adenomyosis with good ovarian reserve, the ultra-long protocol was worth considering, while more data was needed to verify a better choice between ultra-long and long protocol.

Considering the possible choosing bias, we analyzed the usage of different protocols and pregnancy outcomes among women ≥35 years. In fresh ET cycles, the antagonist protocol had older age than

the ultra-long protocol, while no difference in age existed in other intragroup comparisons. AMH and the final number of high-quality embryos had no statistical significance. Compared with the antagonist and short protocols, CPR in ultra-long and long protocols was higher. LBR in antagonist and short protocols were lower, although no statistical difference existed. In FET cycles, embryos from long and short protocols had a higher proportion of long-acting GnRHa pretreatment, which IR and CPR increased, indicating the possible benefit of long-acting GnRHa.

Strengths and limitations

This was the first study to make direct comparisons of different COS protocols on IVF/ICSI outcomes.Our study indicated that an ultra-long protocol might be a better choice for severer adenomyosis in fresh ET cycles.We observed that the pregnancy outcomes of antagonist and short protocols were poor in fresh ET cycles, however, the outcomes could be reversed by whole embryos being frozen and a FET strategy. The principal limitation of the study was the possible bias of a retrospective cohort study, therefore, we developed strict inclusion and exclusion criteria to minimize the bias. Prospective studies are needed to verify the conclusions.

Conclusion

If fresh ET was decided upon, an ultra-long or long protocol might be appropriate. If antagonist and short protocols were used, whole embryo frozen combed with FET is recommended. In FET cycles, embryo origin had no impact on pregnancy outcomes.

Data availability statement

Data will be made available to the editors of the journal for review or query upon request.

Ethics statement

The studies involving humans were approved by Ethics Committee at the Center for Reproductive Medicine, Shandong University (No. 2021-133). 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

LG and YL analyzed data and drafted the article. SG collected and analyzed data. LC and Z-JC designed, revised, and approved the manuscript.

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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.2023.1198779/full#supplementary-material

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Association between female circulating heavy metal concentration and abortion: a systematic review and meta-analysis

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Objective: This study aimed to evaluate the association between blood heavy metal (zinc (Zn), copper (Cu), lead (Pb), and cadmium (Cd)) concentrations and spontaneous abortion (SA) and recurrent pregnancy loss (RPL) and explore the possible endocrine dysfunction associated with it.

Methods: A literature search was performed in the PubMed, Embase, Cochrane Library, and Web of Science databases up to April 2023. The overall effects were expressed as the standard mean difference (SMD). Subgroup analysis was performed according to the type of abortion (SA or RPL). Stata 16.0 was utilized for data analysis.

Results: Based on the integrated findings, abortion women showed significantly lower Zn (SMD = −1.05, 95% CI: −1.74 to −0.36, $p = 0.003$) and Cu concentrations (SMD = −1.42, 95% CI: −1.97 to −0.87, $p < 0.001$) and higher Pb (SMD = 1.47, 95% CI: 0.89−2.05, $p < 0.001$) and Cd concentrations (SMD = 1.15, 95% CI: 0.45−1.85, $p = 0.001$) than normal pregnant women. Subgroup analysis showed that Zn and Cu deficiency and Cd and Pb exposure were significantly ($p < 0.05$) associated with RPL, whereas Cu deficiency and Cd and Pb exposure were significantly ($p < 0.05$) associated with SA.

Conclusion: Zn and Cu deficiencies and Pb and Cd exposure were associated with abortion. Endocrine dysfunction, such as insulin resistance, vitamin D insufficiency, and abnormal thyroid and sex hormone concentrations, is thought to be involved in heavy metal-related abortion.

KEYWORDS

recurrent pregnancy loss, spontaneous abortion, endocrine dysfunction, zinc, copper, lead, cadmium

1 Introduction

Spontaneous abortion (SA) is a serious reproductive health problem with various definitions. According to the World Health Organization, SA is defined as the involuntary loss of a fetus weighing ≤500 g before the 20th gestational week (GW) (1), whereas the Chinese Medical Association Obstetrics and Gynecology Branch defines it as the involuntary loss of a fetus weighing ≤1,000 g before the 28th GW (2). SA occurs in 10%–15% of pregnancies, and approximately 80% of SA occurs before 12 weeks of pregnancy, which is known as early pregnancy loss (3). Recurrent pregnancy loss (RPL) is a special form of SA that affects 1.4% of women and causes physical and emotional challenges (4). However, the definition of RPL has been inconsistent. The European Society for Human Reproduction and Embryology (ESHRE) defines it as two or more abortions, irrespective of whether they are consecutive (5), while the American Society for Reproductive Medicine defines it as the loss of two or more consecutive pregnancies (6). There is controversy about the quantity and consecutiveness of abortions (7). The etiologies of SA and RPL, including chromosomal abnormalities, uterine malformations, and endocrine dysfunction, are complex (8–10). Exposure to environmental pollutants is also a risk factor for SA and RPL. Most pollutants are endocrine disrupters and early embryonic development is extremely sensitive to them (11, 12).

Heavy metals are among the most harmful environmental contaminants because they are not biologically degradable and can accumulate in organisms along the food chain (13). Heavy metals are mainly absorbed through air, drinking water, and contaminated food (14). They can be classified as essential (e.g., copper [Cu], zinc [Zn]) and non-essential (such as lead [Pb] and cadmium [Cd]). Essential metals play important roles in metabolism, enzymatic synthesis, and signal transduction, and their deficiency or overexposure may affect normal physiological functions of organisms (14). For instance, Zn and Cu are important components of several proteins, including antioxidant enzymes, metalloenzymes, and coenzymes, which are essential for fetal growth. Maternal Zn and Cu deficiency can reduce the fetal Zn and Cu supply through the placenta and cause fetal loss and pregnancy complications (15–17). Non-essential metals are usually toxic to humans, especially to human reproductive health, even at very low concentrations. Among all nonessential metals, Cd and Pb are endocrine-disrupting metals that can interfere with the production and secretion of sex hormones, leading to poor pregnancy outcomes (18).

Several previous studies have investigated the associations between the concentrations of Cd, Pb, Zn, and Cu in the blood and the risk of abortion (1, 19); however, the results have been inconsistent. Some studies have reported that exposure to heavy metals during early pregnancy can increase the incidence of SA and RPL (20–22), and endocrine dysfunction has been suggested as a mediator (23, 24) Other studies have reported contrasting findings (18). Given the increasing interest of clinicians and researchers, stronger evidence on the effect of heavy metal exposure on abortion and its underlying mechanisms is in demand. We performed the present meta-analysis to clarify the associations between abortion and the concentrations of Cd, Pb, Zn, and Cu. We also systematically reviewed the previous literature to explore the relationships between endocrine dysfunction, the four metals, and RPL or SA.

2 Methods

2.1 Study selection

The systematic review was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement. PubMed, Embase, Cochrane Library, and Web of Science databases were searched for relevant studies published up to April 2023. The subject terms included 'Miscarriage,' 'Pregnancy loss,' 'Abortion, Spontaneous,' 'Zinc,' 'Copper,' 'Lead,' and 'Cadmium.' Random combinations of these subject terms and their synonyms were used for retrieval. The detailed literature search strategy is provided in the Supplementary Material. We reached the corresponding authors when the data were missing.

2.2 Inclusion criteria and exclusion criteria

Studies meeting the following criteria were included in the meta-analysis. (a) Study population: Pregnant women without internal and obstetric diseases that impair the normal process of pregnancy, including infectious diseases, gestational hypertension, gestational diabetes mellitus, and infertility. (b) Measurement: Female serum, plasma, or whole blood metal concentrations. (c) Observation group: Women who had experienced abortion, including SA and RPL. SA is defined as the involuntary loss of a fetus before the 28th GW (including the 20th and 24th GW) (2). RPL is defines as two or more abortions, irrespective of whether they are consecutive (5). (d) Control group: Healthy pregnant women with normal pregnancy or delivery. (e) Study type: Observational study.

The exclusion criteria were as follows: (a) article type: review, meta-analysis, meeting, case report, letter, comment, editorial, note, trial registry record, or protocol, (b) studies that focused on non-human cases (e.g., animal studies), (c) unclear definition of SA or RPL, (d) insufficient data on metal concentration; and (e) unavailable full text.

2.3 Quality assessment

The studies that met our inclusion criteria after the initial search were case–control, nested case–control, and cross-sectional studies. Therefore, the Newcastle-Ottawa Scale (NOS) was used to assess the quality of the studies (25). Each included article was independently appraised by two authors (MR and LiqW). Based on the NOS, studies were categorized as high- (8, 9), moderate- (6, 7), or low-quality (<6). Any disagreements regarding the assessment of the studies were discussed with the third author (LiaW).

2.4 Data extraction

Two investigators independently extracted the relevant data from the included studies (MR and LiaW). All data were double-checked by the third author (LiqW). The following information was extracted from the selected studies: first author, publication year, country and continent of the study population, type of detected sample, type of article, type of heavy metal, type of abortion, follow-up endpoint, sample size, concentrations of heavy metals, and analytical method employed.

2.5 Statistical analysis

Meta-analysis was performed using Stata 16.0 (Stata Corp, College Station, TX, USA). The standard mean difference (SMD) was adopted to integrate the data on metal concentration, as it is a continuous variable with different units across various studies. The 95% confidence intervals (CIs) were computed and presented as forest plots. For each study, statistical heterogeneity was assessed using Cochran's Q-test and I^2 statistics, and a random effects model was used to estimate the relationship between metal concentrations and abortion, as there was significant heterogeneity (p <0.05, I^2 >50%). To investigate the impact of metal concentration on the different types of abortions (SA and RPL), a subgroup analysis was performed. To investigate the origin of the heterogeneity, four additional subgroup analyses were performed based on the follow-up endpoints (ongoing pregnancy and live birth) of participants, continent of the study population (Africa, Asia, North America, Oceania, and Europe), type of article (case–control study, cross-sectional study, and nested case–control study), and type of detected sample (serum, plasma, and whole blood). An influence analysis (sensitivity analysis) was conducted to improve the reliability of the meta-analysis results. A funnel plot and Begg's and Egger's tests were used to detect potential publication bias; p-values <0.05 represented significant statistical publication bias for Begg's and Egger's tests.

3 Results

3.1 Literature search

Figure 1 illustrates the PRISMA flow diagram for the selection of studies for inclusion in the systematic review and meta-analysis. A total of 4,222 potential studies were identified through database search. Among them, 136 articles were removed for duplicates, 1,209 articles were not observational studies (including reviews, meta-analyses, meetings, case reports, letters, comments, editorials, notes, trial registry records, and protocols), and 2,829 articles were not relevant to our study based on screening of their titles and abstracts by two authors. The literature screening results were double-checked to ensure that the relevant documents were not missed and did not need to be retrieved. After an independent review of the full texts by three authors (MR, LiaW, and LiqW), 12 studies were excluded because they did not meet the inclusion criteria, and eight studies were excluded because they had insufficient data (they only reported mean values without standard deviation of the metal concentration). Twenty-eight relevant studies were subjected to a final quantitative assessment based on the exclusion and inclusion criteria. Among the 28 studies, 14 investigated Zn and Cu, 15 investigated Pb, and eight investigated Cd.

3.2 Study characteristics

Table 1 lists the baseline characteristics of the included articles. The included articles were observational studies published between 1979 and 2023 and involved 1,377 abortion cases (including 1,159 females with SA and 218 females with RPL), together with 3,289

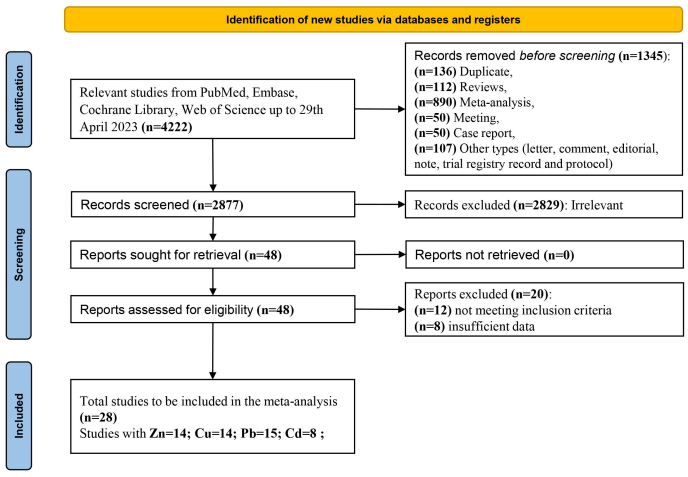

FIGURE 1
Flow chart of the included eligible studies in systematic review and meta-analysis.

TABLE 1 Characteristics of the eligible studies.

										Number of subjects		Metal concentration (mg/dL)				
Sr.No	Author	Year	Country	Continent	Sample	Type of Article	Heavy Metal	Type of Abortion	Follow-up End-point	Cases	Controls	Cases	Controls	*p*	Analytical method employed	Reference
1	Ahmed, M.H.	2007	Egypt	Africa	Serum	Case–control	Zinc	SA	12 weeks	24	14	0.76 ± 0.06 (mg/L)	0.86 ± 0.04 (mg/L)	0.000	AAS	(26)
					Serum	Case–control	Lead	SA	12 weeks	24	14	10.559 ± 1.317 (µg/dL)	7.977 ± 3 (µg/dL)	0.000	AAS	
					Serum	Case–control	Cadmium	SA	12 weeks	24	14	3.2 ± 0.65 (µg/L)	2.74 ± 0.25 (µg/L)	0.039	AAS	
2	Ajayi, O. O.	2012	Nigeria	Africa	Serum	Case–control	Zinc	RPL	20 weeks	35	34	99.25 ± 2.14 (µg/dL)	99.25 ± 2.14 (µg/dL)	0.001	AAS	(27)
					Serum	Case–control	Copper	RPL	20 weeks	35	34	94.25 ± 3.07 (µg/dL)	122.45 ± 2.71 (µg/dL)	0.001	AAS	
					Serum	Case–control	Lead	RPL	20 weeks	35	34	85.96 ± 1.09 (µg/dL)	60.70 ± 1.40 (µg/dL)	0.001	AAS	
					Serum	Case–control	Cadmium	RPL	20 weeks	35	34	4.58 ± 0.77 (µg/dL)	2.49 ± 0.09 (µg/dL)	0.001	AAS	
3	Alebic-Juretic, A.	2005	Croatia	Europe	Plasma	Case–control	Copper	SA	14weeks	17	28	18.2 ± 5.5 (µmol/L)	27.1 ± 7.6 (µmol/L)	<0.001	Photometry	(28)
4	Al-Sheikh, Y. A.	2019	Saudi	Asia	Plasma	Case–control	Zinc	RPL	Delivery	28	28	2.84 ± 0.36 (µmol/l)	3.55 ± 0.49 (µmol/l)	<0.001	ICP-MS	(29)
					Plasma	Case–control	Copper	RPL	Delivery	28	28	19.6 ± 2.75 (µmol/l)	24.5 ± 3.41 (µmol/l)	<0.001	ICP-MS	
5	Attalla, S.M.	2009	Egypt	Africa	Serum	Case–control	Zinc	RPL	12 weeks	40	24	77.01 ± 11.55 (µg %)	90.01 ± 10.77 (µg %)	0.020	AAS	(30)

(Continued)

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TABLE 1 Continued

Sr.No	Author	Year	Country	Continent	Sample	Type of Article	Heavy Metal	Type of Abortion	Follow-up End-point	Number of subjects		Metal concentration (mg/dL)		*p*	Analytical method employed	Reference
										Cases	Controls	Cases	Controls			
					Serum	Case–control	Lead	RPL	12 weeks	40	24	19.78 ± 3.85 (µg/dL)	10.53 ± 1.01 (µg/dL)	<0.0001	AAS	
					Serum	Case–control	Cadmium	RPL	12 weeks	40	24	7.51 ± 1.02 (µg/dL)	5.06 ± 0.81 (µg/dL)	<0.0001	AAS	
6	Bassiouni, B. A.	1979	Egypt	Africa	Plasma	Case–control	Copper	SA	14 weeks	24	14	140.12 ± 15.20 (µg/100 mL)	188.57 ± 14.41 (µg/100 mL)	<0.01	AAS	(31)
7	Borella, P.	1990	Italy	Europe	Plasma	Case–control	Zinc	SA	16 weeks	12	41	13.64 ± 2.35 (µmol/L)	13.13 ± 2.37 (µmol/L)	>0.05	AAS	(32)
					Plasma	Case–control	Copper	SA	16 weeks	12	41	28.22 ± 4.68 (µmol/L)	26.05 ± 6.53 (µmol/L)	>0.05	AAS	
8	Borja-Aburto, V. H.	1999	Mexico	North America	Serum	Case–control	Lead	SA	20 weeks	35	60	12 ± 6.16 (µg/dL)	10.1 ± 5.34 (µg/dL)	0.021	AAS	(33)
9	Dreosti, I. E.	1990	Australia	Oceania	Serum	Case–control	Zinc	SA	12 weeks	35	37	0.75 ± 0.02 (µg/mL)	0.69 ± 0.02 (µg/mL)	>0.05	AAS	(34)
					Serum	Case–control	Copper	SA	12 weeks	35	37	1.36 ± 0.05 (µg/mL)	1.48 ± 0.05 (µg/mL)	>0.05	AAS	
10	Faikoğlu, R.	2006	Turkey	Asia	Serum	Case–control	Lead	SA	20 weeks	20	20	23.2 ± 1 3.77 (µg/dL)	18.04 ± 13.08 (µg/dL)	>0.05	AAS	(35)
11	Ghneim, H. K.	2016	Saudi	Asia	Plasma	Case–control	Zinc	RPL	Delivery	25	25	3.67 ± 0.39 (µmol/L)	4.11 ± 0.49 (µmol/L)	<0.001	ICP-MS	(36)
					Plasma	Case–control	Copper	RPL	Delivery	25	25	25.6 ± 3.25 (µmol/L)	28.8 ± 3.42 (µmol/L)	<0.001	ICP-MS	

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TABLE 1 Continued

Sr.No	Author	Year	Country	Continent	Sample	Type of Article	Heavy Metal	Type of Abortion	Follow-up End-point	Number of subjects		Metal concentration (mg/dL)		*p*	Analytical method employed	Reference
										Cases	Controls	Cases	Controls			
12	Ghosh, A.	1985	China	Asia	Serum	Cross–section	Zinc	SA	12 weeks	45	55	120.18 ± 19.55 (µg/mL)	123.03 ± 18.57 (µg/mL)	>0.05	AAS	(37)
13	Jie, O.	2019	China	Asia	Whole blood	Case–control	Cadmium	SA	12 weeks	95	100	0.32 ± 0.28 (µg/L)	0.22 ± 0.11 (µg/L)	0.002	ICP-MS	(38)
14	Lamadrid-Figueroa, H.	2007	Mexico	North America	Plasma	Case–control	Lead	SA	12 weeks	71	136	0.14 ± 0.13 (µg/L)	0.13 ± 0.13 (µg/L)	0.15	ICP-MS	(39)
15	Lu, Y.	2022	China	Asia	Whole blood	Cross–section	Zinc	SA	12 weeks	92	103	5,082.32 ± 1,030.13 (µg/L)	5,243.88 ± 960.87 (µg/L)	0.251	ICP-MS	(40)
					Whole blood	Cross–section	Copper	SA	12 weeks	92	103	797.36 ± 161.42 (µg/L)	861.77 ± 188.75 (µg/L)	0.008	ICP-MS	
					Whole blood	Cross-section	Lead	SA	12 weeks	92	103	7.27 ± 3.01 (µg/L)	7.61 ± 2.64 (µg/L)	0.165	ICP-MS	
16	Omeljaniuk, W. J.	2015	Poland	Europe	Serum	Case–control	Zinc	SA	Delivery	83	35	0.5865 ± 0.1071 (mg/L)	0.6492 ± 0.1878 (mg/L)	>0.05	AAS	(41)
					Serum	Case–control	Copper	SA	Delivery	83	35	1.1532 ± 0.2980 (mg/L)	1.4450 ± 0.2930 (mg/L)	<0.002	AAS	
17	Omeljaniuk, W. J.	2018	Poland	Europe	Whole blood	Case–control	Lead	SA	Delivery	83	35	35.54 ± 11.0 (µg/L)	27.11 ± 4.6 (µg/L)	<0.0001	AAS	(19)
					Whole blood	Case–control	Cadmium	SA	Delivery	83	35	2.730 ± 2.07 (µg/L)	1.035 ± 0.59 (µg/L)	<0.0004	AAS	
18	Ou, J.	2020	China	Asia	Whole blood	Case–control	Lead	SA	12 weeks	150	150	27.21 ± 31.43 (µg/L)	15.96 ± 12.22 (µg/L)	0.000	ICP-MS	(42)

(Continued)

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TABLE 1 Continued

										Number of subjects		Metal concentration (mg/dL)				
Sr.No	Author	Year	Country	Continent	Sample	Type of Article	Heavy Metal	Type of Abortion	Follow-up End-point	Cases	Controls	Cases	Controls	*p*	Analytical method employed	Reference
19	Popovic, J. K.	2016	Serbia	Europe	Plasma	Case–control	Copper	SA	12 weeks	35	50	20.52 ± 3.76 (μmol/L)	28.43 ± 4.45 (μmol/L)	<0.01	Colorimetry	(43)
20	Sairoz	2023	India	Asia	Serum	Nested Case–control	Zinc	SA	12 weeks	80	100	51.7 ± 10.4 (μg/dL)	81.6 ± 20.3 (μg/dL)	0.0000	Reaction with nitro-PAPS	(44)
					Serum	Nested Case–control	Copper	SA	12 weeks	80	100	222.5 ± 60.5 (μg/dL)	302.5 ± 95.2 (μg/dL)	0.0006	Reaction with Di-Br-PAESA	
21	Shen, P. J.	2015	China	Asia	Serum	Nested Case–control	Zinc	SA	12 weeks	58	1389	72.67 ± 11.98 (μmol/L)	83.25 ± 12.79 (μmol/L)	<0.05	AAS	(45)
					Serum	Nested Case–control	Copper	SA	12 weeks	58	1389	29.96 ± 5.27 (μmol/L)	31.24 ± 5.07 (μmol/L)	>0.05	AAS	
22	Skalnaya, M. G.	2019	Russia	Europe	Serum	Case–control	Copper	SA	28 weeks	75	169	1.12 ± 0.29 (μg/L)	1.60 ± 0.54 (μg/L)	<0.001	ICP-MS	(46)
23	Tabassum, H.	2022	Saudi	Asia	Serum	Case–control	Lead	RPL	12 weeks	30	30	77.96 ± 5.51 (ppb)	38.65 ± 0.20 (ppb)	<0.001	ICP-MS	(47)
					Serum	Case–control	Cadmium	RPL	12 weeks	30	30	0.45 ± 0.04 (ppb)	0.42 ± 0.01 (ppb)	<0.05	ICP-MS	
24	Tousizadeh, S.	2023	Iran	Asia	Serum	Case–control	Zinc	RPL	Delivery	60	60	5.26 ± 1.96 (mg/l)	15.06 ± 7.17 (mg/l)	<0.001	AAS	(48)
					Serum	Case–control	Lead	RPL	Delivery	60	60	3.69 ± 2.48 (mg/l)	0.31 ± 0.61 (mg/l)	<0.001	AAS	
25	Vigeh, M.	2010	Iran	Asia	Whole blood	Case–control	Lead	SA	Delivery	15	336	3.51 ± 1.42 (μg/dL)	3.83 ± 1.99 (μg/dL)	0.41	ICP-MS	(49)

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TABLE 1 Continued

										Number of subjects		Metal concentration (mg/dL)				
Sr.No	Author	Year	Country	Continent	Sample	Type of Article	Heavy Metal	Type of Abortion	Follow-up End-point	Cases	Controls	Cases	Controls	*p*	Analytical method employed	Reference
26	**Vigeh, M.**	**2021**	**Iran**	**Asia**	**Whole blood**	**Case–control**	**Lead**	**SA**	**Delivery**	**25**	**141**	**55.43 ± 54.3 (μg/L)**	**44.97 ± 45.6 (μg/L)**	**0.307**	**ICP-MS**	(50)
					Whole blood	**Case–control**	**Cadmium**	**SA**	**Delivery**	**25**	**141**	**0.51 ± 0.5 (μg/L)**	**0.51 ± 0.5 (μg/L)**	**0.957**	**ICP-MS**	
27	**Wang, R.**	**2020**	**China**	**Asia**	**Serum**	**Cross–section**	**Zinc**	**SA**	**12 weeks**	**56**	**55**	**4.18 ± 0.26 (mg/L)**	**3.24 ± 1.47 (mg/L)**	**>0.05**	**ICP-MS**	(51)
					Serum	**Cross–section**	**Copper**	**SA**	**12 weeks**	**56**	**55**	**1.80 ± 0.58 (mg/L)**	**1.41 ± 0.55 (mg/L)**	**<0.001**	**ICP-MS**	
					Serum	**Cross–section**	**Lead**	**SA**	**12 weeks**	**56**	**55**	**0.17 ± 0.09 (mg/L)**	**0.15 ± 0.10 (mg/L)**	**>0.05**	**ICP-MS**	
28	**Yildirim, E.**	**2019**	**Turkey**	**Asia**	**Whole blood**	**Case–control**	**Lead**	**SA**	**12 weeks**	**29**	**20**	**54.11 ± 17.27 (μ/L)**	**44.45 ± 12.49 (μ/L)**	**0.038**	**AAS**	(52)
					Whole blood	**Case–control**	**Cadmium**	**SA**	**12 weeks**	**29**	**20**	**0.39 ± 0.06 (μ/L)**	**0.40 ± 0.05 (μ/L)**	**0.704**	**AAS**	

SA, Spontaneous Abortion; RPL, Recurrent Pregnancy Loss; AAS, Atomic-Absorption Spectrophotometry; ICP-MS, Inductively Coupled Plasma Mass spectrophotometry.

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normal pregnant females. Of the 28 articles included in this meta-analysis, six were completed in China (37, 38, 40, 42, 45, 51), three in Egypt (26, 30, 31), three in Saudi Arabia (29, 36, 47), three in Iran (48–50), two in Mexico (33, 39), two in Turkey (35, 52), two in Poland (19, 41), one in Nigeria (27), one in Croatia (28), one in Italy (32), one in Australia (34), one in Serbia (43), one in India (44), and one in Russia (46). The sample size of the included studies ranged from 38 to 1,447.

3.3 Quality of included studies

The quality assessment results for all studies are shown in Supplementary Table 1. Studies with quality scores higher than 6, which is the cut-off NOS score for low quality, were considered credible. All of the included articles had quality scores above 6.

3.4 Meta-analysis for Zn

Fourteen studies investigated the association between Zn concentrations and abortion. The pooled effect size showed that the Zn concentration was negatively associated with abortion (SMD = −1.05, 95% CI: −1.74, −0.36, p = 0.003, I^2 = 96.9%; Figure 2A). Subgroup analysis showed that women with RPL had significantly lower Zn concentrations than healthy controls (SMD = −3.44, 95% CI: −5.01 to −1.87, p <0.001), whereas the Zn concentrations of women with SA and healthy controls were not significantly different (SMD = −0.14, 95% CI: −0.86–0.58, p = 0.710). Significant heterogeneity was observed in each subgroup (SA, p <0.001, I^2 = 96.6%; RPL, p <0.001, I^2 = 96.6%; Figure 2B). To investigate the origin of the high heterogeneity, subgroup analyses based on follow-up endpoint, continent, type of article, and type of detected sample were performed. Subgroup analyses revealed persistently high heterogeneity (Supplementary Figure 1). Sensitivity analysis showed that omission of any study did not change the overall effect (Figure 2C). There was no evidence of publication bias among the included studies (Begg, p = 0.511; Egger, p = 0.335; Figure 2D).

3.5 Meta-analysis for Cu

Comparisons of Cu concentrations in women with and without abortion were reported in 14 studies. The pooled effect size of the 14 studies revealed significantly lower Cu concentrations in the abortion group than in the control group (SMD = −1.42, 95% CI: −1.97, −0.87, p <0.001, I^2 = 95.4%) (Figure 3A). Subgroup analysis stratified by the type of abortion (SA and RPL) showed that patients with SA and RPL had lower Cu concentrations than the healthy controls (Figure 3B); the SMD was −0.97 (95% CI: −1.47, −0.48, p <0.001) for women with SA and −3.92 (95% CI: −6.97, −0.87, p = 0.012, respectively). However, subgroup analysis based on abortion type showed obvious heterogeneity (SA, p <0.001, I^2 = 94.0%; RPL, p <0.001, I^2 = 97.8%). Subgroup analyses for the follow-up endpoint, continent, type of article, and type of detected sample showed high heterogeneity (Supplementary Figure 2). Sensitivity analysis showed that omission of any single study did not change the overall effect (Figure 3C). Publication bias was detected in the studies that included Cu (Begg: p = 0.037; Egger: p = 0.012). Visual inspection of funnel plots showed asymmetry (Figure 3D).

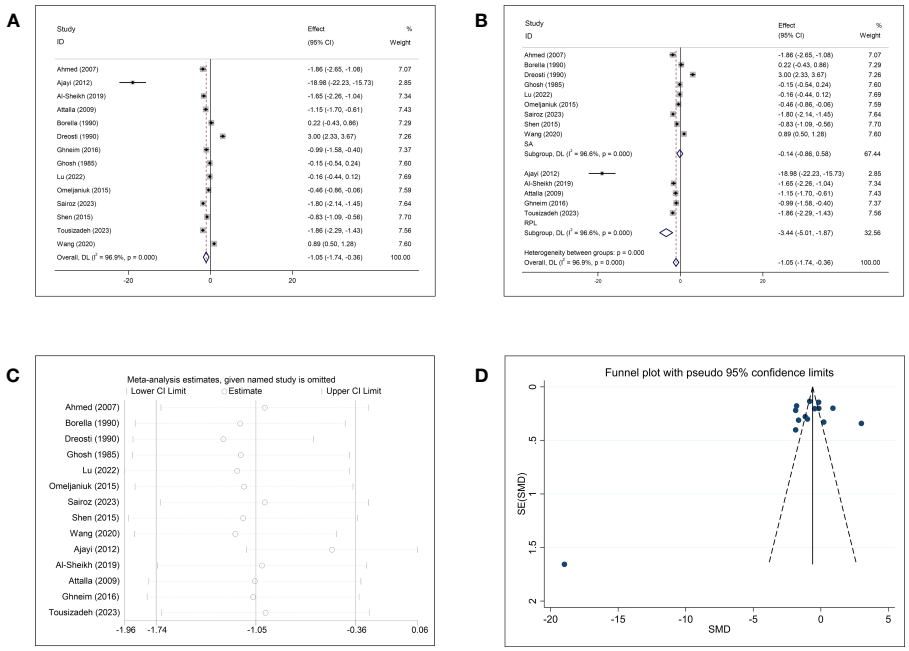

FIGURE 2
Meta-analysis outcomes of zinc. **(A)** Forest plot showing the meta-analysis outcomes between abortion group and normal pregnant women; **(B)** Subgroup analysis based on the type of abortion (SA and RPL); **(C)** Sensitivity analysis; and **(D)** Funnel plot.

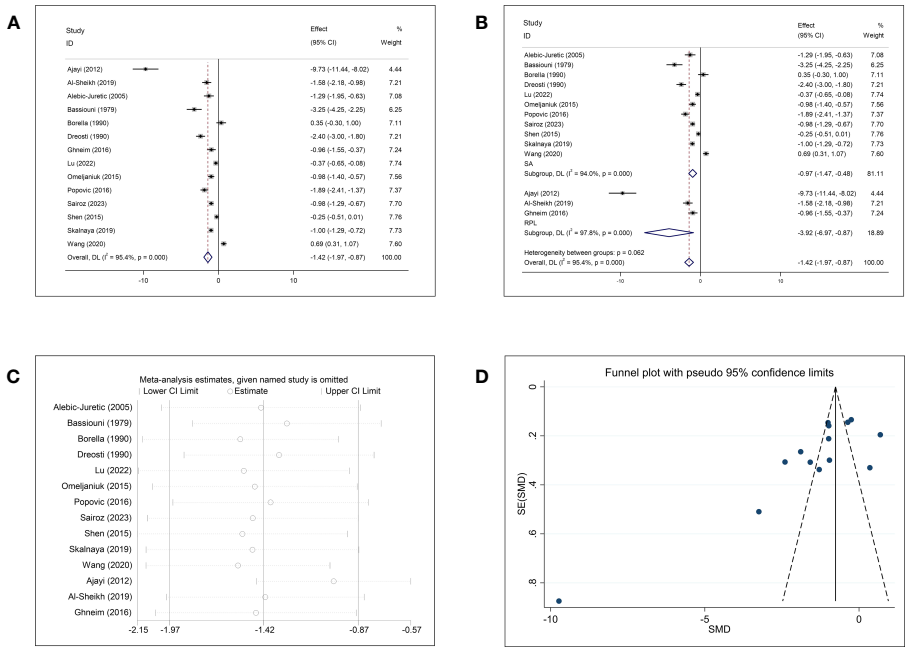

FIGURE 3
Meta-analysis outcomes of copper. **(A)** Forest plot showing the meta-analysis outcomes between abortion group and normal pregnant women; **(B)** Subgroup analysis based on the type of abortion (SA and RPL); **(C)** Sensitivity analysis; and **(D)** Funnel plot.

3.6 Meta-analysis for Pb

The meta-analysis of the association between Pb concentration and abortion included 15 studies (Figure 4A). The pooled circulating Pb concentration was significantly higher in women who had experienced an abortion than in those with normal pregnancies (SMD = 1.47, 95% CI: 0.89–2.05, p <0.001, I^2 = 96.0%). Subgroup analysis for SA and RPL showed significantly higher Pb concentrations in women with SA (SMD = 0.33, 95% CI: 0.12–0.55, p = 0.002) and RPL (SMD = 8.19, 95% CI: 4.52–11.85,

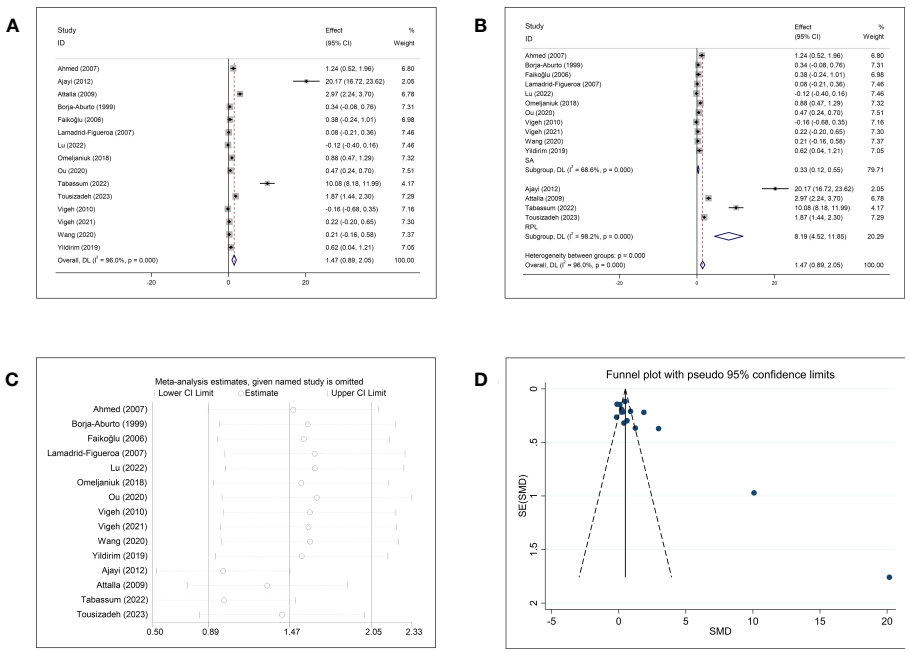

FIGURE 4
Meta-analysis outcomes of lead. **(A)** Forest plot showing the meta-analysis outcomes between abortion group and normal pregnant women; **(B)** Subgroup analysis based on the type of abortion (SA and RPL); **(C)** Sensitivity analysis; and **(D)** Funnel plot.

p <0.001) than in healthy pregnant women (Figure 4B). However, significant heterogeneity was observed (SA: p <0.001, I^2 = 68.6%; RPL: p <0.001, I^2 = 98.2%). Further subgroup analyses based on the follow-up endpoint, continent, type of article, and type of detected sample also showed high heterogeneity (Supplementary Figure 3). Sensitivity analysis showed that omission of any single study did not change the overall effect (Figure 4C). Visual inspection of the funnel plots (Figure 4D) and Begg's and Egger's tests showed publication bias (Begg: p = 0.002; Egger: p = 0.001).

3.7 Meta-analysis for Cd

The pooled results of the meta-analysis of eight studies on Cd and abortion showed significantly higher Cd concentrations in women who underwent abortion than in normal pregnant women (SMD = 1.15, 95% CI: 0.45–1.85, p = 0.001, I^2 = 93.7%) (Figure 5A). Subgroup analysis based on abortion type showed that women with SA and RPL had significantly higher Cd concentrations than the normal controls (SA: SMD = 0.42, 95% CI: 0.02–0.82, p = 0.040; RPL: SMD = 2.45, 95% CI: 0.85–4.04, p = 0.003). However, the heterogeneity was significant in each subgroup (SA: p = 0.003, I^2 = 75.1%; RPL: p <0.001, I^2 = 94.2%) (Figure 5B). Further subgroup analyses based on the follow-up endpoint, continent, and type of detected sample also showed high heterogeneity (Supplementary Figure 4). All studies on Cd and abortion were case–control studies, and subgroup analysis for different article types could not be performed. The sensitivity analysis showed that the exclusion of any single study could change the overall effect (Figure 5C). Both funnel plots and Begg's and Egger's tests showed no publication bias for Cd (Begg: p = 0.174; Egger: p = 0.113; Figure 5D).

4 Discussion

Due to widespread human exposure to (53) and bio-accumulation of heavy metals (54), there are growing concerns about the adverse effects of heavy metals on normal pregnancies. Exposure to toxic metals or deficiency of essential metals has long been suspected to lead to abortion (55). However, the results of previous studies have not been consistent (1, 19). To provide stronger evidence for this important clinical issue, we conducted the present meta-analysis, focusing on two common toxic metals (Pb and Cd) and two essential metals (Zn and Cu) (56). This study is first to investigate the overall association between blood Zn, Cu, Pb, and Cd concentrations and abortion, including RPL and SA. Zn or Cu deficiency was associated with the prevalence of abortion in women, and exposure to Pb or Cd increased the risk of abortion (SA and RPL). Only one relevant meta-analysis was carried out in 2021, showed that exposure to Cd and Pb increased the incidence of abortion (undistinguished threatened abortion, SA, and RPL) (23). Subgroup analysis based on abortion type was not performed (23). In the present study, we recruited more studies to reinforce the association between exposure to Cd and Pb and the increased risk of abortion and performed subgroup analysis based on the type of abortion to investigate the effect of Cd and Pb exposure on patients with SA and RPL. In addition, the exploration of Zn and Cu in women with RPL and SA provides a basis for clinicians who tend to intervene early against RPL in women with Zn and Cu deficiencies.

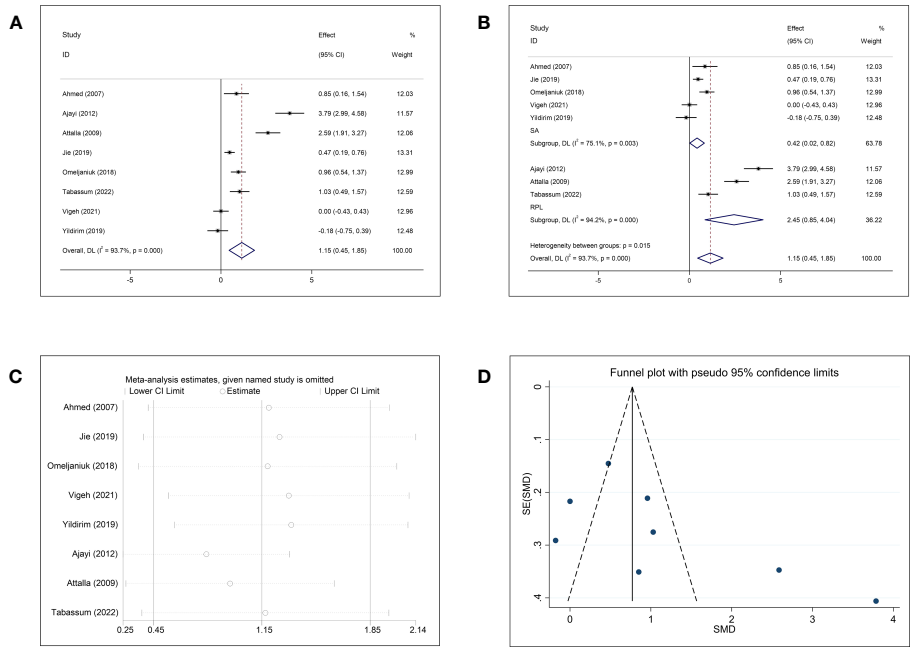

FIGURE 5
Meta-analysis outcomes of cadmium. **(A)** Forest plot showing the meta-analysis outcomes between abortion group and normal pregnant women; **(B)** Subgroup analysis based on the type of abortion (SA and RPL); **(C)** Sensitivity analysis; and **(D)** Funnel plot.

The exact mechanisms underlying the induction of SA and RPL by Pb and Cd exposure and Zn and Cu deficiency are unknown. Studies have shown that heavy metals are common environmental endocrine disruptors. Previous studies have reported that exposure to toxic metals and deficiencies of essential metals lead to abortion mainly through endocrine dysfunction, such as insulin resistance, vitamin D insufficiency, and abnormal thyroid and sex hormone concentrations, among others. The details of this process are discussed below.

4.1 Female Zn concentration and abortion

In our study, we found that Zn inadequacy tended to increase the chances of abortion, especially for RPL; however, it may not increase the incidence of SA. The underlying mechanism of Zn inadequacy-related RPL remains unknown. However, it may also be associated with endocrine dysfunction caused by Zn deficiency.

Zn deficiency has been reported to decrease insulin sensitivity and cause insulin resistance (IR) (57, 58), whereas Zn supplements can decrease IR (59, 60). IR, defined clinically as a decreased biological response to exogenous or endogenous insulin, can cause mitochondrial dysfunction in the placenta, diminished trophoblast invasion, a subclinical inflammatory state, and oxidative stress. These factors are all considered crucial in the pathophysiology of RPL (61–64). Zn can reinforce glucose transport into cells and potentiate insulin-induced glucose transport via the insulin signaling pathway (65). Zn can also act as an insulin mimetic to maintain glucose homeostasis, which may also be a mechanism underlying Zn deficiency-induced IR (66).

Apart from IR, Zn deficiency is also closely related to vitamin D deficiency, as Zn regulates the transcriptional activation of hormone-related genes via a cysteine-rich Zn-finger region in vitamin D receptors (VDRs) (67–69). Vitamin D plays a vital role in maintaining normal biological functions, such as calcium homeostasis, and cell proliferation, differentiation, and apoptosis, all of which are crucial for immunomodulation and normal pregnancy (70). Vitamin D inadequacy was reported to be associated with SA and RPL in a recent meta-analysis (71, 72). Supplementation with vitamin D can suppress inflammatory cytokine production and elevate the secretion of cathelicidin in decidual cells and trophoblasts, which can reduce the risk of abortion (73–75).

Zn deficiency appears to interfere with sex hormone synthesis and further causes RPL. Zn can affect the biosynthesis and function of sex hormones, such as progesterone and prolactin, by altering LH and FSH levels and inducing oxidative stress (17, 76–78). Zn may also promote estrogen release by forming ligand bonds with metal-binding sites on the estrogen receptor (ER) (79). Insufficient secretion of sex hormones, such as progesterone, testosterone, estrogen, and prolactin, can reduce endometrial receptivity and oocyte quality in women, which is related to RPL (55, 80, 81).

Furthermore, Zn is an essential trace element for thyroid function and homeostasis (82), and its deficiency can lead to hypothyroidism (82–84). Hypothyroidism and subclinical hypothyroidism can lead to poor pregnancy outcomes such as SA and RPL (85, 86). Zn supplements can elevate thyroxine (T4) concentrations and reduce triiodothyronine (T3) concentrations by altering the expression of key genes (*nis*, *tpo*, *thrα*, *dio1*, *dio2*, and *ugt1ab*) in the hypothalamic–pituitary–thyroid (HPT) axis (87).

4.2 Female Cu concentration and abortion

We found that women undergoing abortion (both SA and RPL) had lower Cu concentrations, indicating that Cu deficiency may be closely related to the incidence of abortion (SA and RPL). However, the underlying mechanism remains unknown. However, previous studies have reported that Cu deficiency can induce endocrine dysfunction, such as IR, vitamin D insufficiency, and abnormal thyroid and sex hormone concentrations, which may be involved in the pathogenesis of SA and RPL. Insufficient Cu can cause IR by upregulating cytochrome c oxidase 1 (SCO1) and vascular adhesion protein-1 (VAP-1) (88–90); it can also reduce progesterone synthesis by regulating the expression of steroidogenic factor 1 (SF-1) (91). Cu deficiency can also reduce Cu/Zn superoxide dismutase (Cu/Zn-SOD) and cause oxidative stress in the ovary, ultimately leading to dysfunctional luteal formation and insufficient progesterone secretion (17). In addition, Cu deficiency can decrease the expression of estrogen synthetases such as aromatase (CYP19A1) and 17β-hydroxysteroid dehydrogenase (17β-HSD) (92). Furthermore, Cu deficiency can lead to hypothyroidism (82–84, 93) by inducing oxidative stress and decreasing thyroxine synthesis by limiting tyrosinase availability (82, 93–95).

4.3 Female Pb concentration and abortion

Our study found that women who experienced abortion (SA and RPL) had higher Pb concentrations, suggesting that Pb exposure could increase the risk of abortion (SA and RPL). Our results are consistent with those of the meta-analysis by Kaur et al. (23). Pb can substitute polyvalent cations, such as calcium (Ca^{2+}), and affect various cellular processes, such as apoptosis, cell adhesion, and cell signaling (96). However, the mechanism underlying Pb-induced abortion remains unclear. Animal studies have shown that Pb exposure can downregulate IR-related genes in the P13K and Akt signaling pathways, which are involved in hepatic gluconeogenesis and glucose production (97, 98). Low-level Pb exposure promotes the gene expression of key enzymes involved in hepatic gluconeogenesis and eventually induces hyperglycemia and impaired fasting plasma glucose, which is known as hepatic insulin resistance (99). Additionally, Pb appears to be involved in the pathology of vitamin D deficiency. Pb can diminish the activity of vitamin D by blocking the normal renal synthesis of active 1,25-dihydroxy vitamin D (1,25(OH)2D) and reduce the generation of vitamin D binding protein (DBP) (56, 100, 101). Pb can also promote degradation and block the synthesis of 1,25(OH)2D3 by upregulating the hepatic expression of Cyp24a1 enzymes and inhibiting 25-hydroxylase (CYP2R1) and 1-α-hydroxylase (CYP27B1) at the gene and protein levels (100, 102). Previous studies have suggested that Pb may be closely associated with luteal phase deficiencies. Pb can directly inhibit the expression of several key enzymes involved in progesterone synthesis, such as StAR, CYP11A1, and 3β-HSD (103, 104). Pb also appears to indirectly

interfere with progesterone synthesis by inhibiting the cAMP-PKA-dependent signaling pathway that regulates the expression of these key enzymes (104–106). Pb has adverse effects on sex hormone concentration. Pb exposure is associated with increased testosterone and prolactin concentrations and appears to reduce estrogen concentrations by decreasing the expression of estrogen synthases such as 17β-HSD (103, 107, 108). Furthermore, Pb accumulation negatively affects thyroid function, which is also related to abortion. Excessive exposure to Pb may lead to hypo- or hyperthyroidism (109). As an oxidant, Pb can negatively impact thyroid cells by promoting oxidative stress, and it can also interact with other essential elements such as Cu, Zn, and Fe to indirectly affect thyroid function (82, 109).

4.4 Female Cd concentration and abortion

We found that the Cd concentration was significantly higher in women who experienced abortion (SA and RPL) than in normal pregnant women. Our results are in line with those of Kaur et al., who revealed that Cd exposure could increase the risk of abortion (23). Cd is a highly potent environmental pollutant that causes indirect oxidative damage to DNA, leading to the induction of cellular proliferation and inhibition of DNA repair mechanisms, causing cytotoxicity (110). However, research on the mechanisms of Cd exposure-related abortion is lacking. In recent years, an increasing number of studies have found a strong relationship between Cd and endocrine dysfunction, which is the main reason for abortions (both SA and RPL). Epidemiological surveys have shown that Cd can cause IR through perturbations in gluconeogenesis, pancreatic islet dysfunction, and metabolic and mitogen impairments in the liver and adipose tissue (111, 112). Epidemiological studies have also demonstrated that high blood Cd concentrations are negatively correlated with vitamin D concentrations (113, 114), which may be due to the interaction of Cd with renal mitochondrial hydroxylases (115). Cd is also involved in the pathogenesis of luteal phase deficiencies. It can directly or indirectly inhibit the expression of several key enzymes (StAR, CYP11A1, and 3β-HSD) involved in progesterone synthesis by regulating the cAMP-PKA-dependent signaling pathway (24, 116, 117). Cd can also interfere with the balance of sex hormone concentrations. Cd exposure appears to decrease the expression of estrogen synthetases (CYP19A1 and 17β-HSD), and it is also a potent xenoestrogen that can mediate the proliferation of anterior pituitary cells and prolactin secretion by mimicking estrogen (118). Cd can also negatively affect thyroid cells by promoting oxidative stress, ultimately leading to thyroid dysfunction (82, 109).

4.5 Strengths

The strength of our study is that we comprehensively investigated the relationships between blood Zn, Cu, Pb, and Cd concentrations and abortion rates (SA and RPL). In addition, we systematically reviewed previous publications on the endocrine mechanisms of metal exposure-related abortions. We propose that IR, vitamin D insufficiency, and abnormal thyroid and sex hormone concentrations may be involved in Zn and Cu deficiencies and Pb and Cd exposure-associated abortions. Third, the large sample size of 4,666 pregnant women from 14 countries makes our estimates reliable. Fourth, most included studies were of good quality. In the included studies, the definitions of cases and controls were adequate, and the selection of controls and assessment of exposure were consistent. Fifth, a sensitivity analysis was conducted to verify the associations between the four metals and abortion.

4.6 Limitations

This study has some limitations. First, several dated documents that appeared to meet our inclusion criteria were not included because we were unable to reach the authors. Second, despite our best efforts, we were only able to find 28 related papers because of the relatively large number of animal studies and case reports. Third, most meta-analyses included in our study had high heterogeneity. To ascertain its sources, we performed a subgroup analysis based on the type of abortion, follow-up endpoint, continent, type of observational study, and type of detected sample. However, we failed to find sources of heterogeneity by subgroup analysis, as most subgroup analyses showed high heterogeneity. After carefully reviewing the included articles, we found that different diagnoses of SA or RPL may have led to clinical heterogeneity. In addition, regional variations in metal concentrations of the study participants were considered another source of heterogeneity. Local mineral deposits and their exploitation affect the metal concentrations in the environment (air, water, and soil), and different terrains can impact the diffusion of pollutants (119). Thus, participants had different risks of metal exposure. Moreover, participants of different races with different genetic backgrounds have various sensitivities to metal exposure (120). Owing to the limited information regarding the region and race of the investigated subjects in the original literature, we were only able to perform a subgroup analysis based on different continents to investigate the heterogeneity caused by regional differences. The age of the study population and the time point of blood collection in each study were also considered potential sources of heterogeneity, as heavy metals could accumulate in the human body, and older adults may have higher blood metal concentrations. Furthermore, metal concentrations can change during different trimesters and the time points of blood collection may lead to heterogeneity (121, 122). Fourth, the literature regarding Cu and Pb had obvious publication and reporting bias, although the publication and reporting the bias of literature regarding Zn and Cd were acceptable. Language and multiple publication biases were considered primary problems as only the English literature was included, and two studies had outcomes from the same study population.

4.7 Implications for treatment

The findings of the present study broaden our understanding of the effects of toxic and essential metals on the RPL and SA. Endocrine dysfunction can lead to metal exposure and abortions. It will be helpful to screen blood Zn, Cu, Pb, and Cd concentrations in females. However, well-designed prospective cohort studies are

needed to clarify the causal relationship between endocrine dysfunction and heavy-metal-induced abortion.

5 Conclusion

In the present study, we found that higher blood Pb and Cd concentrations and lower Zn and Cu concentrations in females may be associated with SA and RPL. Exposure to toxic metals, as well as deficiencies in essential metals, may cause SA and RPL through endocrine dysfunction, such as insulin resistance, vitamin D insufficiency, and abnormal thyroid and sex hormone concentrations. However, further prospective cohort and experimental studies are required to provide stronger evidence.

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

XS and MR proposed the subject and designed the protocol for this systematic review. MR, LTW, LQW and JC conducted literature screening and data extraction. MR, LQW, and LTW assessed the quality of all studies. LTW and MR performed statistical analysis. MR, LTW, and LQW produced the tables, figures. MR, LQW and LTW drafted the manuscript. XS and SQ gave overall supervision, critical revisions, and final approval of the article. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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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.2023.1216507/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Subgroup analysis of circulating Zn level in abortion women or healthy pregnant women. **(A)**. Subgroup analysis based on the follow-up endpoint (ongoing pregnancy and live birth); **(B)**. Subgroup analysis based on the continent of study population (Africa, Asia, Europe, and Oceania); **(C)**. Subgroup analysis based on the type of article (case-control study, cross-section study, and nested case-control study); **(D)**. Subgroup analysis based on the type of detected sample (serum, plasma, and whole blood).

SUPPLEMENTARY FIGURE 2
Subgroup analysis of circulating Cu level in abortion women or healthy pregnant women. **(A)**. Subgroup analysis based on the follow-up endpoint (ongoing pregnancy and live birth); **(B)**. Subgroup analysis based on the continent of study population (Africa, Asia, Europe, and Oceania); **(C)**. Subgroup analysis based on the type of article (case-control study, cross-section study, and nested case-control study); **(D)**. Subgroup analysis based on the type of detected sample (serum, plasma, and whole blood).

SUPPLEMENTARY FIGURE 3
Subgroup analysis of circulating Pb level in abortion women or healthy pregnant women. **(A)**. Subgroup analysis based on the follow-up endpoint (ongoing pregnancy and live birth); **(B)**. Subgroup analysis based on the continent of study population (Africa, North America, Asia, and Europe); **(C)**. Subgroup analysis based on the type of article (case-control study and cross-section study); **(D)**. Subgroup analysis based on the type of detected sample (serum, plasma, and whole blood).

SUPPLEMENTARY FIGURE 4
Subgroup analysis of circulating Cd level in abortion women or healthy pregnant women. **(A)**. Subgroup analysis based on the follow-up endpoint (ongoing pregnancy and live birth); **(B)**. Subgroup analysis based on the continent of study population (Africa, Asia, and Europe); **(C)**. Subgroup analysis based on the type of detected sample (serum and whole blood).

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Impact of thyroid-stimulating hormone levels after controlled ovarian hyperstimulation on *in vitro* fertilization/intracytoplasmic sperm injection outcomes in women with fresh embryo transfer: a prospective cohort study

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Objective: Maternal hypothyroidism before and during pregnancy is associated with an increased risk of adverse pregnancy outcomes; many studies have evidenced that controlled ovarian hyperstimulation (COH) triggers a significant increase in the levels of TSH; however, no large-scale prospective studies have evaluated the impact of TSH levels after COH on assisted reproductive technology outcomes. The aim of this prospective study was to investigate whether *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) outcomes are affected by TSH levels after COH in women with fresh embryo transfer (ET).

Methods: A total of 664 patients who underwent IVF/ICSI treatment and received fresh ET at the Peking University Third Hospital were included in this study. The rates of clinical pregnancy, miscarriage, live birth, and preterm delivery were analyzed.

Results: The patients were categorized into two groups based on serum TSH levels after COH (0.55 mIU/L < TSH < 2.5 mIU/L: n= 449, 2.5 mIU/L ≤ TSH ≤ 4.78 mIU/L: n= 215). There were no significant differences in the rates of clinical pregnancy, miscarriage, and live birth between the two groups, even after adjusting for age, body mass index (BMI), thyroid antibody positivity, and COH protocols. However, the preterm delivery rate was significantly higher in women with TSH < 2.5 mIU/L than in those with TSH ≥ 2.5 mIU/L, even after adjusting for

relevant confounding factors. There was no significant difference in live birth weight between the two groups.

Discussion: Mildly elevated TSH levels (TSH ≥ 2.5 mIU/L) after COH did not affect IVF/ICSI outcomes, and strict control of TSH levels within 2.5 mIU/L after COH might not be necessary. Additionally, strictly controlled TSH levels (TSH < 2.5 mIU/L) may increase preterm delivery risk.

KEYWORDS

in vitro fertilization, intracytoplasmic sperm injection, controlled ovarian hyperstimulation, clinical pregnancy, miscarriage, live birth, preterm delivery

1 Introduction

Adequate thyroid hormone levels are necessary for normal pregnancy and fetal development. Since the fetal thyroid gland is non-functional during early pregnancy, fetal growth and development are completely dependent on maternal thyroid hormone transfer. Thus, maternal hypothyroidism before and during pregnancy is associated with an increased risk of adverse pregnancy outcomes, including pregnancy loss, premature birth, and low birth weight (1, 2). Furthermore, intelligence quotient scores were reportedly lower in children of women with hypothyroidism than in children of women with normal thyrotropin concentrations, suggesting that hypothyroidism was detrimental to fetal neurocognitive development (3).

Given the potential danger of hypothyroidism, many studies suggest routine screening for thyroid function before and during pregnancy. In particular, serum thyroid-stimulating hormone (TSH) measurement is the most accurate assay for hypothyroidism evaluation because elevated levels of serum TSH are the earliest abnormal laboratory indicator of the occurrence of hypothyroidism (4). Although hypothyroidism is defined as an increase in serum TSH levels above the upper limit of normality, several guidelines suggest that the upper limit of TSH levels before pregnancy should not exceed 2.5 mIU/L; the Endocrine Society recommends that TSH should not exceed 2.5 mIU/L before pregnancy and during the first trimester (5). Furthermore, the 2011 American Thyroid Association guidelines recommend that the upper reference limit for serum TSH concentration in the first trimester of pregnancy should be defined as 2.5 mIU/L (6). The 2021 European Thyroid Association Guideline suggests levothyroxine (LT4) treatment in subfertile women with TSH levels >4.0 mIU/L to maintain serum TSH levels <2.5 mIU/L (7). Recently, large-scale cohort studies also indicated that mildly elevated TSH levels (TSH ≥2.5 mIU/L) before pregnancy may increase the risk of adverse pregnancy outcomes (8, 9). However, whether to monitor and control TSH levels during assisted reproductive treatment remains unclear.

An increasing number of studies reported a higher incidence rate of hypothyroidism in women with infertility, a population that needs to achieve pregnancy using assisted reproductive technology (ART) (10). Controlled ovarian hyperstimulation (COH) is a necessary procedure in ART, and whether thyroid function should be monitored during and after COH is controversial. The most important question is whether the changes in thyroid hormone levels during COH affect ART and pregnancy outcomes. In a meta-analysis of 15 cohort studies, Li et al. found significantly increased serum TSH levels and significantly decreased free thyroxine (FT4) levels during COH (11). Another meta-analysis of 14 cohort studies, which failed to detect differences in serum FT4 levels, also showed a significant increase in serum TSH levels in patients after COH (12). Although alteration in serum FT4 levels during COH remains debatable, a significant increase in serum TSH levels after COH has been observed in many studies.

Most published studies focused on the association between TSH levels before COH on ART outcomes. However, no large-scale prospective studies have evaluated the impact of TSH levels after COH on ART outcomes, which may be very important in fresh embryo transfer (ET) cycles. To address this clinical question, we performed this prospective study to assess whether TSH levels after COH affect ART outcomes in women undergoing fresh ET.

2 Methods

2.1 Study population

We recruited 1698 infertile women who underwent the first or second *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) treatment and received fresh ET at Peking University Third Hospital between June and September 2021. Patients were eligible for this study if they were 20–40 years of age and scheduled for their first or second IVF/ICSI-ET cycle. Patients were excluded if they had a history of thyroid surgery, uterine malformations, hypothalamic or pituitary diseases, diabetes mellitus or other endocrinologic or metabolic diseases, recurrent spontaneous abortion (defined as three or more previous spontaneous pregnancy losses), and abnormal results on parental karyotyping. A total of 690 women were ultimately included (Figure 1). All patients were treated using

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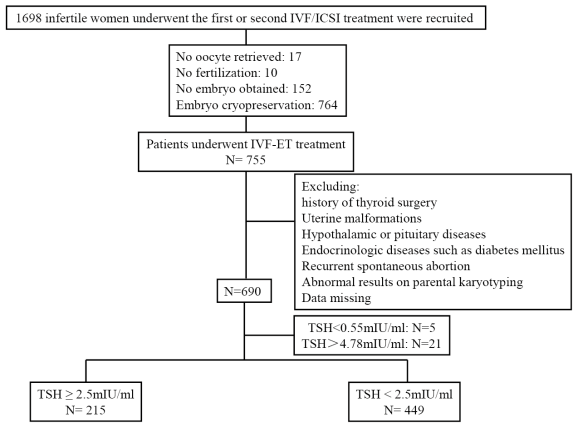

FIGURE 1
Flow chart of study cohort selection.

standard COH protocols, including the ultralong gonadotropin-releasing hormone (GnRH) agonist protocol, long GnRH agonist protocol, or the GnRH antagonist protocol according to the patients' conditions. Oocytes were retrieved 34–36 hours after administration of recombinant human chorionic gonadotropin (HCG). Insemination was performed 4–6 hours after oocyte retrieval by conventional IVF or ICSI, according to sperm quality. Up to two Day 3 embryos were transferred three days after oocyte retrieval.

2.2 Laboratory tests

Serum samples for thyroid hormone testing were collected after COH. Thyroid function measurements, including TSH, FT4, thyroid peroxidase antibody (TPOAb), and thyroglobulin antibody (TGAb) levels, were performed using a fully automatic chemiluminescence immunoassay analyzer (ADVIA Centaur XP, Siemens Healthcare Diagnostics). The reference range were 0.55–4.78 mIU/L for TSH and 0.89–1.80 ng/dL for FT4. The level below 60 IU/mL indicated negative for TPOAb and TGAb. Five patients with TSH <0.55 mIU/L and 21 patients with TSH >4.78 mIU/L were excluded. The patients were divided into a low-normal TSH group (TSH <2.5 mIU/L) and a high-normal TSH group (TSH ≥2.5 mIU/L).

2.3 Study outcomes

Clinical pregnancy was defined as the presence of at least one gestational sac in the uterus, identified using ultrasonography 35 days after ET. Miscarriage was defined as the loss of clinical pregnancy before 28 weeks of gestation. Live birth was defined as the delivery of at least one live fetus. Preterm delivery was defined as delivery before 37 weeks of gestation.

2.4 Statistical analyses

Continuous variables were expressed as means (standard deviations, SDs) for normally distributed data and as medians (interquartile ranges, IQRs) for data without a Gaussian distribution. Categorical variables were presented as numbers (percentages). Student's t-test was used to compare the differences in continuous variables with normal distribution between the two groups, and the Mann-Whitney U test was performed for continuous variables without a Gaussian distribution. Comparisons between categorical variables were performed using the Chi-squared test. Logistic regression analysis was conducted to calculate the odds ratios (ORs) with 95% confidence intervals (CIs) after adjusting for relevant factors. A two-sided P<0.05 indicated statistical significance. All statistical analyses were performed using IBM SPSS Statistics for Windows version 24.0 software (Armonk, NY: IBM Corp).

3 Results

A total of 664 women who underwent IVF/ICSI treatment and fresh ET were included in this study. According to the TSH levels on HCG trigger day, 449 women with TSH <2.5 mIU/L were defined as the low-normal TSH group, and 215 women with TSH ≥2.5 mIU/L were defined as the high-normal TSH group. The baseline features of the two groups were compared, and the results were summarized in Table 1. Women with TSH ≥2.5 mIU/L had significantly higher body mass index (BMI, P=0.034) than women with TSH <2.5 mIU/L. No statistically significant differences were noted between the two groups in age, type of infertility, primary cause of infertility, rate of thyroid antibody positivity, or markers relevant to ovarian reserve, such as basal follicle-stimulating hormone, luteinizing hormone, estradiol, and anti-Mullerian hormone levels. We also collected data on thyroid function within six months before COH. While there was no significant difference in the basal FT4 level between two groups, a significantly higher basal TSH level was observed in the high-normal TSH group than in the low-normal TSH group (P<0.001).

The group characteristics of COH and IVF are presented in Table 2. We observed a significant difference in the terms of COH protocols between the two groups. The number of patients using GnRH antagonist protocols was significantly higher in the low-normal TSH group than in the high-normal TSH group. In contrast, the proportion of patients using ultralong GnRH agonists in the high-normal TSH group was higher than that in the low-normal TSH group. Although the gonadotropin dose and the days of ovarian stimulation were significantly higher in women with TSH ≥2.5 mIU/L than in those with TSH <2.5 mIU/L (P=0.007 and 0.041, respectively), no significant differences were observed between the two groups in the hormone levels on the HCG trigger day, the number of retrieved oocytes, and the number of good-quality embryos.

There were no significant differences between the low-normal TSH and high-normal TSH groups in the rates of clinical pregnancy (50.1% vs. 44.2%, P=0.153), miscarriage (16.4% vs. 14.7%, P=0.703), and live birth (41.9% vs. 37.7%, P=0.303) (Table 3). Multiple logistic regression was performed to adjust for relevant confounding factors, and the results showed no significant differences between the two groups in the rates of clinical pregnancy (OR: 0.80, 95% CI:

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TABLE 1 Baseline characteristics of patients.

Characteristics	Low-normal TSH (n = 449)	High-normal TSH (n = 215)	P value
Age, mean (SD), years	31.9 (3.7)	32.3 (3.8)	0.194
BMI, mean (SD), kg/m^2	22.8 (3.5)	23.4 (3.6)	0.034*
Type of infertility, no. (%)			
Primary	281 (62.6)	130 (60.5)	0.599
Secondary	168 (37.4)	85 (39.5)	
Primary cause of infertility, no. (%) [a]			
Male factors	166 (37.0)	83 (38.6)	0.448
Female factors			
Tubal factor	142 (31.6)	71 (33.0)	
Polycystic ovary syndrome	41 (9.1)	25 (11.6)	
Endometriosis	37 (8.2)	11 (5.1)	
Unknown factors	63 (14.0)	25 (11.6)	
Basal FSH, mean (IQR), mIU/mL [b]	6.9 (5.7-8.2)	6.8 (5.6-8.5)	0.719
Basal LH, mean (IQR), mIU/mL	3.7 (2.5-5.0)	3.5 (2.3-5.0)	0.546
Basal estradiol, mean (IQR), pmol/L	146.5 (109.0-179.0)	141.0 (102.0-169.0)	0.136
Basal FT4, mean (SD), ng/dL [c]	1.2 (0.2)	1.2 (0.2)	0.628
Basal TSH, mean (SD), mIU/L	1.8 (0.8)	2.8 (0.9)	< 0.001*
AMH, mean (IQR), ng/mL	2.1 (1.2-3.4)	1.8 (1.1-3.3)	0.284
No. of thyroid antibody positivity	64 (14.3)	28 (13.0)	0.668

AMH, anti-Mullerian hormone; BMI, body mass index; COH, controlled ovarian hyperstimulation; FSH, follicle-stimulating hormone; FT4, free thyroxine; IQR, interquartile range; LH, luteinizing hormone; no., number; SD, standard deviation; TSH, thyroid-stimulating hormone.
[a]Primary cause of infertility indicates the most important cause for patients seeking *in vitro* fertilization/intracytoplasmic sperm injection treatment.
[b]Testing for basal FSH, LH, and estradiol levels was performed between day 2 and day 4 of the menstrual cycle.
[c]Data of basal FT4 and TSH were collected within 6 months before COH.
*P<0.05.

0.58–1.12, P=0.193), miscarriage (OR: 0.84, 95% CI: 0.42–1.68; P=0.619), and live birth (OR: 0.87, 95% CI: 0.62–1.23, P=0.434) after adjusting for age, BMI, thyroid antibody positivity, and COH protocols (Table 4). Surprisingly, a significantly higher rate of preterm delivery was observed in women with TSH <2.5 mIU/L compared with those with TSH ≥2.5 mIU/L (22.3% vs. 11.1%, P=0.031), and this difference existed after adjusting for age, BMI, thyroid antibody positivity and COH protocols (OR: 0.39, 95% CI: 0.18–0.86, P=0.020) (Tables 3, 4). In singleton or twin pregnancies, birth weight was not significantly different between the two groups.

4 Discussion

Currently, whether screening for thyroid function after COH is needed to initiate appropriate management and intervention remains controversial. To our knowledge, this is the first large-scale prospective study to assess the impact of maternal TSH levels after COH on IVF/ICSI outcomes in women undergoing fresh ET. In this cohort of women undergoing IVF/ICSI-ET treatment, women with TSH ≥2.5 mIU/L after COH had similar pregnancy outcomes to those of women with TSH <2.5 mIU/L. Unexpectedly, women with TSH <2.5 mIU/L had a higher preterm delivery risk, even after adjusting for relevant confounders, such as age, BMI, thyroid antibody positivity, and COH protocols. Our study suggests that TSH ≥2.5 mIU/L after COH is not a risk factor for poorer reproductive outcomes, and there is no need to strictly control TSH levels below 2.5 mIU/L after COH.

The impact of mildly elevated TSH levels on pregnancy outcomes has been debated for several years; most studies have focused on the impact of preconception TSH values on pregnancy outcomes. Two large-scale population-based cohort studies classified women into different groups according to TSH levels within 6 months before pregnancy and showed that even slightly elevated preconception TSH levels were associated with various adverse maternal outcomes, and recommended an optimal preconception TSH range between the lower reference limit and 2.50 mIU/L (8, 9). However, no large-scale study has investigated the effect of TSH levels after COH treatment on pregnancy outcomes.

COH is an important part of ART, which causes estradiol to rapidly rise to supraphysiological levels. Many studies have shown

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TABLE 2 Protocols of COH and data of IVF and ET.

Characteristics	Low-normal TSH (n = 449)	High-normal TSH (n = 215)	P value
Protocols of COH, no. (%)			
Ultralong GnRH agonist	25 (5.6)	24 (11.2)	0.035*
Long GnRH agonist	78 (17.4)	34 (15.8)	
GnRH antagonist	346 (77.1)	157 (73.0)	
Gonadotropin dose, median (IQR), IU	2625.0 (1875.0-3300.0)	2850.0 (2025.0-3900.0)	0.007*
No. of days of ovarian stimulation, median (IQR)	10.0 (9.0-12.0)	11.0 (9.0-13.0)	0.041*
LH level on HCG trigger day, median (IQR), mIU/mL	1.1 (0.5-2.3)	1.2 (0.5-2.4)	0.443
Estradiol level on HCG trigger day, median (IQR), pmol/L	6099.0 (4649.0-8449.0)	6637.0 (4883.0-8427.0)	0.186
Progesterone level on HCG trigger day, median (IQR), nmol/L	1.5 (1.3-1.9)	1.6 (1.3-1.9)	0.721
No. of retrieved oocytes per cycle, median (IQR)	9.0 (6.0-12.0)	9.0 (6.0-11.0)	0.632
No. of good-quality embryos, median (IQR)[a]	4.0 (2.0-5.0)	3.0 (2.0-5.0)	0.383
No. of embryo transferred, no. (%)			
1	57 (12.8)	21 (9.9)	0.278
2	389 (87.2)	192 (90.1)	

COH, controlled ovarian hyperstimulation; IVF, *in vitro* fertilization; ET, embryo transfer; GnRH, gonadotropin-releasing hormone; HCG, human chorionic gonadotropin; IQR, interquartile range; LH, luteinizing hormone; no., number; SD, standard deviation; TSH, thyroid-stimulating hormone.
[a]The embryos were evaluated on the third day after fertilization. Good-quality embryos were developed from two pronuclei zygotes and met the following criteria: (1) had more than five blastomeres; (2) size difference <20%; and (3) fragmentation <50%.
*P<0.05.

an association between COH and changes in thyroid function (11, 12). The underlying mechanism involves an increase in thyroid-binding globulin levels triggered by supraphysiologic estradiol levels, which reduces free thyroid hormone concentrations and, in turn, triggers serum TSH elevation. Unlike previous studies, our study analyzed TSH levels after COH instead of before, which may better reflect thyroid function before fresh ET. We did not find statistically significant differences in the rates of clinical pregnancy, miscarriage, or live birth between the low-normal and high-normal TSH groups. Our study agrees with several previous studies that showed an association between TSH levels before COH and IVF/ICSI outcomes in infertile women. Based on a secondary data analysis of 1468 infertile women, Seungdamrong et al. showed that preconception TSH ≥2.5 mIU/L did not affect conception, clinical pregnancy, miscarriage, and live birth rates in infertile women without reproductive treatment (13). Two other studies, which restricted the patient populations to infertile women undergoing IVF/ICSI treatment, also found no significant

TABLE 3 Pregnancy outcomes of women with low-normal TSH levels and those with high-normal TSH levels.

Outcomes	Low-normal TSH (n=449)	High-normal TSH (n=215)	P value
Clinical pregnancy [a], no. (%)	225/449 (50.1)	95/215 (44.2)	0.153
Miscarriage [b], no. (%)	37/225 (16.4)	14/95 (14.7)	0.703
Live birth [c], no. (%)	188/449 (41.9)	81/215 (37.7)	0.303
Preterm delivery [d], no. (%)	42/188 (22.3)	9/81 (11.1)	0.031*
Birth weight, g			
Singleton	3300.0 (3000.0-3600.0)	3350.0 (2990.0-3600.0)	0.908
Twin	2550.0 (2300.0-2745.0)	2525.0 (2150.0-2703.8)	0.365

No., number; TSH, thyroid-stimulating hormone.
[a]Clinical pregnancy was defined as the presence of at least one gestational sac in the uterus identified using ultrasonography 35 days after embryo transfer.
[b]Miscarriage was defined as the loss of a clinical pregnancy before 28 weeks of gestation. The miscarriage rate was defined as the proportion of women with miscarriage among women with clinical pregnancy.
[c]Live birth was defined as the delivery of at least one living fetus.
[d]Preterm delivery was defined as delivery before 37 weeks. The preterm delivery rate was defined as the proportion of women with preterm delivery among women with live birth.
*P < 0.05.

TABLE 4 Multivariate logistic regression analysis of factors associated with pregnancy outcomes.

Factors	Clinical pregnancy		Miscarriage		Live birth		Preterm delivery	
	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value
Age, years	0.95 (0.91-0.99)	0.011*	1.11 (1.01-1.21)	0.023*	0.93 (0.89-0.97)	0.001*	0.97 (0.88-1.06)	0.435
BMI, kg/m^2	0.98 (0.94-1.03)	0.455	1.07 (0.98-1.16)	0.160	0.97 (0.93-1.02)	0.200	1.07 (0.98-1.17)	0.146
TSH on HCG trigger day								
Low-normal (reference)	NA	NA	NA	NA	NA	NA	NA	NA
High-normal	0.80 (0.58-1.12)	0.193	0.84 (0.42-1.68)	0.619	0.87 (0.62-1.23)	0.434	0.39 (0.18-0.86)	0.020*
Thyroid antibody positivity	1.00 (0.64-1.56)	0.998	0.31 (0.09-1.05)	0.060	1.25 (0.80-1.96)	0.332	1.54 (0.68-3.46)	0.300
Protocols								
Ultralong GnRH agonist	1.30 (0.72-2.36)	0.383	1.17 (0.40-3.42)	0.774	1.17 (0.64-2.15)	0.613	1.44 (0.43-4.77)	0.554
Long GnRH agonist	1.27 (0.84-1.92)	0.252	0.99 (0.44-2.21)	0.974	1.25 (0.82-1.90)	0.296	1.09 (0.49-2.41)	0.834
GnRH antagonist	NA	NA	NA	NA	NA	NA	NA	NA

BMI, body mass index; CI, confidence interval; GnRH, gonadotropin-releasing hormone; HCG, human chorionic gonadotropin; NA, not available; OR, odds ratio; TSH, thyroid-stimulating hormone.
*P<0.05.

differences in the rates of clinical pregnancy, miscarriage, and live birth between the TSH <2.5 mIU/L and TSH ≥2.5 mIU/L groups (14, 15).

Most previous studies focused on the impact of the mildly elevated TSH levels on adverse ART outcomes and some guidelines recommended to maintain TSH levels below 2.5 mIU/L before pregnancy. In our study, no significant difference was found in the rates of clinical pregnancy, miscarriage and live birth between women with TSH ≥2.5 mIU/L and women with TSH <2.5 mIU/L, however, we observed a significantly higher rate of preterm delivery in women with TSH <2.5 mIU/L than in those with TSH ≥2.5 mIU/L, even after adjusting for age, BMI, thyroid antibody positivity, and COH protocols, which suggest that strictly control TSH levels below 2.5 mIU/L may trigger a detrimental effect on assisted reproductive outcomes. Our results were supported by a recent large-scale cohort study, which enrolled 175,112 women and performed a restricted cubic spline (RCS) regression model with multiple percentiles of TSH level to analyze the association between preconception TSH levels and adverse pregnancy outcomes. The study showed lower TSH was associated with a higher OR of preterm delivery (nonlinear P<0.001) (16). However, some previous studies based on TSH levels before COH showed inconsistent results. In a retrospective study, Zhang et al. reported no significant difference in the rates of preterm delivery between women with low TSH levels (TSH <2.5 mIU/L) and those with mildly elevated TSH levels (TSH ≥2.5 mIU/L) (14). Another retrospective study showed a significantly lower gestational age at delivery and lower birth weight in women with TSH ≥2.5 mIU/L than in those with TSH <2.5 mIU/L before COH (17). The controversial conclusions may result from the different timing of TSH test and screening for TSH levels after COH may be necessary in fresh ET cycles.

In our study, mildly elevated TSH levels (TSH ≥2.5 mIU/L) after COH did not affect IVF/ICSI outcomes. However, the rate of preterm delivery significantly increased in women with TSH <2.5 mIU/L. Based on our study, TSH levels after COH should not be strictly reduced to below 2.5 mIU/L. All assays for TSH and antithyroid antibodies in our study were performed in the same standardized laboratory, which reduced the potential inter-assay variability. However, our study also had some limitations. First, we only focused on TSH levels after COH and did not collect longitudinal TSH measurements throughout pregnancy. However, many previous studies have already shown the impact of TSH levels during different stages of pregnancy on reproductive outcomes (18). Second, we did not investigate the impact of the use of levothyroxine because of marked differences in the initiation time of thyroid hormone replacement. As such, further randomized controlled trials may be needed. Furthermore, we did not analyze additional pregnancy complications, such as gestational hypertension and gestational diabetes mellitus, because of the relatively small sample size. Further prospective studies with larger sample sizes are required to confirm these findings.

5 Conclusions

To our knowledge, our study is the first large-scale prospective study to demonstrate that mildly elevated TSH levels after COH may not adversely affect IVF/ICSI outcomes in women who receive fresh ET, that TSH ≥2.5 mIU/L after COH is not a risk factor for poorer reproductive outcomes, and that strict control of TSH levels to levels below 2.5 mIU/L after COH may not be necessary.

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 Peking University Third Hospital Medical Science Research Ethics Committee. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

NH took part in patient follow-up and wrote the initial draft of the paper. NH, LC and YL contributed to the data analysis. HC and JQ contributed to the conception and design of the study. All authors contributed to the research discussion and manuscript revision.

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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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Sequential embryo transfer versus double cleavage-stage embryo or double blastocyst transfer in patients with recurrent implantation failure with frozen-thawed embryo transfer cycles: a cohort study

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Background: Recurrent implantation failure (RIF) is more common among patients receiving assisted reproductive treatment. Many efforts have been made to increase the incidence of clinical pregnancy among patients with RIF. The effect of the sequential transfer procedure, a two-step interval transfer of a cleavage-stage embryo followed by a blastocyst in one transfer cycle, on the clinical outcomes of RIF patients remains controversial.

Methods: In total, 1774 frozen-thawed embryo transfer (FET) cycles in RIF patients were included. Of these cycles, 302 were sequential embryo transfer (ET) cycles, 979 were double day 3 cleavage-stage ET cycles, and 493 were double blastocyst ET cycles. The primary outcomes were the rates of implantation, clinical pregnancy and multiple pregnancy, and the secondary outcomes were the rates of hCG positive, early miscarriage and ectopic pregnancy.

Results: The implantation, hCG positive, and clinical pregnancy rates in the sequential ET group (32.1%, 58.9%, 50.7%) were significantly higher than those in the day 3 cleavage-stage ET group (24.9%, 46.5%, 40.4%) and were similar to those in the blastocyst transfer group (30.1%, 56.4%, 47.1%). The early miscarriage rate in the blastocyst transfer group was significantly higher than that in the cleavage-stage ET group (17.2% vs. 8.1%, P <0.05), while the ectopic pregnancy rate in the blastocyst transfer group was significantly lower than that in the cleavage-stage ET group (0.4% vs. 3.0%, P <0.05). The multiple pregnancy rate in the sequential ET group was significantly lower than that in the cleavage-stage ET group (17.0% vs. 25.5%, P

<0.05) and the blastocyst transfer group (17.0% vs. 27.6%, *P* <0.05). When cycles of blastocyst culture failure were excluded, the clinical pregnancy rate was significantly higher (55.7% vs. 47.1%, *P* <0.05), and the early miscarriage rate and multiple pregnancy rate were significantly lower (8.5% vs. 17.2%, 17.7% vs. 27.6%; *P* <0.05, respectively) in the sequential ET group than in the double blastocyst ET group.

Conclusions: Sequential embryo transfer in FET cycles could improve the clinical outcomes of patients with RIF.

KEYWORDS

repeated implantation failure, frozen-thawed embryo transfer, sequential embryo transfer, cleavage-stage embryo transfer, blastocyst transfer

Introduction

Since 1978, many infertile couples have benefited from assisted reproductive technology (ART). As of 2019, more than 8 million children had been born after ART worldwide. Over 2.5 million *in vitro* fertilization (IVF) cycles are performed every year, resulting in over 500,000 deliveries annually (1). However, the best embryo implantation rate ranges from 25-40% (2, 3). Improving the success rate is a challenging problem associated with ART treatment programs.

Repeated implantation failure (RIF) refers to a situation when the transferred embryos fail to implant after at least three IVF-embryo transfer (IVF-ET) cycles with 1-2 high-quality embryos in each cycle (4, 5). The prevalence of RIF is 8-15% (6–8), which poses great difficulties and challenges to clinicians and embryologists. Recurrent failures of IVF-ET also bring psychological, physical, and financial distress to patients.

High-quality embryos, a receptive endometrium, and good synchrony between the embryo and endometrium are necessary conditions for successful implantation (9). The implantation process involves three phases: apposition, adhesion, and invasion. During these stages, the cross-talk between the endometrium and embryo is significant, and suboptimal endometrial receptivity is the most critical cause of RIF (10).

In recent years, scientists have proposed a sequential transfer procedure, a two-step interval transfer of a cleavage-stage embryo followed by a blastocyst in one transfer cycle to help RIF patients increase the chance of pregnancy. Several studies have suggested that sequential ET significantly improves the clinical outcomes of IVF-ET (11–13). However, the effect of sequential ET is still controversial, and its effectiveness and potential biological mechanisms have not been proven.

To further confirm the effect of sequential ET, this study analyzed the data of sequential ET at our reproductive center to evaluate the effect of sequential ET on the clinical outcomes of patients with a history of RIF in frozen-thawed embryo transfer (FET) cycles. The primary outcome measures were implantation rate, clinical pregnancy rate and multiple pregnancy rate. The secondary outcome measures were hCG positive rate, early miscarriage rate and ectopic pregnancy rate.

Patients and methods

This retrospective observational study was performed at the Reproductive Center of Peking University Third Hospital from January 2020 to June 2022. Patients who had not conceived after three or more ET cycles and undergone sequential ET (one day 3 cleavage-stage embryo followed by a day 5/6 blastocyst in one FET cycle) were included (302 cycles). Two groups based on the ET strategy were adopted as the control groups: the double cleavage-stage embryo (day 3) transfer group (979 cycles) and the double blastocyst (day 5/6) transfer group (493 cycles). Among these participants, those employing PGT for chromosomal structural rearrangements (PGT-SR) or monogenic/single gene defects (PGT-M), those using egg donor cycles, those with a thin endometrium (thickness less than 6 mm) and those with autoimmune diseases were excluded.

Frozen-thawed embryo transfer procedure

All the included cycles were FET cycles. Endometrial preparation for FET was performed as previously described (14), and the preparation method was the artificial (hormone replacement) cycle, natural cycle, or stimulation cycle. On the transfer day, embryo grading was performed. Cleavage-stage embryos were evaluated according to the criteria of the Istanbul Embryo Evaluation Symposium (15), and blastocysts were evaluated using the Gardner grading system (16). The embryos were transferred using the Cook Sydney IVF catheter (k-jets-7019-SIVF). In the sequential ET group, one of the frozen-thawed embryos was transferred on day 3, whereas

the rest were cultured; then, one blastocyst was transferred on day 5 or day 6. In the cleavage-stage ET group, double embryos were transferred on day 3, while in the blastocyst ET group, double blastocysts were transferred on day 5.

Outcome measures

Serum β-hCG levels were measured 14-21 days after ET, with β-hCG levels ≥25 IU/L being defined as biochemical pregnancy, also named hCG positive. Clinical pregnancy is defined as the presence of an intrauterine gestational sac on ultrasonography. The implantation rate was defined as the number of gestational sacs divided by the total number of embryos or blastocysts transferred. Early miscarriage was defined as loss of the clinical pregnancy within 12 weeks of gestation. Ectopic pregnancy was defined as an extrauterine pregnancy. Multiple pregnancy was defined as the presence of two or more gestational sacs on ultrasound, and the rate was calculated as the number of multiple pregnancy cycles divided by the number of clinical pregnancy cycles.

Statistical analysis

Data analysis was performed using SPSS statistics software version 23 (IBM). The continuous variables are presented as the means ± standard deviations (SDs). One-way ANOVA was used for continuous variables that had a normal distribution, while the Kruskal–Wallis test was performed for nonnormally distributed continuous data. Categorical variables are presented as counts and percentages. The chi-square test was applied to test categorical variables. In the multivariate logistic regression analysis, FET groups, parental age, infertility duration, cycles of implantation failure, insemination methods, endometrial preparation methods and endometrial thickness were included, and adjusted odds ratios (ORs) and 95% confidence intervals (95% CIs) were reported. A P value of < 0.05 was considered statistically significant for all tests.

Results

Characteristics of sequential embryo transfer cycles

A total of 302 sequential ET cycles were included in this study. Of these, there were 5 cycles with no embryo to culture after cleavage-stage ET, resulting in 2 cycles of cleavage-stage ET only and 3 cycles of cleavage-stage ET followed by frozen-thawed blastocyst transfer. There were 44 cycles in which the remaining frozen-thawed cleavage-stage embryos were not cultured into blastocysts after cleavage-stage ET (including 32 cycles of cleavage-stage ET only and 12 cycles of cleavage-stage ET followed by frozen-thawed blastocyst transfer). Overall, 253 cycles were completed successfully with day 3 cleavage-stage ET followed by cultured blastocyst transfer (Table 1). In total, day 3 cleavage-stage embryo and cultured blastocyst transfer cycles, day 3 cleavage-stage embryo and frozen-thawed blastocyst transfer cycles, and day 3 cleavage-stage ET only cycles accounted for 83.8%, 5.0%, and 11.3% of total sequential ET cycles, respectively (Figure 1).

In summary, of the 302 sequential ET cycles performed, cleavage-stage embryos were transferred in only 34 (11.3%) cycles due to the lack of embryos for culture after ET (2 cycles) or failure of the remaining embryos to form blastocysts (32 cycles). There were fifteen (5.0%) cycles of cleavage-stage ET followed by frozen-thawed blastocyst transfer. In addition, 253 (83.8%) cycles were completed successfully with day 3 cleavage-stage ET followed by cultured blastocyst transfer.

TABLE 1 Cycle characteristics of sequential embryo transfer of FET.

Sequential embryo transfer	Cycles (n)	Clinical pregnancy n (%)
Total cycles	**302**	**153(50.7%)**
No embryo to culture after a cleavage-stage embryo transfer	**5**	**1(20%)**
A cleavage-stage embryo transfer only	2	0 (0)
A cleavage-stage embryo transfer followed by a frozen-thawed blastocyst transfer	3	1(33.3%)
Frozen-thawed cleavage-stage embryos did not form blastocysts after a cleavage-stage embryo transfer	**44**	**11(25%)**
A cleavage-stage embryo transfer only	32	8(25%)
A cleavage-stage embryo transfer followed by a frozen-thawed blastocyst transfer	12	3(25%)
A cleavage-stage embryo transfer followed by a cultured blastocyst transfer	**253**	**141(55.7%)**

The bold values in Table 1 indicate the primary classification; non-bold values are the further subgroups.

Demographic characteristics of the three groups

We compared the baseline data, including parental age, female BMI, infertility duration, primary infertility ratio, previous failed cycles, insemination method, endometrial preparation protocols, and endometrial thickness on FET among the three groups. The infertility duration was significantly shorter (4.91 ± 3.29 years vs.

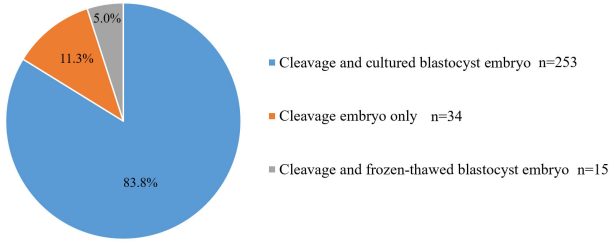

FIGURE 1
The cycles of different transferred embryos in the sequential ET.

5.52 ± 3.27 years & 5.73 ± 3.46 years), and the number of cycles of previous failure were significantly lower (3.14 ± 1.69 vs. 4.83 ± 2.36 & 4.07 ± 2.00) in the cleavage-stage ET group than in the sequential ET and blastocyst transfer groups. The proportion of artificial cycles was significantly higher (65.9% vs. 53.7% & 56.2%) in the sequential ET group than in the cleavage-stage ET and blastocyst transfer groups. There were no significant differences in parental age, female BMI, primary infertility ratio, insemination method, and endometrial thickness on FET among the three groups (Table 2).

FET outcomes

Compared to those in the cleavage-stage ET group, the implantation, hCG positive, and clinical pregnancy rates were significantly higher in the sequential ET group and the blastocyst transfer group. There was no significant difference in the implantation rate, hCG positive rate, or clinical pregnancy rate between the sequential ET group and the blastocyst transfer group. The early miscarriage rate in the blastocyst transfer group was significantly higher than that in the cleavage-stage ET group, while the ectopic pregnancy rate in the blastocyst transfer group was significantly lower than that in the cleavage-stage ET group. The early miscarriage rate and ectopic pregnancy rate in the sequential ET group were not significantly different from the rates in the other two groups. The multiple pregnancy rate in the sequential ET group was significantly lower than that in the cleavage-stage ET group and the blastocyst transfer group (Figure 2).

We then compared the clinical outcomes of cycles in which sequential ET was completed successfully (a day 3 cleavage-stage ET followed by a cultured blastocyst transfer) with those of cycles in which blastocyst transfer had been completed successfully. The clinical pregnancy rate in the successfully completed sequential ET group was significantly higher than that in the blastocyst transfer group, while the early miscarriage rate and multiple pregnancy rate in the successfully completed sequential ET group were significantly lower than those in the blastocyst transfer group. There was no significant difference in the implantation rate, hCG positive rate, or ectopic pregnancy rate between the two groups (Figure 3).

Multiple logistic regression analysis with adjustments for possible confounders for clinical pregnancy, early miscarriage, and multiple pregnancy was used to evaluate the effectiveness of sequential ET (Table 3). Adjustments were made for confounding variables including FET group, parental age, infertility duration, cycles of implantation failure, insemination methods, endometrial preparation methods, and endometrial thickness. Compared to sequential ET and double blastocyst transfer, double cleavage-stage ET had a significantly lower clinical pregnancy rate (OR 0.610, 95% CI 0.432-0.861, *P*=0.005; OR 0.697, 95% CI 0.531-0.914, *P*=0.009, respectively). Endometrial thickness was associated with clinical pregnancy (OR 1.087, 95% CI 1.016-1.164, *P*=0.016). Double cleavage-stage ET had a lower early miscarriage rate than double blastocyst transfer (OR 0.439, 95% CI 0.240-0.800, *P*=0.007), female age had a significantly negative effect on early miscarriage (OR 1.162, 95% CI 1.040-1.299, *P*=0.008), and double blastocyst transfer had a significantly higher multiple pregnancy rate than sequential ET (OR 1.860, 95% CI 1.012-3.418, *P*=0.046).

TABLE 2 Demographic and cycle characteristics among the three groups according to embryo transfer of FET.

Variable	Sequential embryo transfer (n = 302)	Cleavage-stage embryo transfer (n = 979)	Blastocyst embryo transfer (n = 493)	*P* value
Female age (years)	34.05 ± 4.51	33.63 ± 4.27	33.67 ± 4.01	0.302
Male age (years)	35.13 ± 6.82	34.94 ± 5.34	34.88 ± 5.13	0.291
Body mass index	22.39 ± 3.48	22.37 ± 3.45	22.36 ± 3.23	0.993
Infertility duration (years)	5.52 ± 3.27	4.91 ± 3.29	5.73 ± 3.46	**<0.001***
Primary infertility ratio (n, %)	201 (66.6%)	650 (66.4%)	320 (64.9%)	0.831
Previous failed cycles (n)	4.83 ± 2.36	3.14 ± 1.69	4.07 ± 2.00	**<0.001***
Insemination method (n, %)				0.246
IVF	202 (66.9%)	603 (61.6%)	312 (63.3%)	
ICSI	100 (33.1%)	376 (38.4%)	181 (36.7%)	
Endometrial preparation (n, %)				**0.006***
Artificial cycle	199(65.9%)	526 (53.7%)	277(56.2%)	
Natural cycle	88 (29.1%)	378 (38.6%)	185 (37.5%)	
Stimulation cycle	15 (5.0%)	75 (7.7%)	31 (6.3%)	
Endometrial thickness on FET (mm)	9.99 ± 1.73	9.98 ± 1.79	10.07 ± 1.67	0.757

IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; FET, frozen-thawed embryo transfer; *P<0.05.
The bold values in Tables 2, 3 indicate significant statistical differences among groups (P <0.05).

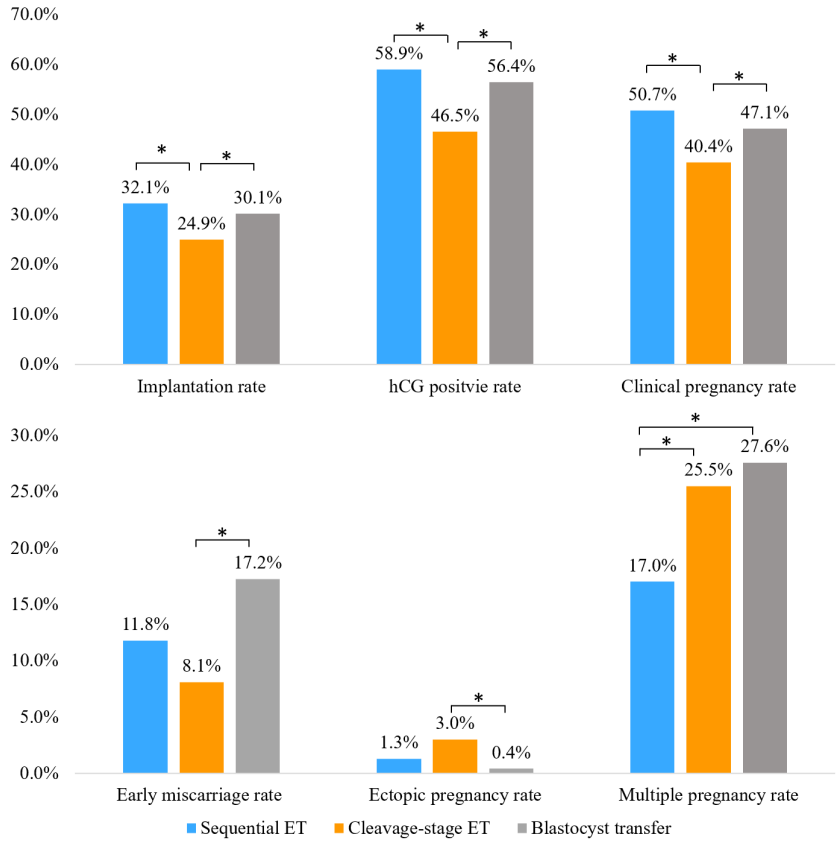

FIGURE 2
Clinical outcomes of patients in each group. *P<0.05.

Discussion

Sequential ET was first performed by Abramovici et al. in 1988 since embryo freezing was not an option in their IVF-ET program at that time (17). Since then, some clinicians have tried to use a sequential ET approach to help patients with RIF increase their chances of pregnancy. Some studies showed that sequential ET did not improve clinical outcomes for patients with RIF (18–20), while other studies suggested that it was more effective toward increasing the clinical pregnancy and live birth rates than day 3 or day 5 ET in these patients (11–13). The study by Ji et al. included patients with a history of RIF undergoing FET cycles (18), and studies by Tehraninejad et al. (20) and Kyono et al. (19) included patients undergoing IVF fresh cycles and showed that sequential embryo transfer failed to increase the chance of pregnancy. A retrospective cohort study by Stamenov et al. (12) and a prospective and

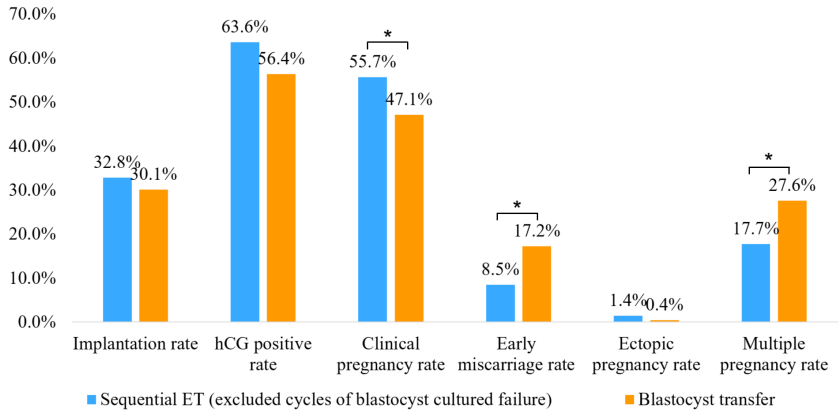

FIGURE 3
Comparison of clinical outcomes between successfully completed sequential embryo transfer group (day 3 cleavage-stage embryo transfer followed by a cultured blastocyst transfer) and double blastocyst transfer group. *P<0.05.

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TABLE 3 Multivariate logistic regression analysis of clinical outcomes after adjustment.

	Adjusted OR	95% CI	*P* value
Clinical pregnancy			
FET group (cleavage vs. sequential)	0.610	0.432-0.861	**0.005***
FET group (blastocyst vs. sequential)	0.875	0.612-1.251	0.465
FET group (cleavage vs. blastocyst)	0.697	0.531-0.914	**0.009***
Female age	0.965	0.924-1.007	0.104
Male age	0.983	0.951-1.016	0.317
Infertility duration	0.964	0.927-1.003	0.069
Previous failed cycles	1.005	0.942-1.072	0.880
Insemination method (ICSI vs. IVF)	0.979	0.766-1.252	0.866
Endometrial preparation (natural vs. artificial)	1.169	0.911-1.500	0.222
Endometrial preparation (stimulation vs. artificial)	0.864	0.528-1.415	0.562
Endometrial preparation (natural vs. stimulation)	1.352	0.815-2.243	0.242
Endometrial thickness on FET	1.087	1.016-1.164	**0.016***
Early miscarriage			
Group of FET (cleavage vs. sequential)	0.790	0.349-1.788	0.572
Group of FET (blastocyst vs. sequential)	1.802	0.824-3.942	0.140
Group of FET (cleavage vs. blastocyst)	0.439	0.240-0.800	**0.007***
Female age	1.162	1.040-1.299	**0.008***
Male age	0.929	0.850-1.017	0.110
Infertility duration	0.992	0.904-1.089	0.868
Previous failed cycles	0.862	0.731-1.018	0.079
Insemination method (ICSI vs. IVF)	1.197	0.694-2.063	0.518
Endometrial preparation (natural vs. artificial)	0.595	0.329-1.076	0.086
Endometrial preparation (stimulation vs. artificial)	1.596	0.599-4.322	0.358
Endometrial preparation (natural vs. stimulation)	0.373	0.129-1.073	0.067
Endometrial thickness on FET	0.978	0.842-1.137	0.776
Multiple pregnancy			
Group of FET (cleavage vs. sequential)	1.315	0.714-2.421	0.380
Group of FET (blastocyst vs. sequential)	1.860	1.012-3.418	**0.046***
Group of FET (cleavage vs. blastocyst)	0.707	0.448-1.115	0.135
Female age	0.950	0.872-1.034	0.234
Male age	0.994	0.929-1.065	0.871
Infertility duration	1.008	0.935-1.086	0.837
Previous failed cycles	1.025	0.916-1.147	0.665
Insemination method (ICSI vs. IVF)	0.786	0.513-1.205	0.270
Endometrial preparation (natural vs. artificial)	0.950	0.623-1.449	0.811
Endometrial preparation (stimulation vs. artificial)	0.319	0.093-1.089	0.068
Endometrial preparation (natural vs. stimulation)	2.980	0.860-10.326	0.085
Endometrial thickness on FET	0.951	0.844-1.075	0.434

IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; FET, frozen-thawed embryo transfer; OR, odds ratio; CI, confidence interval; *P<0.05.
The bold values in Tables 2, 3 indicate significant statistical differences among groups (P <0.05).

randomized trial by Torky et al. (13) showed that the implantation and clinical pregnancy rates were significantly higher in patients who underwent sequential embryo transfer than in those who underwent cleavage transfer on day 3 or blastocyst transfer on day 5 in IVF fresh cycles. Arefi et al. (11) conducted a prospective study to evaluate the improvement of pregnancy rate in sequential FET on day 3/day 5 in individuals who suffered from RIF and suggested that sequential transfer was more effective than regular day 5. A systematic review by Zhang et al. demonstrated that the clinical pregnancy rate and live birth rate were higher in the sequential ET group than in the cleavage-stage ET group for women who experienced RIF, and there were no significant differences between sequential ET and blastocyst ET (21). Even in patients with poor ovarian response, a report showed that sequential transfer had a higher live birth rate than day 3 ET and had a similar live birth rate to blastocyst transfer in the FET cycle (14). However, the sample size in each study was less than 150 subjects. In the present retrospective study, we included 302 cycles as the observation group. We also included 979 cycles of double day 3 ET and 493 cycles of double blastocyst transfer as the control groups. All of the patients had a history of three consecutive implantation failures and underwent FET cycles. We found that sequential ET had higher rates of implantation and pregnancy than conventional cleavage-stage ET. In addition, the rates of implantation and pregnancy in the sequential ET group were similar to those in the double blastocyst transfer group without increasing the risk of early miscarriage or ectopic pregnancy. This finding suggests that sequential ET may be effective in improving pregnancy outcomes in patients with RIF.

There are several advantages and potential mechanisms in sequential ET. First, successful embryo implantation requires synchronous interactions between endometrial receptivity and embryos with high developmental potential. During the implantation process, many molecular mediators, including cytokines, lipids, adhesion molecules, growth factors, and others, support the establishment of pregnancy (9). Endometrial injury by biopsy catheters during the luteal phase of the menstrual cycle has been shown to improve implantation and pregnancy rates in subsequent treatment cycles (22). During the window of implantation, endometrial preparation is guided not only by maternal factors but also by molecules secreted by the embryo, such as chorionic gonadotropin and interleukin-1β (IL-1 β) (23). Therefore, after the first ET procedure of sequential ET, mechanical microstimulation caused by catheter insertion and cytokines produced by the embryo and endometrium may not only be a benefit for the implantation of the first transferred embryo but could also promote better implantation conditions and increase the implantation probability following blastocyst transfer (24). Second, the window of implantation is transient in humans, and implantation beyond this window results in pregnancy failure (25). Mechanical stimulation of the endometrium may slightly alter the implantation window for personalized ET, which has a beneficial effect on the receptive endometrium (26). In addition, sequential ET can probably extend the availability time for transferred embryos to access the implantation window. Moreover, compared with double cleavage-stage day 3 ET, cleavage-stage embryos cultured *in vitro* from 3 days to 5-6 days could be screened to identify embryos with higher implantation potential, resulting in a higher pregnancy rate (27).

Compared with double blastocyst transfer, sequential ET can decrease the risk of ET cycle cancelation since prolonged culture may result in a lack of available blastocysts for transfer. In this study, the clinical pregnancy rate was approximately 25%, even when only a single day 3 embryo was transferred during sequential ET cycles. For patients with many embryos that have good developmental potential, sequential ET is likely to help them improve clinical outcomes. Our study showed that the clinical pregnancy rate was significantly higher and that the early miscarriage rate was significantly lower in the successfully completed sequential ET treatment (a day 3 cleavage-stage ET followed by transfer of a cultured blastocyst) than in the double blastocyst ET treatment. This further suggests that the first day 3 cleavage-stage ET procedure in sequential ET could probably improve the clinical outcomes of ART treatment.

The prevalence of multiple pregnancy is higher with ART than with natural pregnancy (28). This is related to the number of ETs, and there is a consequent impact on maternal and newborn outcomes (29, 30). In the last two decades, controlling the number of embryos transferred (single ET per cycle) has been advocated to reduce the risks of multiple gestations (28, 31). However, compared to the successive failure of IVF-ET, increasing the probability of pregnancy is more beneficial to patients economically and psychologically, even though it sometimes increases the risk of multiple pregnancy. In the case of multiple pregnancy, multifetal pregnancy reduction is also an option for patients to reduce the risk of maternal and fetal complications (32). Therefore, transferring two embryos is a strategy used for patients with RIF. In contrast to previous studies, this study showed that the multiple pregnancy rate in the sequential ET group was significantly lower than that in the double cleavage-stage ET group and the double blastocyst group. This seems to be another benefit of sequential ET. However, further studies are needed to investigate and define multiple pregnancy occurrence in sequential ET.

The larger population and two types of ET (double cleavage-stage embryos and double blastocysts) as controls are the strengths of our study. However, it also had some limitations, including its retrospective nature and lack of data related to live birth. Prospective studies are needed to identify this scientific issue to meet the clinical demand.

In conclusion, this study investigated the value of sequential ET in patients with RIF in FET cycles. Sequential ET was associated with a higher implantation rate, hCG positive rate, and clinical pregnancy rate than double cleavage-stage ET and comparable to those of the double blastocyst-stage ET group without increasing the risk of early miscarriage or ectopic pregnancy. Sequential ET had a lower multiple pregnancy rate than double cleavage-stage ET and double blastocyst-stage ET. In addition, the clinical pregnancy rate was significantly higher, and the early miscarriage rate was significantly lower in the

sequential ET than in the double blastocyst-stage ET when cycles of blastocyst culture failure in the sequential ET group were excluded. These findings suggested that sequential ET is an effective and beneficial option for patients with RIF.

Data availability statement

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

Ethics statement

The studies involving humans were approved by Peking University Third Hospital Medical Science Research Ethics Committee (M2022128). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

HW and XL conceived the idea. JG and JL reviewed the literature and designed the study. YY and TT collected the data and conducted the analysis. JG designed the figures and tables and wrote the manuscript. YL, PL, RL, JQ, HW and XL coordinated 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

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Diagnostic workup of endocrine dysfunction in recurrent pregnancy loss: a cross-sectional study in Northeast China

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Objective: To evaluate the prevalence of abnormal endocrine dysfunction for recurrent pregnancy loss (RPL) amongst patients with two versus three or more pregnancy losses.

Methods: This cross-sectional study retrospectively collected pre-pregnancy data of 537 women diagnosed with RPL in Shengjing Hospital of China Medical University from 2017 to 2022, including the baseline data of patients and the test results of endocrine factors. Several endocrine dysfunction included in this study were: thyroid dysfunction, obesity, hyperprolactinemia, polycystic ovary syndrome and blood glucose abnormality. Furthermore, vitamin D level were collected to study its relationship with endocrine dysfunction. Finally, we subdivided the patients according to the number of previous pregnancy loss and compared the prevalence of endocrine dysfunction between subgroups.

Results: Among 537 RPL patients, 278 (51.8%) patients had abnormal endocrine test results. The highest incidence of endocrine dysfunction was thyroid dysfunction (24.39%, 131/537), followed by hyperprolactinemia (17.34%, 85/490), obesity (10.8%, 58/537), polycystic ovary syndrome (10.50%, 56/533), and abnormal blood glucose (5.29%, 27/510). Only 2.47%(13/527) of patients have vitamin D level that reach the standard. After subdividing the population according to the number of pregnancy loss, we did not find that the incidence of endocrine dysfunction (P=0.813), thyroid dysfunction (P=0.905), hyperprolactinemia (P=0.265), polycystic ovary syndrome (P=0.638), blood glucose abnormality (P=0.616) and vitamin D deficiency (P=0.908) were different among patients with two versus three or more pregnancy losses. However, obesity (P=0.003) was found more frequently observed in patients with more times of pregnancy loss.

Conclusion: The prevalence of endocrine dysfunction in RPL population is high. There is no difference in the prevalence of endocrine dysfunction, except for obesity, among patients with two or more pregnancy losses, which may suggest investigations of endocrine dysfunction when patients have two pregnancy losses.

KEYWORDS

recurrent miscarriage, PCOS, hyperprolactinemia, obesity, vitamin D, thyroid dysfunction, blood glucose abnormality

Introduction

Recurrent miscarriage can be devastating for women who wish to have children, with a global prevalence ranging from 1 to 3% (1). Individuals who experience recurrent miscarriage are at increased risk of many obstetric complications, as are the emotional and psychological harms of miscarriage. This is not the end of the physical and psychological consequences of recurrent miscarriages, since complex etiologic screening and expensive systemic therapy can also be financially stressful for a family (2). Endocrinological factors are now a critical component of the screening process used by clinicians to screen patients for recurrent miscarriage. The secretion of hormones such as thyroid stimulating hormone and prolactin has an irreplaceable impact on pregnancy outcomes. Such as prolactin, it may play an important role in maintaining corpus luteum function and progesterone secretion, with potential impact on the establishment of pregnancy (3). Hyperprolactinaemia plays a critical role in infertility because it suppresses the production of GnRH and thus pituitary gonadotropins (4). The most common endocrine abnormalities seen in patients with recurrent miscarriage are luteal phase defect, polycystic ovary syndrome, thyroid dysfunction, obesity, and hyperprolactinaemia. The incidence and risks of these endocrine abnormalities in recurrent miscarriage have been the subject of many independent studies, but studies systematically describing the proportion of endocrine factor abnormalities in patients with recurrent miscarriage are still lacking.

Not only are screening factors varied and complex, but the timing of screening is also a challenge in current research on recurrent miscarriage (5). Due to regional differences in the definition of recurrent miscarriage and clinicians' own practice experience, some have begun routine etiologic screening for recurrent miscarriage following two pregnancy losses, whereas others wait for a third or more pregnancy losses before initiating etiologic screening. Based on these two points, the aims of this study include (1): To describe the proportion of endocrine dysfunction in patients with recurrent miscarriages (2). To compare whether there are differences in endocrine dysfunction between patients with recurrent miscarriages with different numbers of miscarriages.

Materials and methods

Study sample

The China Medical University Birth Cohort is an ongoing prospective cohort study that includes a sub-cohort of patients with recurrent miscarriage specifically enrolled in the Recurrent Miscarriage Clinic. In this cross-sectional study, data from 2017 to 2022 are collected from the recurrent miscarriage clinic at Shengjing Hospital, a local tertiary center, with patients being enrolled according to the following criteria: 1) Patients with two or more pregnancy losses and have completed a detailed history taking form of pregnancy losses. 2)Patients who have received a comprehensive aetiological screening prior to conception for recurrent miscarriage regarding endocrine factors (including thyroid dysfunction, obesity, hyperprolactinemia, polycystic ovary syndrome and blood glucose abnormality) at our hospital. 3) Patients did not take medicines that may affect the test results (including traditional Chinese medicine) before screening. Whereas patients with abnormal results of the endocrine factors on the initial examination will need at least a second repeat examination to make the diagnosis. Simple random sampling was adopted for the study.

Pregnancy loss as defined in this study was urine/blood β-hCG positive or ultrasound-confirmed pregnancy sacs. Pregnancy loss was defined as any spontaneous pregnancy loss or fetal weight ≤ 500g before 20 weeks. Molar pregnancy, ectopic pregnancy, implantation failure and pregnancy terminations were excluded from the analysis.

Sample-size calculation

The sample size was calculated using an online sample calculation website, http://riskcalc.org:3838/samplesize/. The collection of data from 100 cases in the preliminary stage allowed us to roughly determine the proportion of various endocrine abnormalities in the three populations (patients with two/three/four and more pregnancy losses). Glucose abnormalities were the type of endocrine abnormality that had the lowest prevalence and had a prevalence of approximately 5%. The Type I error rate set in this study was 0.05, the degree of certainty of the study was 0.8, and the approximate ratio of

the sample size between the three populations was 1. A minimum sample size of 480 was calculated for this study.

Data collecting

A patient history collection form is used to collect baseline characteristics, which asks the patient to describe in detail her menstrual history and maternal history, including the number of miscarriages, the cause of the miscarriages, and the presence of ultrasound images to determine the occurrence of early intrauterine pregnancy. The clinician reviews this information (usually with more detailed questioning) in order to clarify the accuracy of the data collected. Whereas outcome-related data are collected primarily through the collection of laboratory test results obtained from the hospital's electronic medical record system. We do not capture data from tests conducted by patients at other hospitals, since differences in the kits can lead to inaccurate results.

The baseline characteristics collected were: age, height, weight, number of pregnancies and number of pregnancy losses. Outcome data collected included PCOS, thyroid dysfunction, hyperprolactinaemia, blood glucose abnormality, obesity, and levels of vitamin D. Premature ovarian failure was removed from the outcome events because of a small number of cases; luteal phase defect was removed from the outcome events because of diagnostic challenges. All baseline data were collected from the patients on their first visit to the recurrent miscarriage clinic. Data on all disease diagnoses were obtained in the non-pregnant state.

Diagnostic criteria

The diagnosis of PCOS is based on the revised Rotterdam diagnosis (6). Thyroid dysfunction is categorized as abnormal thyroid autoimmune antibodies alone, elevated or reduced TSH levels alone, and abnormal TSH levels in combination with abnormal levels of autoimmune antibodies; An abnormal TSH is diagnosed as a TSH of less than 0.3 μIU/ml or more than 4.8 μIU/ml (Specific reference values established for non-pregnant local normal individuals). Hyperprolactinaemia was defined as PRL greater than 26.72 ng/ml. abnormal blood glucose was classified as impaired glucose tolerance (6.1 mmol/L ≤ fasting glucose <7.0 mmol/L), and diabetes mellitus (fasting glucose ≥7.0 mmol/L). Diagnosis of obesity is based on the criteria for diagnosis of obesity developed by the working group on obesity in China (7). Endocrine dysfunction is determined by a combination of PCOS, abnormalities in thyroid function, hyperprolactinaemia, glucose abnormalities, and obesity; if any of these abnormalities are present, the patient is considered to have an endocrine dysfunction. Furthermore, because vitamin D may affect many endocrine factors in patients with recurrent miscarriages, vitamin D levels were also included in the present study and examined as a separate outcome; the concentration of Vitamin D was measured as 25 hydroxyvitamin D. 25-OH Vit D ≤ 20ng/ml was diagnosed as a vitamin D deficiency; A diagnosis of vitamin D insufficiency was made if 20 < 25-OH Vit D ≤ 30ng/ml. Patients were asked to undergo tests related to recurrent pregnancy loss when they are nonpregnant and at least three months after their last pregnancy loss. Patients undergoing these laboratory tests are advised to avoid cold or menstrual periods, which can affect the results of laboratory tests. Screening time for hormones is 1-3 days of menstruation.

Analyzed data

For comparisons of baseline data, the information about the measure is expressed as the mean ± the standard deviation. Counts are shown as quartiles. Comparisons of endocrine abnormality rates between those with two and three miscarriages, and those with more than three miscarriages, were made using a two-sided Pearson chi-square test. Since multiple group comparisons were performed, we used Bonferroni correction. The vitamin D comparisons were analyzed by ANOVA. All data analysis was carried out in SPSS, windows, version 25.

Results

General situation of patients and the proportion of endocrine dysfunction

The total number of RPL patients included in this study was 537, including 278 patients with a diagnosis of endocrine dysfunction, comprising 51.8% of RPL patients. Fifty-six of these patients were diagnosed with PCOS (10.50%, 56/533); 85 patients were diagnosed with hyperprolactinaemia (17.34%, 85/490); Twenty-seven patients had a diagnosis of blood glucose abnormality (5.29%, 27/510); 58 patients were diagnosed with obesity (10.80%, 58/537). A total of 537 patients had their vitamin D levels tested, and the average vitamin D value was 15.11 ± 6.42ng/ml. 83.49% of these patients, (440/527) had a diagnosis of vitamin D deficiency, 14.04% (74/537) were diagnosed with vitamin D insufficiency, and only 2.47% (13/527) of patients had vitamin D levels that were up to the standard. A total of 131 patients (24.39%) were diagnosed with thyroid function abnormalities (131/537). 101 of the patients with abnormal thyroid function were found to have abnormal autoantibodies alone, representing 77.1% of patients with abnormal thyroid function (101/131); Eighteen had an abnormal TSH level alone, representing 13.74% of patients with an abnormal thyroid function (13/131); and 12 had combined autoantibodies and TSH levels, accounting for 9.16% of patients with abnormal thyroid. Among 113 patients with positive autoimmune thyroid antibodies, 39.82% (45/113) were found to be positive for anti-Tg alone; Of these, 17.70% (20/113) were positive for anti-Tpo antibody alone; and 42.48% (48/113) were positive for both antibodies. Further details can be found in Tables 1 and 2.

TABLE 1 Baseline characteristics and the proportion of endocrine disorders.

Characteristic	n(%)
Age	
20-29	133(24.77)
30-34	272(51.03)
35-45	132(24.58)
BMI	
<18.5	29(5.40%)
18.5-23.9	321(59.78%)
24-28	129(24.02%)
>28 (obesity)	58(10.8%)
Times of pregnancy loss	
2	297(55.31)
3	184(34.52)
More than 3	56(10.51)
PCOS	
No	477(89.50)
Yes	56(10.50)
Thyroid Dysfunction	
No	406(75.61)
Yes	131(24.39)
HPRL	
No	405(82.66)
Yes	85(17.34)
Blood glucose abnormality	
No	483(94.71)
IGT	23(4.51)
DM	4(0.78)
Vitamin D level	
Normal	13(2.47)
Insufficient	74(14.04)
Deficiency	440(83.49)

Multiple endocrine dysfunctions

When analyzing whether patients presented with a combination of multiple endocrine disorders, we found that 74.46% (207/278) of patients with RPL had only one endocrine disorder;

23.02% (64/278) had a combination of two endocrine disorders; 2.15% (6/278) had a combination of three endocrine disorders, and only 1 patient had a combination of 4 endocrine disorders. To further examine whether there was an interaction between the various endocrine disorders, or what types of endocrine disorders were seen more frequently together, the association between each of the endocrine factors was analyzed. Although we did not find a significant association between any two endocrine disorders (results not shown), there was a tendency for PCOS to be associated with obesity (p=0.059), which is consistent with our clinical knowledge.

Endocrine dysfunction and the number of pregnancy losses

Comparisons of subgroups revealed that PCOS, thyroid dysfunction, hyperprolactinaemia, blood glucose abnormality, and vitamin D levels were not significantly different between groups. There was a significant difference in obesity between patients with different numbers of pregnancy losses (p=0.003). We then stratified the patients by age for different numbers of pregnancy losses and showed that all endocrine disorders were not associated with the number of miscarriages in the subgroups less than 30 years and greater than or equal to 35 years. On the other hand, among those above or equal to age 30 and below age 35, obesity was the only factor that was significantly different between patients with different numbers of pregnancy losses (p=0.017).

Discussion

Main findings

This cross-sectional study describes the proportion of endocrine disorders in the recurrent miscarriage population and compares whether there are differences in the distribution of endocrine disorders among those with two, three, and more pregnancy losses. Except for obesity, our results did not find a significant association between the number of pregnancy losses and the distribution of endocrine disorders. This finding may suggest that clinicians need to begin screening for endocrine factors at the beginning of two miscarriages in order to intervene early and prevent patients from experiencing further miscarriages.

Thyroid dysfunction

Currently, abnormal thyroid function is a hot topic in recurrent miscarriage research. Abnormalities in thyroid function typically include both abnormal TSH levels and abnormal autoimmune antibodies. Many clinical studies have been conducted to examine whether abnormal thyroid function can lead to pregnancy loss and other adverse pregnancy outcomes. Recent research suggests that abnormal levels of TSH alone or the presence of thyroid antibodies are associated with pregnancy loss (8). In a meta-analysis of 22 studies, high serum thyroid antibody levels have been shown to lead to recurrent miscarriage and the use of T4 replacement therapy is beneficial in pregnant patients with recurrent miscarriage (9). However, a randomized clinical trial carried in 2019 concluded that that the use of levothyroxine in euthyroid women with thyroid peroxidase antibodies did not result in a higher rate of live births than placebo in normal population (10), and the preconception use of

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TABLE 2 Comparison of endocrine disorders among patients with different numbers of pregnancy loss.

	Times of pregnancy loss			P value
	2	3	≥4	
Endocrine dysfunction				0.817
No	146(56.4%)	88(34.0%)	25(9.7%)	
Yes	151(54.3%)	96(34.5%)	31(11.2%)	
PCOS				0.638
No	261(54.7%)	166(34.8%)	50(10.5%)	
Yes	34(60.7%)	16(28.6%)	6(10.7%)	
Thyroid Dysfunction				0.905
No	225(55.4%)	140(34.5%)	41(10.1%)	
Yes	72(55.0%)	44(33.6%)	15(11.5%)	
HPRL				0.265
No	220(53.9%)	143(35.0%)	45(11.0%)	
Yes	54(63.5%)	24(28.2%)	7(8.2%)	
Blood glucose abnormality				0.616
No	272(56.3%)	161(33.3%)	50(10.4%)	
IGT	10(43.5%)	9(39.1%)	4(17.4%)	
DM	2(50.0%)	2(50.0%)	0(0.0%)	
Vitamin D level				0.908
Normal	7(53.8%)	4(30.8%)	2(15.4%)	
Insufficient	38(51.4%)	27(36.5%)	9(12.2%)	
Deficiency	248(56.4%)	147(33.4%)	45(10.2%)	
Obesity				**0.003***
No	277(57.8%)	155(32.4%)	47(9.8%)	
Yes	20(34.5%)	29(50.0%)	9(15.5%)	

* means P value<0.05.

levothyroxine in recurrent miscarriage population still need further research. The mechanism by which thyroid antibodies contribute to poor pregnancy outcomes may be linked to their action on immune cells in the endothelium; studies have shown that the secretion of IL-4 and IL-10 is significantly reduced in endothelial T cells and that the expression of interferon-γ is significantly increased in antibody-positive patients (11). Likewise, polyclonal B cells were overexpressed in patients with autoimmune thyroid disease, and toxic NK cell migration was significantly enhanced (12). There were no significant differences in the prevalence of thyroid dysfunction according to the number of pregnancy losses reported in this paper. These findings are also similar to previous studies.

PCOS

The second endocrine abnormality that is the focus of this paper is PCOS, whose prevalence in the recurrent miscarriage population remains a mystery. The reported incidence of polycystic ovarian changes on ultrasound imaging in the RPL population has been reported to range from 4.8 to 82% (13–16). In a meta-analysis published in 2016, the authors included 15 articles that used the Rotterdam diagnosis as a diagnostic criterion for PCOS and concluded that the average prevalence of PCOS in the general population was 10% (17). In the RPL population, the prevalence of PCOS were reported to be 14.3% (18). Common symptoms of PCOS include insulin resistance and hyperinsulinaemia, both of which are independent risk factors for pregnancy loss (13, 19, 20). Therefore, it is important to intervene before pregnancy for PCOS patients to improve miscarriage rates. Unfortunately, there is still a lack of research on how to manage during pregnancy in PCOS patients. Researchers have found that metformin treatment might reduce the risk of miscarriage in PCOS patients (21). However, more research is needed on the safety of medication and its use in the population with recurrent miscarriage.

Obesity and glucose abnormality

Of note, about 35% of PCOS patients have a combination of obesity, which is believed to be associated with pregnancy loss (22, 23). The prevalence of obesity has been increasing in various countries in recent decades (24). The prevalence of obesity in women of reproductive age (20-39 years) in the United States rose from 28.4% to 34% between 1999 and 2008 (25). In China, the prevalence of obesity in adults rose from 3.6% in 1992 to 14.0% in 2014 (26). The hypothalamic-pituitary-ovarian axis is disrupted by obesity, and overweight women have a shorter luteal phase and lower levels of follicle-stimulating hormone, luteinizing hormone, and progesterone (27). However, in the current study, we found that obese patients were more common among women with a greater number of pregnancy losses. Obesity remained associated with the number of pregnancy losses among those aged thirty to thirty-four years even after stratifying for age. It is noteworthy that the subgroup sample sizes of the study population did not achieve the minimum sample size considered for validity following patient stratification, therefore, a larger sample size is required to confirm the association of obesity with pregnancy loss. As with obesity, blood glucose abnormality is also highly associated with pregnancy loss. In a recently published cross-sectional study, the authors suggest that women with recurrent miscarriages are more likely to have impaired β-cell function and abnormal glucose metabolism (28). Hyperglycaemia can inhibit the differentiation of trophoblasts and thereby interfere with implantation, increase oxidative stress, and affect the expression of key genes that are essential for embryogenesis (29). Hyperglycaemia promotes pregnancy loss through the promotion of premature programmed cell death of key progenitor cells within blastocysts (30). In this study, 5.29% of patients had abnormal blood glucose levels. Whereas the prevalence of diabetes among Chinese women of all ages was as high as 11%, the lower prevalence in this study may be due to the occurrence of diabetes being more prevalent in the elderly and in more economically developed regions.

Hyperprolatinaemia

Prolactin is a hormone secreted from the lactotrophic cells in the anterior pituitary. In a randomized trial, Hirahara et al. found that high levels of prolactin increased the risk of pregnancy loss in women with RPL (31). A cross-sectional study of 69 women with RPL and 31 women of reproductive age and 30 women with infertility found that the prevalence of hyperprolactinaemia was similar across groups, though it was highest in the infertility arm and not in the RPL group (32). While the deleterious effects of hyperprolactinaemia in the recurrent miscarriage population remain unclear, due to its potential risk of producing infertility and the patient's desire for children, pre-pregnancy medication is still needed.

Vitamin D

Lastly, the article also analyzed the incidence of vitamin D levels in individuals with different numbers of pregnancy losses and found no significant differences. Vitamin D concentrations are now considered essential for maintaining pregnancy. Endometrium with a greater number of vitamin D receptors is more likely to conceive, while vitamin D deficiency is more likely to result in miscarriage (33). Vitamin D deficiency can result in the development of numerous endocrine defects including PCOS, autoimmune thyroid disease, diabetes, and obesity (34). Despite such an important role of vitamin D in pregnancy, vitamin D insufficiency or deficiency is currently prevalent in the population. In this study, only 2.47% of patients had a normal vitamin D level, whereas 83.49% were diagnosed with a vitamin D deficiency level. Consistent with our results, Li et al. found that as many as 70% of women during pregnancy were deficient in vitamin D levels, and only 1.6% reached normal levels (35). Though the study population is different, the results of our study and those of Li et al. may suggest the high prevalence of insufficient and deficient vitamin D levels in both the pregnant and non-pregnant women in China. This study also suggests that vitamin D insufficiency and deficiency are not associated with the number of pregnancy losses in patients with recurrent miscarriage. Additional studies are needed to determine if vitamin D supplementation can ameliorate miscarriage.

Bias

Bias in cross-sectional studies includes many aspects and a variety of classifications of bias are summarised in the study by Wang et al (36). Because only baseline data collection involved question-based or questionnaire-based data collection (and this data was reviewed by clinicians), non-response bias, loss-to-follow-up bias, observer bias, interviewer bias, and recall bias are all relatively minor contributors to the total bias in this study. Furthermore, because data collectors were not the originators of the study, i.e. data collectors were unaware of the study objective; therefore, the study would have generated less sampling bias, as well as less allocation bias. Prevalence bias is likely to be the largest source of bias in this study, also referred to as Neyman bias, in which some patients with mild or severe diseases will be missing from the data collection process. In this study, the situation that emerged was the lack of patients with mild diseases. Because study data were collected from regional tertiary medical centers throughout the country, they were referred to patients with complex etiology and relatively severe diseases. Furthermore, because inclusion in the study required that patients undergo at least 1 complete etiologic screen for endocrine factors, these inclusion criteria also led to the loss of a proportion of patients with milder diseases. To address this bias, the study's conclusions should be similarly qualified. Tertiary care centers were more likely than local primary care to use the etiologic distribution of endocrine

factors for recurrent miscarriage derived from this study. Furthermore, a significant confounding factor between the number of pregnancy loss and endocrine dysfunction was age. In this study, we propose to use a stratified approach to remove the influence of confounding factors.

Conclusion

In summary, this study describes the proportion of endocrine factor abnormalities in patients with recurrent miscarriages and finds no significant differences in endocrine factor abnormalities other than obesity between patients with recurrent miscarriages depending on the number of pregnancy losses. The findings of this study may support the screening of patients for endocrine-related aetiology after two miscarriages.

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 China Medical University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

LZ and YD designed the study. JZ and CL were involved in the data collection. YL and HS did the data analysis. JL advised on the conduct of the study. CQ had the conception for the study. All authors listed made important intellectual contribution to the work and approved the final version of the manuscript for publication. 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.2023.1215469/full#supplementary-material

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Impact of the number of previous embryo implantation failures on IVF/ICSI-ET pregnancy outcomes in patients younger than 40 years: a retrospective cohort study

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Objective: The objective of this study was to examine the influence of repeated embryo implantation failures on pregnancy outcomes among patients under 40 years of age undergoing *in vitro* fertilization/intracytoplasmic sperm injection embryo transfer (IVF/ICSI-ET).

Materials and methods: A retrospective analysis was conducted on the clinical data of 13,172 patients who underwent 16,975 IVF/ICSI-ET treatment cycles at Henan Reproductive Hospital between January 1, 2015, and December 31, 2018. Patients were categorized into four groups based on the number of previous embryo implantation failure cycles: Group A=no implantation failure, Group B= 1 implantation failure, Group C=2 implantation failures, Group D=≥3 implantation failures. Baseline characteristics and pregnancy outcomes were compared among the four groups. The impact of the number of previous embryo implantation failures on pregnancy outcomes among IVF/ICSI-ET patients was investigated using univariate and multiple regression analyses.

Results: Univariate logistic regression analysis demonstrated that factors such as the number of previous embryo implantation failures, female age, basal follicle count, endometrial thickness, total number of oocytes retrieved, type of cycle, number of high-quality embryos transferred, and stage of embryo development significantly affected implantation rate, clinical pregnancy rate, early spontaneous abortion rate, and live birth rate (all $P < 0.05$). The duration of infertility and anti-Mullerian hormone (AMH) levels were also found to influence implantation rate, clinical pregnancy rate, and live birth rate (all $P < 0.05$). Upon conducting multivariate logistic regression analysis and adjusting for confounding factors such as age, AMH levels, basal follicle count, endometrial thickness, total number of oocytes obtained, cycle type, number of high-quality embryos transferred, ovarian stimulation protocol, and stage of embryo development, it was revealed that, compared to Group A, Groups B, C, and D exhibited significantly lower implantation and live birth rates, as well as a significantly higher risk of early spontaneous abortion (all $P < 0.05$).

Conclusions: The number of previous embryo implantation failures is an independent factor affecting implantation rate, clinical pregnancy rate, spontaneous abortion rate and live birth rate of patients underwent IVF/ICSI-ET. With the increase of the number of previous embryo implantation failures, the implantation rate, clinical pregnancy rate and live birth rate of patients underwent IVF/ICSI-ET decreased significantly, and the rate of early spontaneous abortion gradually increased.

KEYWORDS

IVF/ICSI, implantation rate (IR), Live birth rate (LBR), pregnancy outcome, recurrent implantation failure (RIF)

Introduction

Infertility has increasingly become a significant global public health and sociological issue that profoundly impacts human development and health (1). The World Health Organization reports that infertility affects approximately 8–14% of women of reproductive age in western developed countries, with the prevalence rising to 25–30% in some developing regions, such as Africa and the Middle East (2). Currently, the infertile population in China exceeds 40 million, with an incidence of 12.5% among women of childbearing age (3). With the rapid advancement of assisted reproductive technology (ART), the implantation rate for a single high-quality blastocyst transfer has improved to as high as 65% (4). The *in vitro* fertilization/intracytoplasmic sperm injection-embryo transfer (IVF/ICSI-ET) technique has helped numerous infertile families achieve a healthy baby. However, approximately 10% of these individuals are unable to attain a clinical pregnancy even after three or more embryo transfers (5). Despite facing considerable emotional and financial strain, most of these patients still desire to continue their IVF/ICSI-ET treatment in hopes of increasing the "take-home-baby" rate.

Numerous factors influence the embryo implantation rate, including embryo chromosomal abnormalities, diminished endometrial receptivity, asynchrony between embryo and endometrium, maternal endocrine disorders, and thrombotic tendencies. The number of previous embryo implantation failures also plays an essential role in the embryo implantation rate (6). As the number of previous embryo implantation failures rises, the financial and psychological burden on patients grows as well. These individuals require clinicians to help predict the success probability of subsequent embryo transfer cycles based on the number of previous embryo implantation failures. However, the impact of the number of previous embryo implantation failures on the pregnancy outcome of the next IVF/ICSI-ET for infertile patients remains a contentious issue (7–11). Consequently, our study aimed to investigate the effect of the number of prior embryo implantation failures on the pregnancy outcome of the subsequent IVF/ICSI-ET treatment for patients aged <40 years.

Materials and methods

Patient and cycle characteristics

The present study analyzed the clinical data of 16,975 cycles of IVF/ICSI from 13,172 patients who were recruited from the Center of Henan Reproductive Hospital between January 2015 and December 2018. The inclusion criteria comprised the presence of indications for IVF/ICSI-ET and the absence of contraindications, in addition to an age < 40 years. The exclusion criteria included incomplete data or loss to follow-up, ultrasound-identified abnormal uterine structure, such as single or bicornuate uterine diaphragm, chromosomal abnormalities in either partner, previous pregnancy from IVF/ICSI-ET cycles, and the use of donor eggs or sperm. The IVF/ICSI-ET cycles were categorized into four groups according to the number of previous embryo implantation failures: Group A included 13172 cycles without any implantation failures; Group B included 2989 cycles with one embryo implantation failure; Group C included 658 cycles with two embryo implantation failures; and Group D included 156 cycles with three or more embryo implantation failures. The Ethics Committee of Henan Provincial People's Hospital approved the study (No. (28) Len Audit (2022).

Controlled ovarian stimulation protocol

The appropriate protocol for controlled ovulation was selected based on the patient's age, body mass index (BMI), basal follicle count, basal sex hormone levels, anti-müllerian hormone (AMH) levels, and previous IVF/ICSI-ET protocols. Patients underwent controlled ovulation stimulation (COS) and the dosage of gonadotropin was adjusted according to follicular size and hormonal levels. When the follicular diameter was ≥18 mm in three or more follicles, 4000–10000 IU of human chorionic gonadotropin (hCG) (Lizhu Pharmaceutical Company, China) was administered to induce ovulation. Oocyte retrieval guided by vaginal ultrasound was performed 36–37 h later. Routine luteal

support was performed after oocyte retrieval, and one to two available embryos were transferred on either the third or fifth day after oocyte retrieval.

Preparation protocols for frozen-thawed ET

Patients with regular menstrual cycles and normal ovulation underwent natural cycle preparation of the endometrium. Transvaginal ultrasound was used to monitor follicular development and endometrial condition starting from day 11 of the menstrual cycle. Endometrial transformation occurred on the day of ovulation, and the endometrium was transformed 3 or 5 days before the transfer of cleavage-stage or blastocyst-stage embryos, respectively. For patients with irregular menstruation, hormone replacement therapy was employed using estradiol valerate (Progynova, 1 mg/tablet, Bayer, Germany). Oral administration of 4–8 mg/day was started on day 2–4 of the menstrual cycle. The dose of Progynova was maintained when the endometrial thickness was ≥8 mm, and the duration of administration was ≥11 days, while luteal support was given at the same time. After progesterone was administered, embryos were transferred on the third or fifth day. Endometrial transformation was performed on day 4 or 6 for the transfer of cleavage-stage or blastocyst-stage embryos, respectively.

Conventional luteal phase support regimen

Luteal support was provided through daily administration of vaginal progesterone gel (Crinone, 90 mg/unit, Merck & Serono) at a dosage of 90 mg, along with a once-daily oral dose of 20 mg dydrogesterone. The estrogen and progesterone doses remained unchanged after embryo transfer until blood β-hCG levels were measured on the 14th day post-embryo transfer. In case of pregnancy (β-hCG > 50 U/L), the original luteal support was maintained and the dosage was gradually reduced until the 10th week of pregnancy.

Clinical observation indicators

Clinical pregnancy was defined as the presence of an intrauterine gestational sac, confirmed by vaginal ultrasonography 4–6 weeks after embryo transfer. Spontaneous miscarriage was defined as pregnancy termination before 28 weeks of gestation with a fetal weight of less than 1000g. When spontaneous miscarriage occurs before 12 weeks of gestation, it is considered early pregnancy loss. Newborns delivered after 28 weeks of gestation and surviving are considered live births.

Other observed indicators included age, duration of infertility, BMI, type of infertility, cycle type, stage of embryo development, number(No.) of embryos transferred, endometrial thickness, No. of good-quality embryos, AMH levels, antral follicle count (AFC), infertility factors, and ovulation protocol.

Statistical analysis

Statistical analysis was conducted using Empower Stats software based on R language. Continuous variables were presented as mean ± standard deviation (SD), and categorical variables were presented as N (%). Intergroup comparisons were performed using one-way analysis of variance (ANOVA) and χ^2 tests for categorical variables. Univariate regression analysis was used to identify potential factors affecting pregnancy outcomes. Furthermore, multivariate Logistic regression model with generalized estimating equation (GEE) was chosen to conduct univariate and multivariate analysis of the association of effect of the number of previous embryo implantation failures on pregnancy outcomes to account for the correlation between cycles from the same patient and to obtain ORs and 95% CIs for the risk of the number of implantation failures associated with pregnancy outcomes, while adjusting for confounding factors. A value of $P < 0.05$ was considered statistically significant.

Results

A total of 13,172 patients who underwent 16,957 IVF/ICSI cycles were recruited and categorized into four groups based on the number of previous embryo implantation failures. The patient demographics and characteristics are presented in Table 1. The four groups exhibited significant differences in terms of age, duration of infertility, AMH levels, AFC, endometrial thickness, number of eggs retrieved, stage of embryo development, infertility factors, No. of high-quality embryos transferred, cycle type, and ovarian stimulation protocol ($P < 0.05$). However, no significant differences were observed in BMI, type of infertility, No. of embryos transferred, or No. of quality embryos transferred among the groups ($P > 0.05$).

Comparison of clinical outcomes among the four groups

The pregnancy outcomes of the four groups are presented in Table 2. The implantation rate, clinical pregnancy rate, and live birth rate exhibited significant decreases with an increase in the number of previous embryo implantatfailures ($P<0.001$). Conversely, the rate of early spontaneous abortion increased significantly ($P<0.001$).

Univariate analysis for clinical outcomes

The univariate logistic regression analysis revealed that the number of previous embryo implantation failures, female age, AFC, endometrial thickness, total number of eggs obtained, cycle type, No. of high-quality embryos transferred, and stage of embryo development had an impact on the implantation rate, clinical pregnancy rate, early spontaneous abortion rate, and live birth rate ($P < 0.05$). The factors affecting implantation, clinical

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TABLE 1 Comparison of demographic and clinical characteristics of the four groups of patients.

Item	group A (n = 13172)	group B (n = 2989)	group C (n = 658)	group D (n = 156)	*P-value*
Age(y)	30.25 ± 4.15	31.09 ± 4.29[a]	31.84 ± 4.18[ab]	33.41 ± 4.10 [abc]	<0.001
BMI (kg/m2)	23.32 ± 4.69)	23.45 ± 3.84	23.22 ± 3.54	23.37 ± 5.29	0.591
Duration of infertility((y))	3.95 ± 2.77	2.88 ± 3.11 [a]	3.04 ± 3.04 [a]	3.47 ± 3.09[ab]	<0.001
AMH(ng/mL)	4.48 ± 3.76	3.20 ± 1.77 [a]	3.04 ± 1.90[a]	2.96 ± 1.92[a]	<0.001
AFC	12.85 ± 6.24	8.52 ± 7.85[a]	8.68 ± 7.37 [a]	7.92 ± 6.31[a]	<0.001
Endometrial thickness(mm)	10.25 ± 2.64	9.83 ± 2.38[a]	9.69 ± 1.92[a]	9.59 ± 2.09[a]	<0.001
No. of Oocytes retrieved	8.58 ± 3.89	8.22 ± 3.49	4.58 ± 5.11[ab]	5.61 ± 4.94[ab]	<0.001
Types of Infertility					0.078
Primary infertility	4750(53.59%)	697 (51.63%)	109 (45.99%)	25 (53.19%)	
Secondary Infertility	4114(46.41%)	653 (48.37%)	128 (54.01%)	22 (46.81%)	
Cause of infertility					0.014
PCOS	968 (12.02%)	197 (10.35%)	29 (11.37%)	3 (4.84%)	0.117
Tubal factors	4715 (58.54%)	1140 (61.37%)	155 (60.78%)	38 (61.29%)	0.193
Ovulatory dysfunction	909 (11.29%)	212 (11.37%)	27 (10.59%)	7 (11.29%)	0.987
Endometriosis	255 (3.17%)	51 (2.74%)	7 (2.75%)	1 (1.61%)	0.686
Male factors	785(9.75%)	191(10.25%)	32 (12.54%)	10 (16.12%)	0.160
Other factors	375 (4.66%)	63 (3.38%)	3 (1.19%)	1 (1.61%)	0.001
Unexplained infertility	47 (0.57%)	10 (0.54%)	2 (0.78%)	2(3.24%)	0.282
Ovarian stimulation protocol					0.004
long protocol	7756 (60.138%)	1776 (60.801%)	390 (60.278%)	87 (57.237%)	0.877
antagonist protocol	1642 (12.732%)	340 (11.640%)	92 (14.219%)	14 (9.211%)	0.121
mild ovarian stimulation protocol	759 (5.885%)	158 (5.409%)	32 (4.946%)	2 (1.316%)	0.063
PPOS	438 (3.396%)	91 (3.115%)	26 (4.019%)	12 (7.895%)	0.012
Other protocol	2302 (17.849%)	556 (19.035%)	107 (16.538%)	37 (24.342%)	0.061
stage of embryo development					<0.001
Cleavage embryo	10267(77.97%)	1657 (55.49%)	308 (47.17%)	82 (52.90%)	
Blastocyst	2901(22.03%)	1329 (44.51%)	345 (52.83%)	73 (47.10%)	
No. of embryos transferred					0.817
1	3417 (25.94%)	767 (25.69%)	175 (26.80%)	36 (23.23%)	
2	9754 (74.06%)	2219 (74.31%)	478 (73.20%)	119 (76.77%)	
No. of high-quality embryos transferred	1.68 ± 0.51	1.54 ± 0.50	1.51 ± 0.49	1.59 ± 0.50	0.213
No. of high-quality embryos transferred(%)	66.98%(15334/22921)	59.11%(3077/5205)[a]	57.17% (646/1131)[a]	47.31% (130/276)[abc]	p<0.001
Type of cycle					p<0.001
Fresh cycle	4267(50.5%)	417(20.5%)[a]	91(17.9%)[a]	30 (21.3%)[a]	
Frozen cycle	4184(49.5%)	1616(79.5%)[a]	416 (82.1%)[a]	111(78.7%)[a]	

overall comparison between the four groups *P < 0.05; a: compared with group A, *P < 0.05; b: compared with group B, *P < 0.05; c: compared with group C, *P < 0.05.

TABLE 2 Comparison of clinical pregnancy outcomes among the four groups of patients.

Item	group A	group B	group C	group D	*P -value*
Implantation rate (n%)	11457/22928 (49.97%)	2085/5211 (40.01%)[a]	436/1132 (38.52%))[a]	72/276 (26.09%))a[bc]	<0.001
Pregnancy rate (n%)	8491/13172 (64.46%)	1561/2989 (52.22%)[a]	336/658 (51.06%)[a]	57/157 (36.54%)[abc]	<0.001
Early spontaneous abortion rate (n%)	1152/8491 (13.57%)	265/1561 (16.97%)[a]	74/336 (22.02%)[ab]	16/57 (28.07%)[abc]	<0.001
Live birth rate (n%)	7261/13172 (54.78%)	1275/2989 (42.66%)[a]	258/658 (39.21%)[a]	39/156 (25.00%)[abc]	<0.001

overall comparison between the four groups *P < 0.05; a: compared with group A, *P < 0.05; b: compared with group B, *P < 0.05; c: compared with group C, *P < 0.05.

pregnancy, and live birth rates were duration of infertility, AMH levels, and AFC ($P < 0.05$). (Table 3)

Multiple logistic regression analysis

Multiple logistic regression was performed to analyze the impact of the number of previous embryo implantation failures on the implantation, clinical pregnancy, early spontaneous abortion, and live birth rates after IVF/ICSI-ET, while adjusting for confounding factors such as age, duration of infertility, AMH levels, AFC, endometrial thickness, total number of oocytes retrieved, infertility factors, embryonic developmental stage, and cycle type. The implantation rate, clinical pregnancy rate, and live birth rate of patients in groups B, C, and D exhibited a gradual decrease compared to group A, while early spontaneous abortion rates showed a significant increase. (Table 4)

Discussion

The impact of the number of previous implantation failures on pregnancy outcomes remains a controversial topic that requires further investigation. Cimadomo Danilo et al (12) reported in an observational study that the embryo implantation rate and early spontaneous abortion rate in the first four cycles of IVF/ICSI-ET treatment could not be predicted based on the number of previous embryo implantation failures. However, they found that the live birth rate decreased significantly in patients with a history of ≥3 implantation failures compared to those who underwent their first

TABLE 3 Univariate analysis of clinical outcomes affecting the number of previous embryo implantation failures.

Item	Implantation rate		Pregnancy rate		Early spontaneous abortion rate		Live birth rate	
	OR (95% CI)	*P -value*	OR (95% CI)	*P -value*	OR (95% CI)	*P -value*	OR (95% CI)	*P-value*
Duration of infertility (y)	0.98 (0.97,1.00)	0.009*	0.98 (0.97, 1.00)	0.008*	1.02 (1.00,1.04)	0.120	0.98 (0.97,0.99)	0.002*
AMH (ng/mL)	1.05 (1.03, 1.06)	<0.000*	1.05 (1.03, 1.06)	<0.000*	0.99 (0.97,1.01)	0.301	1.04 (1.03,1.05)	<0.000*
AFC	1.03 (1.03, 1.04)	<0.000*	1.03 (1.03, 1.04)	<0.000*	0.99 (0.98,1.00)	0.045	1.03 (1.02,1.03)	<0.000*
Endometrial thickness (mm)	1.07 (1.05, 1.08)	<0.000*	1.07 (1.05, 1.08)	<0.000*	0.94 (0.92,0.97)	<0.000*	1.07 (1.06,1.09)	<0.000*
No. of Oocytes retrieved	1.04 (1.03, 1.05)	<0.000*	1.04 (1.03, 1.05)	<0.000*	0.95 (0.94,0.97)	<0.000*	1.05 (1.04,1.06)	<0.000*
Cause of infertility (n%)								
PCOS	1	1	1	1	1	1	1	1
Tubal factors	0.79 (0.69, 0.90)	0.000*	0.79 (0.69, 0.90)	0.001*	0.77 (0.63,0.95)	0.013*	0.91 (0.80,1.03)	0.139
Ovulatory dysfunction	0.77 (0.65, 0.91)	0.002*	0.78 (0.66, 0.93)	0.005*	0.78 (0.59,1.04)	0.088	0.88 (0.75,1.04)	0.128

(Continued)

TABLE 3 Continued

Item	Implantation rate		Pregnancy rate		Early spontaneous abortion rate		Live birth rate	
	OR (95% CI)	*P -value*	OR (95% CI)	*P -value*	OR (95% CI)	*P -value*	OR (95% CI)	*P-value*
Endometriosis	0.73 (0.57, 0.95)	0.019*	0.72 (0.56, 0.94)	0.015*	0.80 (0.51,1.25)	0.333	0.86 (0.67,1.10)	0.228
Male factors	1.25 (1.01, 1.55)	0.043*	1.23 (0.99, 1.53)	0.056	0.59 (0.42,0.84)	0.003*	1.44 (1.18,1.76)	0.001*
Other factors	0.98 (0.78, 1.24)	0.882	1.02 (0.81, 1.30)	0.845	0.55 (0.36,0.84)	0.006	1.19 (0.95,1.49)	0.122*
Unexplained infertility	1.04 (0.59, 1.84)	0.882	1.03 (0.59, 1.82)	0.914	0.86 (0.35,2.07)	0.730	1.06 (0.62,1.79)	0.836
Ovarian stimulation protocol								
long protocol	1	1	1	1	1	1	1	1
antagonist protocol	1.01 (0.92, 1.11)	0.826	1.01 (0.92, 1.11)	0.870	0.95 (0.80,1.13)	0.586	0.64 (2.92,7.38)	<0.000*
mild ovarian stimulation protocol	1.07 (0.93,1.22)	0.358	1.07 (0.93, 1.22)	0.357	0.83 (0.64,1.06)	0.138	0.58 (1.96,6.57)	<0.000*
PPOS	1.07 (0.89, 1.27)	0.458	1.08 (0.91, 1.29)	0.370	1.13 (0.89,1.57)	0.247	0.67 (1.92,1.37)	0.001*
Other protocol	1.02 (0.94, 1.11)	0.708	1.03 (0.95, 1.12)	0.529	0.86 (0.74,1.00)	0.053	0.48 (0.32,0.44)	<0.000*
Type of cycle (n%)								
Fresh cycle	1	1	1	1	1	1	1	1
Frozen cycle	0.81 (0.75, 0.88)	<0.000*	0.81 (0.75, 0.88)	<0.000*	1.43 (1.24,1.64)	<0.000*	0.77 (0.71,0.83)	<0.000*
No. of high-quality embryos transferred (%)	1.24 (1.41,1.52)	<0.000*	1.23 (1.31,1.65)	<0.000*	0.95 (1.31,1.52)	<0.000*	1.18 (1.15,1.57)	<0.000*
Stage of embryo development								
Cleavage embryo	1	1	1	1	1	1	1	1
blastocyst	1.25 (1.16,1.34)	<0.000*	1.23 (1.15, 1.32)	<0.000*	1.27 (1.13,1.43)	<0.000*	1.11 (1.04,1.19)	0.002*

OR: ratio; CI is confidence interval, *P<0.05.

IVF/ICSI cycle. A retrospective cohort study in Israel reported that the live birth rate per IVF/ICSI-ET cycle decreased significantly with the increasing number of embryo transfer cycles. The study found that the live birth rate was almost zero in patients who underwent the fourth embryo transfer cycle, suggesting that infertile patients who did not achieve a clinical pregnancy with four consecutive embryo transfer cycles should discontinue treatment or consider alternative options such as donor eggs and sperm (7). Conversely, a large multicenter study by Andrew D. A. C. Smith et al (8) showed that the success rate of IVF/ICSI-ET tended to decrease with the number of embryo transfer cycles until the ninth cycle, after which live birth was almost impossible. They support the notion that, although the live birth rate decreased after 3–4 cycles of repeated treatment, there was still a possibility of live birth. Wang et al. (10) found no significant correlation between clinical pregnancy rate and the number of the first three transfer cycles, but observed significantly lower implantation and clinical pregnancy rates from the fourth embryo transfer cycle. The inconsistencies among these studies may be due to differences in study population characteristics, inclusion and exclusion criteria, quality of transferred embryos, level of assisted reproductive technology in each country, and sample size. Our findings demonstrate that the number of previous embryo implantation failures is an independent factor affecting implantation rate, clinical pregnancy rate, spontaneous abortion rate and live birth rate of patients underwent IVF/ICSI-ET. With the increase of the number of previous embryo implantation failures, the implantation rate, clinical pregnancy rate and live birth rate of patients underwent IVF/ICSI-ET decreased significantly, and the rate of early spontaneous abortion increased markedly.

TABLE 4 Multifactorial logistic regression analysis of the number of previous embryo implantation failures on pregnancy outcome of IVF/ICSI-ET patients.

Item	Unadjusted		Adjusted	
	OR (95% CI)	*P* -value	OR (95% CI)	*P*- value
Implantation rate				
group A	1	1	1	1
group B	0.61 (0.56, 0.66)	<0.001*	0.57 (0.51, 0.63)	<0.001*
group C	0.58 (0.50, 0.68)	<0.001*	0.53 (0.44, 0.64)	<0.001*
group D	0.32 (0.23, 0.45)	<0.001*	0.33 (0.23, 0.46)	<0.001*
Pregnancy rate				
group A	1	1	1	1
group B	0.60 (0.56, 0.65)	<0.001*	0.57 (0.51, 0.63)	<0.001*
group C	0.58 (0.49, 0.67)	<0.001*	0.53 (0.44, 0.63)	<0.001*
group D	0.32 (0.23, 0.44)	<0.0001*	0.32 (0.23, 0.46)	<0.0001*
Early spontaneous abortion rate				
group A	1	1	1	1
group B	1.27 (1.10, 1.46)	0.009*	1.40 (1.16, 1.70)	< 0.001*
group C	1.71 (1.32, 2.22)	<0.001*	1.84 (1.36, 2.48)	<0.001*
group D	2.61 (1.49, 4.58)	0.008*	2.24 (1.24, 4.02)	0.073*
Live birth rate				
group A	1	1	1	1
group B	0.61 (0.57, 0.67)	<0.001*	0.56 (0.50, 0.63)	<0.001*
group C	0.53 (0.45, 0.62)	<0.001*	0.49 (0.41, 0.58)	<0.001*
group D	0.28 (0.19, 0.40)	<0.001*	0.29 (0.20, 0.42)	<0.001*

Group A was used as the reference group. *P < 0.05 after adjusting for confounding factors, including age, duration of infertility, AMH levels, AFC, endometrial thickness, the total number of eggs obtained, infertility factors, embryo development stage, cycle type, ovarian stimulation regimen, and the number of good-quality embryos transferred.

Effect of the number of previous embryo implantation failures on implantation, clinical pregnancy, and live birth rates in IVF/ICSI-ET patients

Embryo implantation is a crucial step in the success of IVF/ICSI-ET treatment. However, recurrent embryo implantation failure (RIF) is a challenging condition that hinders the improvement of clinical pregnancy rates in these patients. Diagnostic and therapeutic challenges arise due to the diverse etiologies of RIF. Improving embryo implantation rates has become a major challenge in improving the clinical outcomes of IVF/ICSI-ET patients. Therefore, further research on RIF patients is necessary. The definition of RIF lacks a unified international standard. Currently, most experts accept the criteria based on the patient's age, the number of failed IVF/ICSI cycles, and the number of good-quality embryos transferred (13). In China, the 2023 expert consensus defines RIF as the failure to achieve clinical pregnancy after transferring at least three good-quality embryos in three fresh or frozen cycles (14). The etiology of embryo implantation failure is complex, diverse, and partially unknown (15). Some studies have reported that the number of previous embryo implantation failures is an independent risk factor affecting implantation rates (5, 8, 9, 12). However, other studies have suggested that patient outcomes in IVF/ICSI cycles are not significantly related to the number of embryo transfer cycles (16). In our study, we found that patients without any implantation failures had significantly higher implantation rates, clinical pregnancy rates, and live birth rates, and a lower rate of early spontaneous abortion compared to those in the other three groups. The pregnancy outcomes were comparable between one and two previous embryo implantation failure with no significant differences in implantation rate, clinical pregnancy rate, and live birth rate (see attachment for details). However, the outcomes of patients with ≥3 previous implantation failures were worse than those of the other three groups of patients.

It is well known that age is the most critical factor affecting the development of oocytes and the quality of embryos. As female age increases, the decline in ovarian function and the increased probability of aneuploidy, she is prone to embryo developmental delay and stagnation, which can lead to embryo implantation failure, and the implantation rate decreases gradually (17, 18). We selected patients less than 40 years old and adjusted age by multiple logistic

regression to avoid the impact of age on pregnancy outcomes. Impaired endometrial receptivity is also an important factor that causes embryo implantation failure (19). Therefore, patients underwent hysteroscopy after the first cycle of implantation failure to exclude interference from endometrial polyps, uterine adhesions, and chronic endometritis on implantation rates (20).

The success of ART largely depends on the quality of the embryo and the receptivity of the endometrium. In this study, the implantation rate, the clinical pregnancy rate and the live birth rate of patients underwent first embryo transfer cycle were 49.97%, 64.46% and 54.78% respectively, which was a higher rate of successful pregnancy compared to those who had previous failed cycles, which is similar to Shapiro B S study (21). Patients with one or two previous embryo implantation failures exhibited comparable pregnancy outcomes, while patients with three or more previous failures showed significantly lower rates of embryo implantation, clinical pregnancy, and live birth. Patients with three or more previous failures was RIF patients, whose infertility is often caused by multiple factors, including maternal endocrine and immune disorders and thrombophilia (22). Our cohort did not undergo etiology-related investigations and treatments for RIF patient. However, we have since put in place etiologic screening and appropriate treatment for patients with four or more embryo transfer cycles. Our findings highlight the need for further research into RIF patients, as the etiology of this condition is complex and not well understood. Therefore, patients with ≥3 previous implantation failures are recommended to continue subsequent cycles only after examination and treatment to improve pregnancy outcomes.

Effect of the number of previous embryo implantation failures on early spontaneous abortion rate in IVF/ICSI-ET assisted conception

Spontaneous abortion is a common complication of pregnancy in obstetrics and gynecology. It is known that spontaneous abortion after IVF/ICSI-ET-assisted clinical pregnancy reduces the live birth rate. However, it is uncertain how the number of previous embryo implantation failures affects the rate of spontaneous abortion after assisted clinical pregnancy (10, 23). Previous studies have yielded conflicting results regarding the impact of the number of embryo transfer cycles on the rate of spontaneous abortion after clinical pregnancy in IVF/ICSI-assisted conception. Some studies have shown no significant change in the spontaneous abortion rate as the number of cycles increased, while others have demonstrated an increased risk of spontaneous abortion in patients with multiple embryo transfer cycles, which partially supports the results of our study (24, 25). In our study, we found that the early spontaneous abortion rate was lowest in the first embryo transfer group. Patients with ≥3 embryo implantation failures had a significantly higher early spontaneous abortion rate of 28.07%, which may be due to the fact that these patients are RIF population, and the etiology of RIF and recurrent spontaneous abortion (RSA) is similar (26). The etiology of RSA involves chromosomal or genetic abnormalities, anatomical abnormalities, autoimmune diseases, prethrombotic state (PTS), endocrine factors, infectious factors, male factors, and psychological factors (27). However, the etiological examination and correction were not carried out in our study for patients with ≥3 embryo implantation failures, which may explain the high risk of spontaneous abortion in these patients.

However, there are some limitations in this study. First, our study was a single-center, retrospective study and there might be some confounders that we did not control for. In addition, we prioritized some pregnancy outcomes and did not investigate neonatal outcomes. In the future, our findings need to be validated by expanding the sample size or by high-quality randomized clinical trial studies.

In conclusion, the number of previous embryo implantation failures is an independent factor affecting implantation rate, clinical pregnancy rate, spontaneous abortion rate and live birth rate of patients underwent IVF/ICSI-ET. Based on our study, patients with ≥3 previous implantation failures are recommended to undergo etiology-related investigations and treatments to continue subsequent cycles to improve pregnancy outcomes. Investigations for RIF includes: 1. General risk factors such as old age, poor lifestyle, smoking, etc. 2. Immune factors include autoimmunity and alloimmunity; 3. Prethrombotic states include hereditary and acquired thrombophilia; 4 Endometrial receptivity test 5. Factors of infection; 6 Reproductive anatomy; 7. Endocrine factors; 8. Male factor; 9. Chromosomes. Treatments of RIF: 1. General treatment such as weight control, healthy diet and appropriate exercise; 2.IVF-ET program optimization; 3. Regulation of immune disorders (glucocorticoids, low molecular weight heparin, immunoglobulin, etc.); 4. Low molecular weight heparin for treatment of pre-thrombotic state; 5. Doxycycline and metronidazole for chronic endometritis; 6. Treatment of submucosal myoma, polyps and hydrosalpinx and other normal anatomical structure abnormalities; 7. The man controls his weight, reduces smoking, etc. 8. Encourage both couples or those with chromosome abnormalities to perform PGT treatment.

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 Henan Medical Ethics Committee of Provincial People's Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

YF and HQ conception and design, review and final approval of the version to be published. FJ, CT, XH analyses the data. YF and HQ draft and revise the article. YF and FJ 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.

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Lupus and recurrent pregnancy loss: the role of female sex hormones and B cells

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Systemic lupus erythematosus is a debilitating autoimmune disease characterized by uncontrolled activation of adaptive immunity, particularly B cells, which predominantly affects women in a 9 to 1 ratio compared to men. This stark sex disparity strongly suggests a role for female sex hormones in the disease's onset and progression. Indeed, it is widely recognized that estradiol not only enhances the survival of autoreactive B cells but also stimulates the production of autoantibodies associated with systemic lupus erythematosus, such as anti-nuclear antibodies and anti-dsDNA antibodies. Clinical manifestations of systemic lupus erythematosus typically emerge after puberty and persist throughout reproductive life. Furthermore, symptoms often exacerbate during the premenstrual period and pregnancy, as increased levels of estradiol can contribute to disease flares. Despite being fertile, women with lupus face a heightened risk of pregnancy-related complications, including pregnancy loss and stillbirth, which significantly surpass the rates observed in the healthy population. Therefore, this review aims to summarize and discuss the existing literature on the influence of female sex hormones on B-cell activation in patients with systemic lupus erythematosus, with a particular emphasis on their impact on pregnancy loss.

KEYWORDS

recurrent pregnancy loss, lupus, B cells, hormones, pregnancy

1 Introduction

Recurrent pregnancy loss (RPL) is a distressing pregnancy disorder experienced by ~2.5% of women trying to conceive. It is defined as the spontaneous demise of two or more clinically recognized pregnancies before the fetus reaches viability; RPL includes embryonic and fetal losses from the time of conception until 24 weeks of gestation (1, 2).

Autoimmune disorders have been included along with chromosomal errors, anatomical uterine defects, and endometrial dysfunction as the most common etiologies

linked to RPL (3). Indeed, certain features commonly associated with autoimmune diseases, such as inappropriate complement activation (4–6) and the prevalence of specific autoantibodies (4, 7–11) show strong associations with RPL.

Furthermore, systemic autoimmune diseases, including systemic lupus erythematosus (SLE), have been identified as significant risk factors for RPL, similar to other autoimmune conditions (12).

SLE is a chronic autoimmune disease that predominantly affects women of reproductive age compared to men and has the potential to affect any organ in the body (13–15). The intricate clinical presentation and pathogenesis of SLE make its definition exceptionally challenging. According to the European League Against Rheumatism (EULAR) and the American College of Rheumatology (ACR), the classification criteria for SLE consist of a mandatory entry criterion of positive anti-nuclear antibodies (ANAs) at least once, followed by additive weighted criteria grouped into seven clinical domains, namely, constitutional, hematologic, neuropsychiatric, mucocutaneous, serosal, musculoskeletal, and renal, and three immunological domains: antiphospholipid antibodies (aPLs), complement proteins, and SLE-specific antibodies (16). ANAs are a group of autoantibodies that target components of the cell nucleus and can bind to proteins, nucleic acids, and protein–nucleic acid complexes (17).

From an immunological perspective, the intricate interplay of environmental, genetic, and hormonal factors results in dysregulation and abnormal activation of the innate and adaptive immune system. This leads to the generation of pathogenic autoantibodies, such as ANAs, anti-double-stranded DNA antibodies (anti-dsDNA), and aPLs, as well as the deposition of immune complexes, ultimately causing tissue damage (18, 19).

Moreover, the impact of ANAs (20) and the presence of various types of aPLs (21) significantly varies between women with RPL and autoimmune diseases, in comparison to those without autoimmunity (22). Indeed, the rate of pregnancy loss among patients with SLE is substantially higher compared to the general healthy population (3). Furthermore, the stage of SLE that the patient is in at the moment of becoming pregnant, including disease activity and renal involvement, not only impacts the health status of the mother but may also influence fetal and neonatal outcomes (23, 24). In this regard, several studies have found that increased serum levels of IL-6, IL-10, and INF-α in patients with SLE are associated with disease activity (25, 26). Regarding disease activity at the time of conception, numerous prospective studies have recently shown that women with inactive SLE generally experience minimal flares during pregnancy, while those with active SLE face an elevated risk of adverse maternal and fetal outcomes (27–29). These findings are consistent with previous reports, indicating that the rate of live births is lower in patients with clinically active SLE in the 6 months before conception compared to those with inactive disease prior to conception (30). Furthermore, RPL among women with SLE appears to be linked to a higher rate of fetal death, which is associated with the presence of aPLs (31, 32). Furthermore, it is well established that newborn babies born to mothers with SLE can develop neonatal lupus, a rare condition that is not a form of SLE, but rather a condition that affects the newborn due to the transfer of maternal autoantibodies across the placenta during pregnancy (33).

Considering the sex and age predisposition of SLE, female sex hormones are undeniably involved in the pathogenesis of the disease (34). Studies conducted on SLE-prone mice using gonadectomy/hormone deprivation and hormone supplementation have consistently confirmed this association, revealing that estrogen exacerbates the disease, while its removal ameliorates the disease in female subjects [reviewed in (35)]. In the context of pregnancy, the increase in female sex hormone levels may influence or potentiate the abnormal function of the immune cells in patients with SLE, thereby exacerbating the disease symptoms and leading to pregnancy complications, including RPL (36, 37).

Considering all the evidence, the objective of this review is to examine the current state of knowledge regarding the impact of preexisting SLE on the development of RPL, with particular focus on the role of female sex hormones in B cell activation and autoantibody production.

2 Pregnancy in patients with systemic lupus erythematosus: the impact on recurrent pregnancy loss

As mentioned earlier, SLE predominantly affects women during their reproductive age, when individuals may seek to become pregnant. However, while fertility is generally preserved in women with SLE, pregnancy in these patients can be associated with adverse maternal and fetal outcomes, including RPL (38). In a recent meta-analysis of pregnancy studies published from 2017 to 2019, it was shown that patients with SLE had markedly increased risk of stillbirth (risk ratio (RR) 16.49, 95% CI 2.95 to 92.13; $p = 0.001$) and fetal loss (RR 7.55, 95% CI 4.75 to 11.99; $p = 0.00001$) compared to healthy pregnant women (39). Despite substantial declines in rates of pregnancy loss among patients with SLE in recent years, they remain higher compared to the healthy population (40). Indeed, approximately 20% of pregnancies in patients with SLE result in miscarriages (40).

Several biomarkers have been investigated as potential predictors of pregnancy complications in women with SLE. Notably, aPLs, including anticardiolipin antibodies and lupus anticoagulants, have been associated with obstetric complications such as RPL, recurrent implantation failure, pre-eclampsia, and preterm birth (41, 42). Additionally, research has shown that low levels of complement proteins, such as C3 and C4 during the first trimester are associated with an increased risk of pregnancy loss (43) in patients with SLE.

Although the causes behind poor pregnancy outcomes in patients with SLE are diverse, there is a general consensus that active disease, characterized by the activation of autoreactive B cells and production of autoantibodies, at the time of conception and during pregnancy significantly impacts maternal and fetal outcomes (38). This is not surprising, given that a successful pregnancy relies on a precisely regulated balance between maternal immune activation and immune tolerance (44). Any disruptions or imbalances in this delicate equilibrium can lead to pregnancy loss. Conversely, during pregnancy, an increase in the levels of female sex

hormones can promote B cell autoreactivity and exacerbate the symptoms of SLE, creating a negative feedback loop. This phenomenon leads to the activation of various immune mechanisms, which can not only worsen the symptoms of SLE but also contribute to pregnancy loss. Therefore, the hormonal regulation of B cell activation during SLE and its implication in pregnancy loss will be discussed in greater detail below.

3 The impact of female sex hormones on B cell activation in patients with systemic lupus erythematosus

B cells are essential components of the adaptive immune system responsible for antibody production. They can be classified into marginal zone (MZ), B1, and B2 B cells based on their phenotype, localization, and functionality (45). While T cell activation relies on antigen presentation by antigen-presenting cells (APCs), B cells, on the other hand, can directly interact with antigens through their receptor (B cell receptor, BCR) (46). However, apart from the signal provided by antigens through BCR, B cells require a second signal for proper activation, which can be delivered by toll-like receptors (TLRs), BAFF-R, or BCR cross-linking in the case of MZ and B1 B cells (47). On the other hand, upon antigen recognition, B2 B cells migrate to the germinal center, where they receive a second signal from follicular T-helper (Tfh) cells. Subsequently, they mature into either antibody-producing plasma cells or memory B cells.

Female sex hormones play a significant role in the development and activity of the immune system (48). Both innate and adaptive immune cells bear receptors for sex hormones and respond to hormonal cues (49). Women display higher frequencies of B cells (50) along with enhanced B cell survival, maturation, and class switching. They also demonstrate greater antibody responses and higher basal levels of immunoglobulins (Igs) compared to men (51), suggesting the involvement of female sex hormones in controlling diverse B cell functions. Indeed, estrogen has been shown to reduce the production of B cell precursors, impair B cell tolerance, and increase the activation and survival of autoreactive B cells (52, 53). While B cells express both estrogen receptor (ER) α and β, it is ERα that predominantly regulates BCR signal strength (54). Elevated levels of estrogen and ERα engagement result in reduced BCR signal strength and modulation of survival regulators such as Bcl-2, CD22, and SH2-containing protein tyrosine phosphatase (SHP)-2, thereby suppressing apoptosis (52). Moreover, elevated estrogen levels result in increased serum BAFF levels, which, together with reduced BCR signal strength, promote the survival of autoreactive B cells that would otherwise be eliminated from the naive repertoire. Consequently, these autoreactive B cells gain entry into the mature B cell pool (55, 56). In such circumstances, heightened estrogen stimulation on B cells triggers a breakdown of tolerance and uncontrolled proliferation and enhances the survival of high-affinity DNA-reactive B cells, which may potentially lead to autoimmunity (54).

A significant proportion of autoreactive B cells originates from the B2 B cell pool, which requires second signals provided by follicular T helper cells to complete their activation. The significance of these pathways in promoting autoantibody production has been demonstrated in genetically modified lupus-prone mice and using blocking antibodies against various costimulatory molecules, such as inducible costimulatory ligand (ICOS-L) and CD40 ligand. Consequently, T helper cells play a crucial role in the development and progression of SLE disease (57). Furthermore, Tfh cells not only express estrogen receptors, but it has also been demonstrated that estradiol promotes the expansion of Tfh cells and, consequently, enhances the humoral immune response (58). Therefore, in the context of SLE, estradiol appears to exert its effects on the Tfh/B2 B cell axis, promoting the development and survival of autoreactive B cells.

The fact that 90% of patients with SLE are women clearly highlights a strong sex bias in this autoimmune disease. Several hypotheses have been proposed to explain this phenomenon, with the influence of female sex hormones being the most widely accepted (59). In this regard, it is known that the clinical manifestation of the disease typically appears after puberty, affecting women between the ages of 20 to 50, a period during which levels of estradiol and progesterone significantly rise (59). The strongest evidence supporting the role of female sex hormones in SLE comes from the observation that patients with SLE experience disease exacerbation during the premenstrual period and in pregnancy (35, 59). Interestingly, a case report demonstrated that administering cross-gender hormones to a transgender female resulted in lupus nephritis, and the withdrawal of estradiol supplementation upon admission prevented the worsening of symptoms. This provides further support for the role of estradiol in driving SLE (60). Animal studies also provide support for the role of estrogen in SLE. Ovariectomized lupus-prone mice showed ameliorated disease, while estrogen supplementation in castrated male mice worsened the symptoms [reviewed in (35)]. Moreover, targeted deletion of ERα specifically in B cells has been shown to reduce the production of pathogenic autoantibodies and the development of nephritis in lupus-prone mice (61). Additionally, tamoxifen treatment significantly reduced autoantibody production and improved the course of SLE in SLE-prone mice (62).

In pregnant SLE patients, estrogen levels and ERα expression not only mediate the increase in anti-dsDNA but also alter the B-cell repertoire, leading to the expansion of autoreactive clones (63, 64). As a result, hormone levels during pregnancy have a substantial impact on the function of autoreactive B cells, intensifying SLE symptoms and contributing to adverse pregnancy outcomes, including RPL (36, 37). In fact, E2 has been demonstrated to decrease B-cell lymphopoiesis in the bone marrow at the pro–B-cell stages in mice and to alter transitional 2 (T2) B cell maturation, both during pregnancy and in patients with SLE (53, 65). Under SLE conditions, elevated BAFF levels and reduced BCR signal strength can lead to the maturation of transitional B cells into a marginal zone (MZ) B cell expansion. Under specific conditions, marginal zone (MZ) B cells can serve as precursors of unswitched memory B cells without T cell help (66). It has been previously demonstrated that during pregnancy, there is a bias toward the development of marginal zone (MZ) B cells (67). This, along with the abnormal differentiation of unswitched memory B cells

observed in patients with SLE (68) may pose a risk to the successful development of pregnancy in patients with SLE. In fact, an increase in unswitched memory B cells is observed in patients with a history of RPL and obstetric complications (69, 70).

Therefore, the presence of autoreactive B cells, along with increased B cell activation and autoantibody production in patients with SLE, poses significant challenges when it comes to achieving a full-term pregnancy.

4 B-cell activation and autoantibody production in lupus: impact on pregnancy well-being

Upon activation, B cells undergo a series of tightly regulated processes that culminate in the differentiation of highly specialized cells capable of producing antibodies, as well as memory B cells (45). In addition to antibodies, activated B cells can produce a wide range of cytokines, especially when their activation goes through their BCR together with BAFF-R (71). Signaling through the BAFF-R activates several downstream pathways, including NF-KB, ERK, and MAPK, which regulate the survival functions of immature, transitional, and mature B cells (72, 73). Interestingly, it has been demonstrated that B cells from pregnant women show downregulation of transcripts associated with these pathways (74) along with reduced levels of BAFF in serum as pregnancy progresses (75), suggesting that B cells are less susceptible to being activated during pregnancy. Indeed, a transcriptomic analysis performed on B cells isolated from pregnant mice confirmed that several B cell activation pathways, including BCR, TLR, and BAFF-R, are significantly diminished compared to B cells from non-pregnant control animals (44). Furthermore, a study by Valeff et al. (44) found that B cells isolated from pregnant women in the first trimester of pregnancy produced significantly lower levels of inflammatory cytokines when activated through their BCR and TLRs compared to B cells from non-pregnant women, reinforcing the notion of B cells being less susceptible to activation, at least during the early stages of pregnancy.

In the context of SLE, aberrant B-cell activation plays a significant role in the pathogenesis of the disease. Dysregulation of BCR and BAFF-R pathways are common and dominant factors involved in this aberrant B-cell activation (76). Furthermore, patients with SLE exhibit elevated levels of BAFF in their serum (77–79), strongly indicating the involvement of the BAFF-R pathway in B cells as a key component of SLE pathology. Indeed, mice overexpressing BAFF develop a lupus-like disease characterized by the production of ANAs and anti-dsDNA (80).

In the context of pregnancy, while the production of natural and protective antibodies is related to pregnancy success (81, 82), the presence of autoantibodies is associated with RPL (8). There is growing evidence suggesting that ANAs can play a role in both early pregnancy complications, such as embryo implantation, and pregnancy loss (83). While low titers of ANAs are common in healthy women, those with RPL often exhibit high titers of ANAs (>1:160) (83). Moreover, ANAs have been suggested to have a direct effect on the quality and development of oocytes and embryos, leading to reduced implantation rates (84). In the fetal-maternal interface, ANAs can induce the precipitation of immune complexes, attributed to elevated C3 levels, resulting in T cell activation and increased production of inflammatory cytokine (IFN-α), which in turn stimulates the humoral

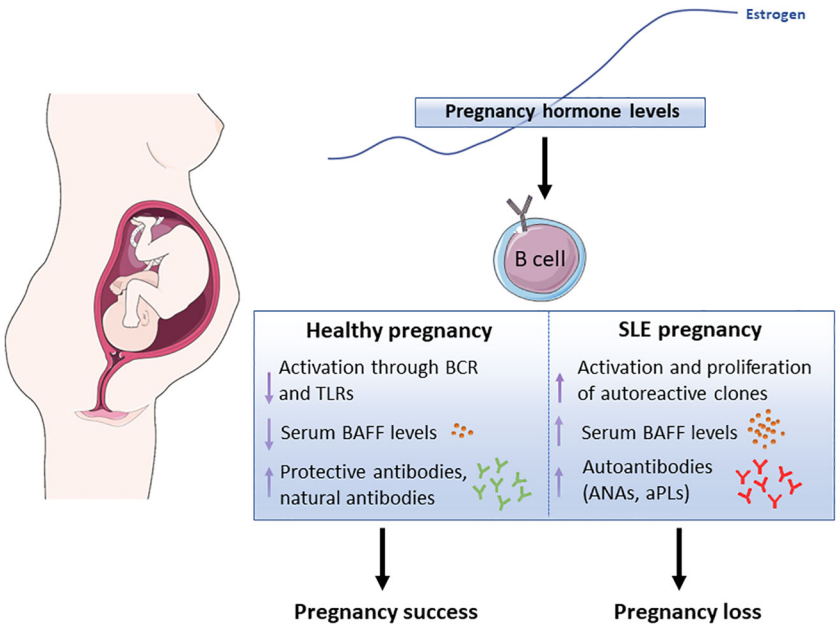

FIGURE 1
Schematic representation showing the potential effect of pregnancy-associated hormones, in particular estrogen, in B cell functions both, in healthy pregnancy and lupus pregnancy. BCR (B cell receptor), TLR (Toll-Like Receptor), BAFF (B Cell-Activating Factor), ANAs (anti-Nuclear Antibodies), aPLs (anti-Phospholipid Antibodies). The Figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/)

immune response (85, 86). Complement activation rapidly increases the production of the pro-inflammatory cytokine TNF, which in turn recruits inflammatory cells into the placenta, ultimately contributing to pregnancy loss (87).

It is well known that imbalances toward a pro-inflammatory milieu are associated with poor pregnancy outcomes (88). Moreover, the TNF/IL-10 ratio in serum is used as an indicator or predictor of pregnancy loss (89). In line with this, the production of IL-10 by B cells is considered essential for successful pregnancies (90). Interestingly, in patients with SLE, there is a significant decrease in IL-10 production by B cells (91). Even though, the elevated serum levels of IL-10 observed in pregnant women with SLE compared to controls (25) would be an advantage in normal pregnancy conditions, the immunosuppressive and anti-inflammatory effects of this cytokine are impaired in patients with SLE compared to healthy individuals (92).

Therefore, it is reasonable to speculate that uncontrolled B cell activation in patients with SLE during gestation may lead to the production of pro-inflammatory cytokines and harmful antibodies, which could potentially compromise the well-being of the pregnancy.

In conclusion, maintaining a balanced B-cell activation is essential for a successful pregnancy. In women with preexisting SLE, hormonal changes may disrupt this balance, leading to the production of inflammatory cytokines and autoantibodies. This dysregulation can exacerbate disease symptoms and contribute to pregnancy complications, including RPL. Therefore, understanding the impact of B-cell activation and its relationship with hormonal changes during gestation is crucial for managing SLE and optimizing pregnancy outcomes (Figure 1).

Author contributions

NV designed, drafted, and revised the work. MV designed and drafted the work. LD drafted and revised the work. FJ designed, drafted, supervised, and revised the work. 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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Low follistatin level is a causal risk factor for spontaneous abortion: a two-sample mendelian randomization study

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Background: Recurrent pregnancy loss is a distressing event during pregnancy, and understanding its causal factors is crucial. Follistatin, a glycoprotein involved in folliculogenesis and embryogenesis, has been implicated as a potential contributor to the risk of spontaneous abortion. However, establishing a causal relationship requires rigorous investigation using robust methods.

Methods: In this study, we utilized mendelian randomization (MR), a powerful genetic epidemiological approach, to examine the causal relationship between follistatin levels and spontaneous abortion. We obtained instrumental variables strongly associated with follistatin levels from large-scale genome-wide association from the IEU database. The inverse variance weighting (IVW) method was taken as gold standard. We also performed sensitivity test to evaluate the robustness of our result.

Results: MR analysis revealed a significant causal relationship between low follistatin levels and spontaneous abortion ($p = 0.03$). Sensitivity analyses, including pleiotropy test, heterogeneity test, and leave-one-out analysis, all supported the robustness of our findings.

Conclusion: Our study provides compelling evidence supporting the causal relationship between low follistatin levels and increased risk of spontaneous abortion. These findings underscore the importance of follistatin in the etiology of spontaneous abortion and suggest potential preventive interventions. Modulating follistatin levels or relevant pathways could hold promise for reducing the incidence of spontaneous abortion and improving reproductive outcomes. The utilization of MRs strengthens the validity of our

results by mitigating confounding and reverse causality biases. Further research is needed to elucidate the underlying molecular mechanisms and explore therapeutic strategies targeting follistatin levels.

KEYWORDS

follistatin, spontaneous abortion, mendelian randomization, abortion, recurrent pregnancy loss

1 Introduction

Spontaneous abortion is a frequently encountered complication during pregnancy, characterized by the loss of pregnancy before 20 weeks of gestation (1). Approximately 9-20% of all recognized pregnancies result in spontaneous abortion. Among these, 3-5% of couples face the challenge of two or more clinically recognized pregnancies ending in failure, known as recurrent pregnancy loss (RPL) (2, 3). RPL can be devastating, bringing great trauma to both the patient and their family (4). Various factors contribute to RPL, with chromosomal abnormality being the most common, responsible for over half of RPL cases (5). Additionally, 10%–15% of women with multiple pregnancy losses exhibit uterine anomalies, such as partial or complete septum (6, 7). Hormonal causes such as luteal phase defect, pregestational diabetes mellitus, and polycystic ovary disease can lead to RPL (8). Moreover, immune disorders may contribute to RPL by dysregulating trophoblast function and endometrial angiogenesis (9). For example, primary antiphospholipid syndrome (APS), present in one-third of RPL patients, is associated with elevated serum anti-phospholipid antibody (aPL) levels. Increased aPL levels reduce placental hormone production, impair trophoblast function, and result in pregnancy loss and other obstetric complications (10). Women with celiac disease often have elevated anti-transglutaminase type 2 (anti-TG2) autoantibodies, leading to reduced blood vessel formation and disrupted endometrial angiogenesis, contributing to RPL (11). Infections, exposure to environmental agents, and elevated homocysteine levels are also implicated in RPL (12). Nevertheless, the understanding of RPL remains significantly limited, as almost 50% of RPL cases are still categorized as unexplained (9).

Follistatin (FST) is a secreted protein that primarily synthesized and secreted by the liver, mainly implicated in suppressing follicle-stimulating hormone (FSH) activity through autocrine or paracrine mechanisms (13–15). Notably, FST serves as a binding protein and regulator in the transforming growth factor-beta (TGF-β) signaling pathway, selectively binding to ligands such as activins and bone morphogenetic proteins (BMP) (13, 16, 17). It restrained granulosa cell proliferation and steroidogenesis by neutralizing the action of activin (18–20). It also enhanced basal estradiol and progesterone production (21, 22), promoting cell invasion *via* the ALK4-SMAD2/3-SMAD4 signaling pathway (23–26).

Serum FST increased significantly throughout gestation until the first day of parturition and declined afterward (27). Evidence showed its possible role as chemokine to induce trophoblast migration and invasion through the enhanced JNK signaling, contributing to maintain trophoblast function and promote placental development (28, 29). FST was upregulated in the decidua during early pregnancy, and women with RPL were observed to have a lower endometrial expression of FST during the luteal phase (30). A lower FST level in endometrium stromal cells of women with RPL was also observed (30). Conditional knockout of mice uterine *Fst* can cause severe fertility defects, reduced responsiveness to estrogen and progesterone signals, impaired artificial decidualization, and an unreceptive environment for embryo attachment. These findings suggest that *Fst* may play a crucial role in facilitating uterine receptivity (31). A decreased FST level was also observed in serum and placenta of women with preeclampsia (PE) (32–34), resulting in impaired trophoblast function through upregulating GDF11 levels in trophoblasts. The dysregulation of the FST-GDF11-Smad2/3 axis may be critical to trophoblast function, which adds more evidence to the essential role of FST on trophoblast during pregnancy (35).

Mendelian randomization (MR) is an epidemiological tool based on genetic variants related to exposure factors, helping to assess the association of these gene variations with outcomes such as disease onset or mortality. Its core relies on using genetic data as a bridge to investigate causal relationships between a particular exposure and a specific outcome (36, 37). Randomized controlled trials (RCTs) have long been recognized as the gold standard for causal inference, yet it is costly and complicated to conduct. Similar to RCTs that randomly assign participants to a trial or control group, MR studies "randomize" participants based on one or more gene alleles that influence risk factors and attempt to determine if carriers of these genetic variations have different disease onset risks compared with non-carriers (38). Traditional observational study designs rely on exposure obtained through questionnaires,

biochemical assays, or imaging, whereas genetic variations exist at birth and remain stable throughout life. Notably, information of genetic variation and diseases is easy to acquire through open-dataset, and since it leaves out the complicated implementation process and ethical restriction, it is much easier to conduct compared with RCTs (39). In view of these incomparable advantages of MR, here we performed a two-sample MR analysis of the GWAS summary data from the UK Biobank and EBI database so as to find whether there is a potential causal association between FST level and spontaneous abortion, trying to provide novel evidence in this field of research.

2 Materials and methods

2.1 Study design

Here, we conducted a two-sample MR analysis to examine the possible causal association between FST level and spontaneous abortion. Figure 1 provides an overview of the study's key factors, including the exposures, outcomes, and genetic instruments. The study was based on previously published materials and public databases and received ethical approval and participant consent from the relevant institutional review committees.

The genetic variants in this study are fully considered based on the three principles below throughout our analysis. First is relevance assumption: the genetic variant must be closely correlated with follistatin levels; second is independence assumption: the genetic variant cannot be associated with any possible confounders of follistatin levels or spontaneous abortion; and third is exclusivity assumption: the genetic variant cannot be related to the relevant outcomes.

2.2 Data sources

2.2.1 Exposure population and data

We extracted exposure data from a previous study (40), downloaded from the website of IEU OPEN GWAS PROJECT (https://gwas.mrcieu.ac.uk/, GWAS ID: ebi-a-GCST90012080).

2.2.2 Outcome population and data

We extracted outcome data from a UK Biobank study (Dataset ID: ukb-d-O03), downloaded from the website of the IEU OPEN GWAS PROJECT (https://gwas.mrcieu.ac.uk/).

2.3 Statistical analysis

All the data processing and statistical analyze are performed using the R4.2.2 software (Lucent Technologies, USA). The MR, heterogeneity test and pleiotropy test were carried by the "Two Sample MR" package (41).

2.4 Extraction of instrumental variables

We performed two-step filtering to eliminate those SNPs that do not satisfy the relevance assumption and obtained the satisfying instrumental variables (IVs). We extracted SNPs that are (1) closely related to the exposure (follistatin level) at a genome-wide significance threshold of $p < 5 \times 10^{-8}$. 2) without linkage disequilibrium (LD) (linkage disequilibrium $r^2 < 0.05$), since we should make sure that no correlation LD between selected IVs and potential confounding factors exist. The R^2 value was calculated for these IVs to assess their association with the exposure (42).

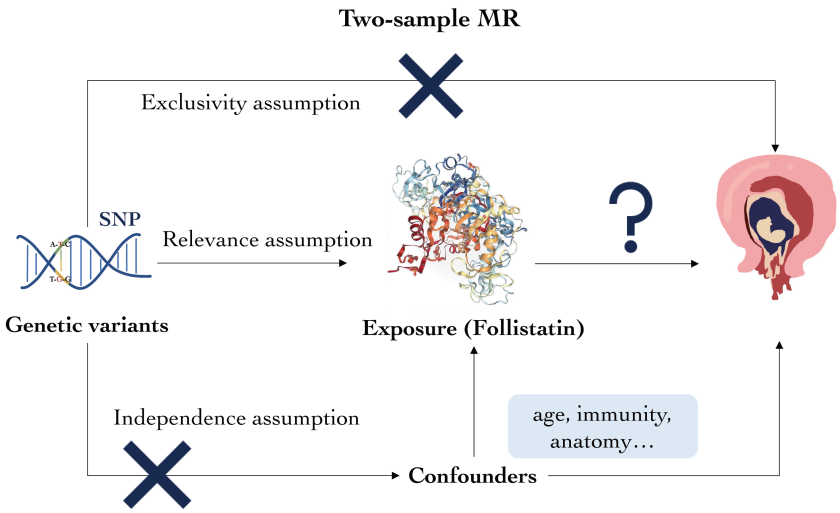

FIGURE 1
Overview of the study's key factors. Genetic variant, exposure, and outcomes are as described. Following the three assumptions, genetic variant must be closely correlated with the exposure but cannot be associated with neither the confounders nor the outcomes.

2.5 Elimination of confounding factors

We used PhenoScanner (Version 2), a database of human genotype–phenotype associations to figure out whether the confounding factors such as anatomical abnormalities, hormonal imbalances (PCOS, luteal phase defect, etc.), and immune disorders (antiphospholipid syndrome, lupus erythematosus, etc.) may influence our result according to the independent assumption (43, 44). Over 65 billion associations and over 150 million unique genetic variants are recorded in PhenoScanner. We applied the "Phenoscanner" R package to investigated each of the five IVs and their corresponding phenotypes (43, 44). Any IVs exhibiting associations with confounding factors were excluded from the analysis, applying stringent criteria ($p < 1 \times 10^{-5}$, $r^2 > 0.8$).

2.6 Sensitivity test

To make sure that our MR results are robust enough for us to come up with a causal conclusion, we extensively performed three aspects of sensitivity test, namely, heterogeneity test, pleiotropy test, and leave-one-out analysis test. R software was used to visualize results with depicting scatter plot, forest plot, etc.

2.7 Two-sample MR analysis

In two-sample MR analysis, five methods are commonly used: MR-Egger, Weighted Median, Inverse Variance Weighted, Simple Mode, and Weighted Mode. Among these methods, Inverse Variance Weighted is widely accepted and considered the most effective, as it accounts for heterogeneity when assessing causality. However, the other four methods also demonstrate robustness to varying degrees. MR-Egger is particularly useful when there is potential violation of instrumental variable assumptions, such as pleiotropy. It estimates the causal effect while allowing for average pleiotropic bias (45). The Weighted Median method provides a robust estimate by considering the median of all possible instrumental variable estimates, even when up to 50% of the instruments are invalid. This method is advantageous in situations where some instrumental variables may be biased or weak. The Simple Mode and Weighted Mode methods combine the estimates from multiple instruments by either taking the mode or using weighted averages. To evaluate the causal risk of FST levels, we performed all five methods. A positive result from any one or more of these methods would indicate a potential causal risk associated with FST levels.

3 Results

3.1 Study population

The exposure population pertains to follistatin levels, sourced from 39 cohorts of European ancestry. These data were thoroughly cleaned and summarized in a previously published study in 2020 (40), where a genome-wide meta-analysis of 90 cardiovascular-related proteins across 15 studies were identified. We extracted identified genetic variants related to follistatin levels from the recorded ebi-a dataset of IEU open GWAS project (GWAS ID: ebi-a-GCST90012080). In this dataset, 21,758 samples are involved, with 13,022,208 SNPs being reported (40). All of them are of European ancestry. The effect allele (EA), other allele (OA), beta coefficients, p value, and standard error (SE) are also included in this dataset for further investigation.

For the outcomes, summary-level data were obtained from the UK Biobank study (46). In the UK Biobank, pregnancy loss was defined as a history of self-reported spontaneous abortion or termination. We utilized the second round of Neale Lab's genome-wide association analyses in the UK Biobank, obtaining 1,150 female patients with spontaneous abortion and 360,044 matched controls of European ancestry and 9,543,298 detected SNPs.

3.2 Five SNPs are validated as instrumental variables

MR relies on the idea of random allocation of genetic traits. If the frequency of SNPs harmonizes with the alteration of the exposure, we can tentatively deduce that the SNP is correlated with the exposure. We screen the total of 13,022,208 SNPs in the exposure dataset based on the relevance assumption and independence assumption mentioned in the "study design" and finally achieved five SNPs that satisfied for IVs. We presented some detailed information for these SNPs such as effect allele frequency,

TABLE 1 General data for five SNPs as instrumental variables.

SNP for IV					Miscarriage (outcome)				Follistatin (exposure)				
Chr	Position	SNP ID	EA	OA	Beta	EAF	SE	*p* value	Beta	EAF	SE	*p* value	R^2
9	92228559	rs10908903	G	T	-1.43E-04	0.4677	0.0106	0.2850	0.0603	0.4587	0.0109	2.76E-08	3.43E-03
2	27730940	rs1260326	C	T	1.05E-04	0.6069	0.0107	0.4381	-0.1323	0.6024	0.0106	9.58E-36	3.21E-03
15	43726625	rs150844304	C	A	-5.26E-04	0.0245	0.0109	0.2187	0.2466	0.0294	0.0321	1.46E-14	6.98E-04
5	53327571	rs31226	C	T	2.62E-04	0.6064	0.0123	0.0541	-0.1289	0.5979	0.0107	1.56E-33	7.87E-04
12	57791833	rs7974833	C	T	9.15E-05	0.2367	0.0321	0.5561	0.0849	0.2350	0.0123	5.61E-12	1.55E-04

Chr, chromosome; SNP, single-nucleotide polymorphism; EA, effect allele; OA, other allele; EAF, effect allele frequency; SE, standard error.

TABLE 2 Evaluation of instrumental variables.

SNPs for IV		Follistatin level (exposure)			
Chr	Position	SNP ID	MAF	beta	SE
9	92228559	rs10908903	0.4587	0.0603	0.0109
2	27730940	rs1260326	0.6024	−0.1323	0.0106
15	43726625	rs150844304	0.0294	0.2466	0.0321
5	53327571	rs31226	0.5979	−0.1289	0.0107
12	57791833	rs7974833	0.235	0.0849	0.0123

IV, instrumental variable; Chr, chromosome; SNP, single nucleotide polymorphism; MAF, minor allele frequency; Beta, the effect size; R^2, IV explains the extent of exposure.

standard error, and effect allele (Table 1). In addition, we calculated the R^2 value for the IVs, which help to explain the extent of exposure (Table 2).

3.3 MR analysis showed that follistatin level is a causal risk for spontaneous abortion

Here, we adopted five methods to evaluate the follistatin level effect on the risk of spontaneous abortion, and the results are shown in Table 3 and Figure 2. Considering the absence of neither heterogeneity nor pleiotropy (which we would describe in detail on the next part), we selected IVW as the main method as well as the gold standard for determining the causal effect of FST level on the risk of spontaneous abortion (47). We found that the IVW method showed a p value of 0.03517563 (<0.05) and b of −0.001282787 (<0), indicating the causal relationship between low FST level in the European population.

3.4 Sensitivity analysis

In this work, sensitivity analysis is performed to (1) evaluate whether the results are robust and the conclusions are reliable; (2) assess whether the results have potential biases (such as genetic pleiotropy and data heterogeneity); and (3) evaluate whether there is a certain instrumental variable that significantly affects the outcome variable.

We detected no heterogeneity in the five IVs that we chose for the spontaneous abortion (MR–Egger Q statistics = 2.738052; Qdf = 3; Qpval = 0.4337995; IVW Q statistics = 2.875518; Qdf = 4; Qpval = 0.5788685) (Table 4). We then focused on the pleiotropy using the MR–Egger method. The intercept with the Y-axis represents the horizontal pleiotropy. Zero horizontal pleiotropy is one of the prerequisites of applying the MR method according to the exclusive assumption. Here, we noticed no horizontal pleiotropy existed in our MR analysis results (Egger_intercept = 7.505441e-05; se = 0.0002024312; p value = 0.7354456) (Table 5). In the "leave-one-out" analysis, we sequentially removed each SNP and calculated the MR effect of the remaining SNPs. We noticed that the removal of any of these individual SNPs did not result in significant changes of the overall causal estimation effect (Figure 3). Taken together, these results suggest that our findings were robust and the exception of single IV exert no difference on the overall estimated causal effect.

The Wald ratio method was used to estimate the causal effect of each individual SNP on the risk of spontaneous abortion. The findings have been presented in a forest plot to provide a visual representation (Figure 4). The threshold of significance for the forest plot remained controversial. It can be defined as either $p < 0.05$ or $p < 0.05/n$ (n refers to the number of SNPs). Here, comprehensively regarding the p values for each single SNP on the outcome (Table 1), the leave-one-out analysis test (Figure 3), and all the SNPs combined (Figure 4), it was quite clear that a causal association existed between follistatin level and the risk of spontaneous abortion.

4 Discussion

In this study, we employed two-sample MR to investigate the causal relationship between FST levels and spontaneous abortion. The evidence from MR analysis indicates that low follistatin level was a causal risk factor for spontaneous abortion and these results

TABLE 3 Causal effect between follistatin level and spontaneous abortion by different MR analysis methods.

Exposure	Outcome	Method	nSNP	p value	beta	R^2
Follistatin levels	Spontaneous abortion	MR–Egger	5	0.36562849	-0.001908907	3.43E-03
Follistatin levels	Spontaneous abortion	Weighted median	5	0.03961415	-0.001535967	3.21E-03
Follistatin levels	Spontaneous abortion	Inverse variance weighted	5	0.03517563	-0.001282787	6.98E-04
Follistatin levels	Spontaneous abortion	Simple mode	5	0.09652113	-0.002171829	7.87E-04
Follistatin levels	Spontaneous abortion	Weighted mode	5	0.10618687	-0.002087265	1.55E-04

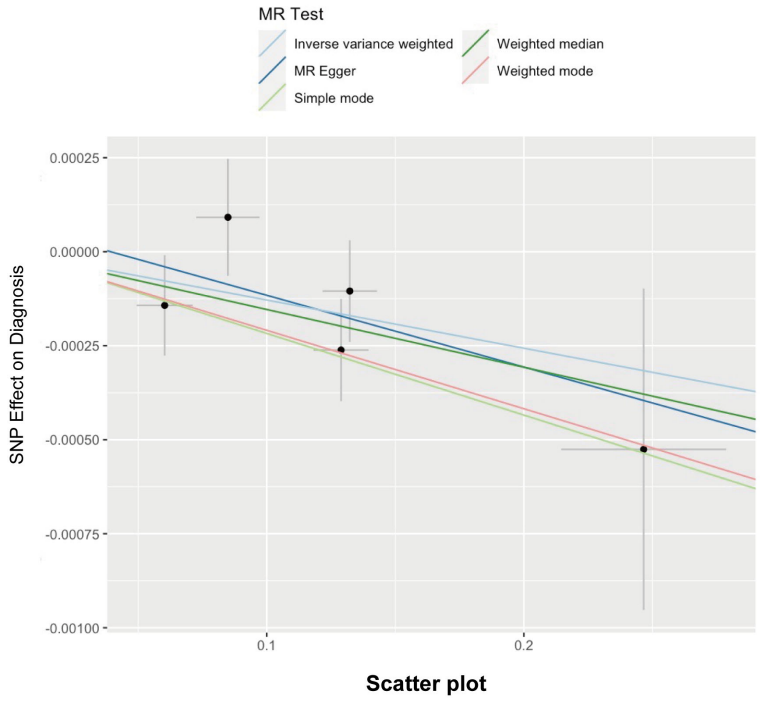

FIGURE 2
Scatter plot illustrating the distribution of individual ratio estimates of follistatin levels with spontaneous abortion as the outcome. Trend lines derived from five different 2SMR methods are also included in each scatter plot to indicate cause and effect.

were generally reliable as the sensitivity analysis strongly supports. These findings have important implications for understanding the pathogenesis of spontaneous abortion and may contribute to the development of potential preventive and therapeutic strategies.

It is widely acknowledged that establishing proper placentation involves sequential processes, notably trophoblast invasion and angiogenesis (48). The orchestrated interplay of angiogenic processes and steroid hormones induces transformative changes in the endometrium, facilitating its receptivity to the blastocyst and initiating the implantation process (49). Successful placentation and the commencement of pregnancy hinge on prerequisites such as endometrial angiogenesis, decidualization, and trophoblast invasion (48, 50, 51). Any dysregulation during the above processes may lead to pregnancy failure and RPL. High levels of anti-annexin V antibody can bind to trophoblast cells, affecting trophoblast invasiveness and causing defective placentation (52). Also, lots of proteins and related pathways have been recognized to be involved in regulating trophoblast function, such as TGF-β which governs the differentiation program of extravillous trophoblasts in the developing human placenta (53).

The underlying biological mechanisms linking low follistatin levels with spontaneous abortion warrant further investigation. Follistatin not only has an inhibitory effect on FSH secretion from cultured anterior pituitary cells (54) but also is involved in trophoblast invasion and embryonic development. FST acts as an antagonist to the TGF-β superfamily and thereby modulates important signaling pathways such as JNK signaling, ALK4-SMAD2/3-SMAD4 signaling, and FST-GDF11-Smad2/3, further affecting trophoblast function (55). It is hypothesized that reduced follistatin levels may disrupt multiple pathways such as JNK signaling, ALK4-SMAD2/3-SMAD4 signaling, and FST-GDF11-Smad2/3, leading to trophoblast dysfunction and causing impaired implantation and defective placental development, ultimately resulting in abortion. Future research should focus on elucidating the specific molecular mechanisms through which follistatin influences pregnancy outcomes.

Our results are consistent with previous studies that have reported a probable association between low follistatin levels and adverse pregnancy outcomes (30, 56–58). For instance, Prakash et al. (30) observed a dramatic decrease of FST expression in endometrial stromal cells of women with spontaneous abortion. However, it should be noted that some studies reported no significant decrease of follistatin in the serum of women with spontaneous abortion (59, 60). These discrepancies may arise

TABLE 4 Heterogeneity statistics.

Method	Q Q_df	Q_pval
MR–Egger	2.738052	0.4337995
Inverse variance weighted	2.875518	0.5788685

TABLE 5 Pleiotropy statistics of MR analysis.

Method	Egger regression of intercept	SE	*p* value
MR–Egger	7.51E-05	0.000202431	0.7354456

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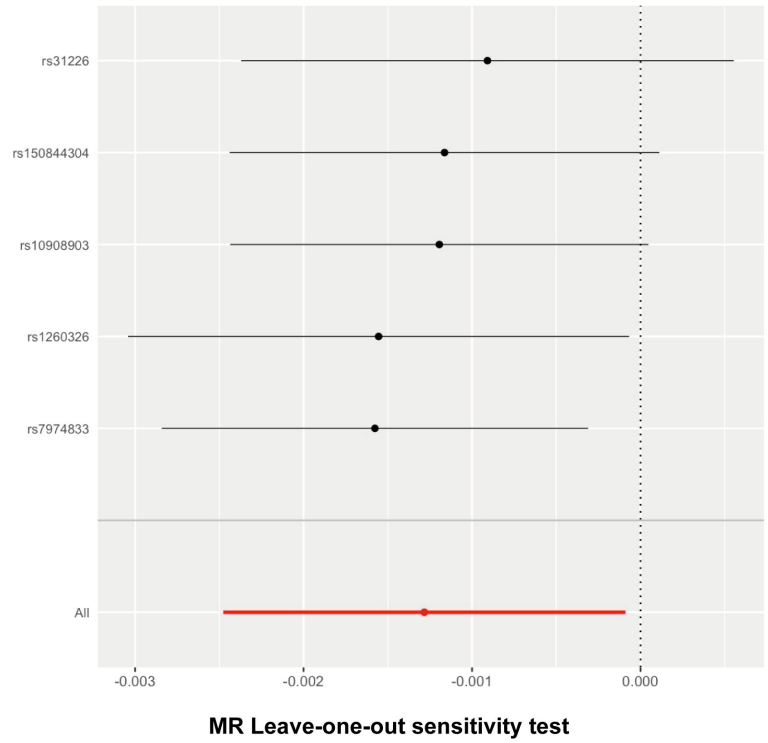

FIGURE 3
Leave-one-out analysis for follistatin levels on spontaneous abortion. The given dark dots indicate effect measures from IVW-MR analysis excluding specific SNPs. Red line indicates pooled analysis including all SNPs by the IVW-MR method (plotted for comparison).

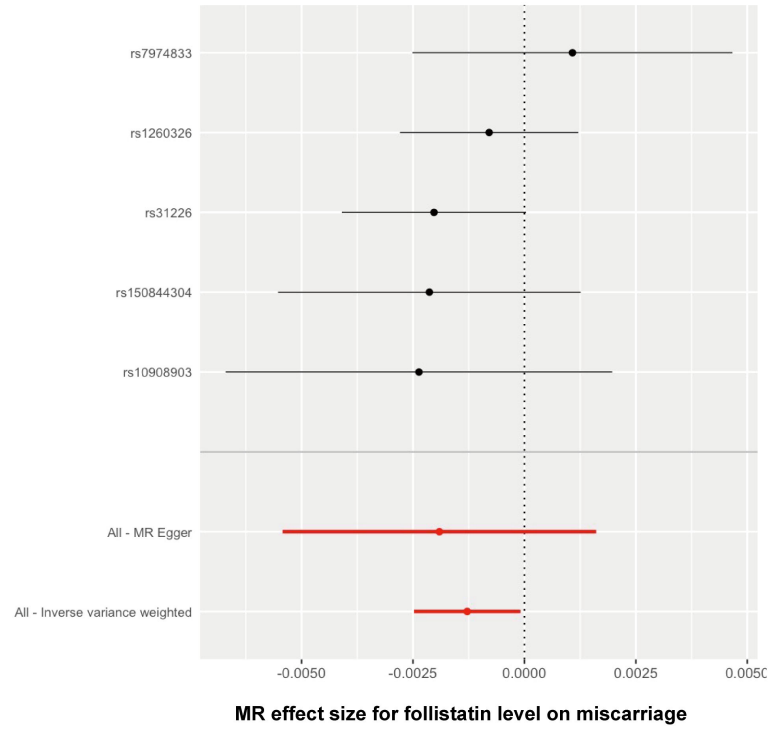

FIGURE 4
Forest plot showing the causal effect of each single SNP on the risk of miscarriage.

from variations in sample size and characteristics, since it only includes around 10 abortion samples and 10 control samples.

Although previous studies have pointed out a possible association between FST and spontaneous abortion, there is no absolute evidence on a genetic aspect. Establishing a direct causal link between follistatin and spontaneous abortion presents challenges due to confounding effects from factors such as lifestyle and environmental influences. To overcome this challenge, a promising approach is the utilization of MR, which leverages genetic variants as IVs to infer causal relationships. In our study, we utilized MR to present novel evidence that demonstrates a causal relationship between low FST levels and spontaneous abortion. This finding holds significant implications for the field of reproductive health. Our results suggest that interventions targeting increased FST levels may have the potential to reduce the incidence of spontaneous abortion, which is a devastating outcome for numerous couples. However, despite the valuable insights gained from this study, several limitations should be acknowledged. We recognize that our analysis only included the European population, introducing the possibility of potential bias associated with differing ancestries.

Our study yields meaningful clinical implications. First, it indicates that FST levels might be integrated into routine antenatal assessments to evaluate the risk of pregnancy failure. This is particularly crucial for individuals with a history of spontaneous abortion, where assessing their FST levels may serve as a predictive indicator for the occurrence of RPL. Concurrently, FST may function as a prospective biomarker for targeted interventions like hormonal therapies or lifestyle adjustments and the development of personalized medical strategies. Future studies could explore interventions aimed at modulating follistatin levels to potentially prevent or mitigate the risk of spontaneous abortion. Moreover, investigations into the long-term effects of low follistatin levels on maternal and offspring health outcomes would be valuable for a comprehensive understanding of the implications.

In conclusion, our study contributes to the growing body of reliable evidence supporting the critical role of FST in successful pregnancy outcomes and highlights it as a promising therapeutic target. We remain hopeful that further research, conducted with larger sample sizes based on our observations, will provide additional insights into the underlying mechanisms that link FST and pregnancy outcomes.

Data availability statement

This study analyzed the exposure data acquired from EBI database and the outcome data sourced from the UK Biobank. Both datasets are publicly accessible and can be found on the IEU Open GWAS Project website (https://gwas.mrcieu.ac.uk/).

Author contributions

CG: Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. WY: Methodology, Writing – review & editing. XL: Writing – review & editing. XLL: Writing – review & editing. YW: Validation, Writing – review & editing. CT: 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.

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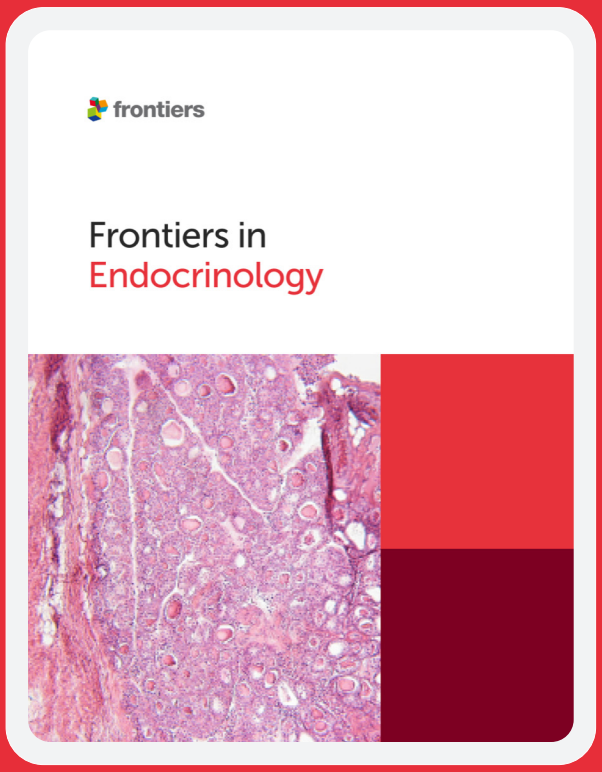

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