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PCOS: From infertility to pregnancy

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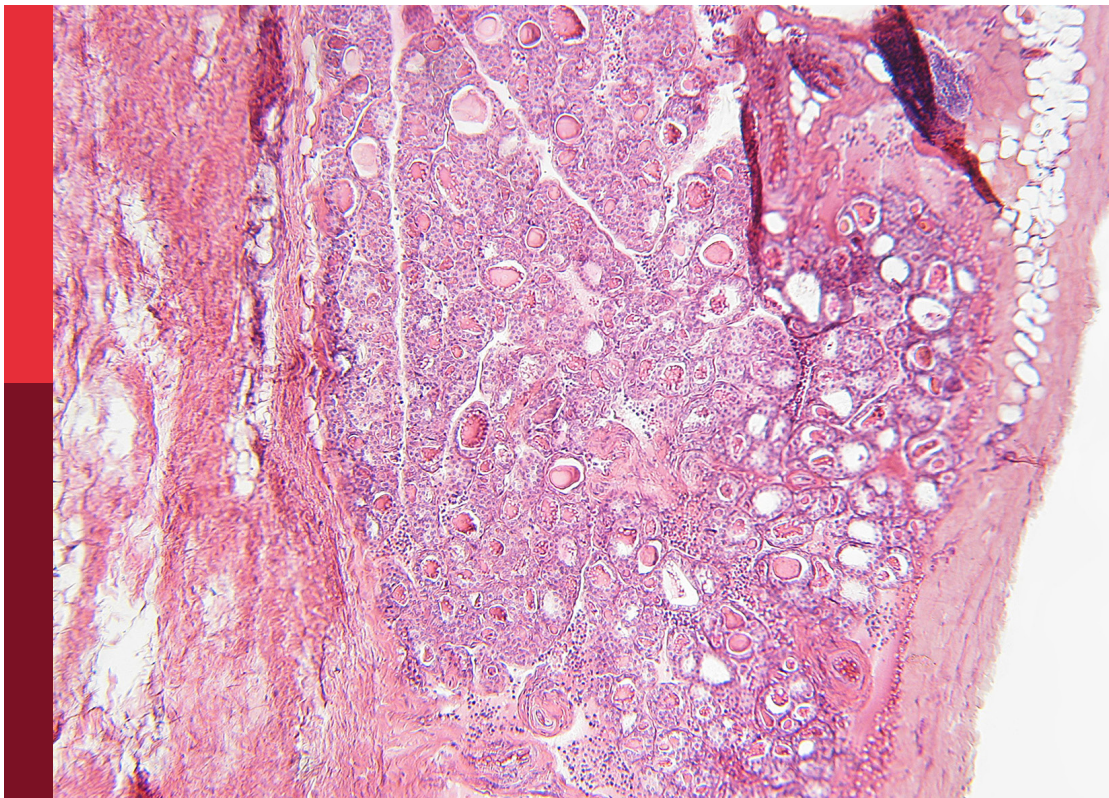

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PCOS: From infertility to pregnancy

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Editorial: PCOS: from infertility to pregnancy

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cardiovascular risk, infertility, PCOS, polycystic ovarian syndrome, pregnancy, reproduction

Editorial on the Research Topic
PCOS: from infertility to pregnancy

Polycystic ovary syndrome (PCOS) is a heterogeneous and very frequent endocrinological disease associated with reproductive alterations (1). Women with PCOS have reduced fertility in the presence of ovulatory dysfunction that is a key element in the pathogenesis of PCOS (2). However, patients with PCOS have alterations in several reproductive athways independently of their ovulatory status (3). These include primary and secondary alterations of the oocyte and embryo quality (4), and of endometrial competence (5). Those endometrial alterations, with subsequent abnormal trophoblast invasion and placentation, are probably the main etiologic factor of the increased incidence of pregnancy complications in PCOS (6). However, PCOS is also associated with an increased cardiometabolic risk (1), albeit clinical evidence about the related mortality is still lacking.

The current Research Topic includes 11 papers, selected after an intense reviewing process. Five articles represent original research, whereas the other 6 consisted of narrative and systematic reviews with or without meta-analysis.

In an experimental animal study, Feng et al. assessed the therapeutic effect and mechanism of action of *Dendrobium officinale* extract, which is commonly administered in China as a dietary supplement, in letrozole-induced PCOS rats. *Dendrobium officinale* extract reduced body weight, and restored estrous cycle by improving follicle development and lowering testosterone levels. These effects were potentially mediated by gut microbiota changes. Shen et al. assessed the effect of curcumin, a phenolic compound with potent anti-inflammatory and antioxidant properties also administered as a dietary supplement. The meta-analysis of 7 randomized controlled trials (RCTs) demonstrated that curcumin is safe and effective to reduce body weight, to improve inflammation, and markers of insulin resistance and lipid profile. Unfortunately, many trials had a high risk of bias, are from the Asiatic area and included a very low number of randomized subjects. Similarly, Rong et al. reported the synthesis of data from 16 RCTs for a total of 1385 patients showing the safety and efficacy of the *Guizhi fuling* pill as an adjuvant treatment for infertile patients with PCOS. When added to Western medicine, including oral contraceptives, insulin-sensitizing drugs, and clomiphene citrate, *Guizhi fuling* pill, a traditional Chinese herbal formula, induced a significant improvement in the ovulation and pregnancy rates of about 24% and 53%, respectively, in PCOS patients. Again the suboptimal certainty of evidence and the unclear risk of bias precluded any clear-cut recommendation for clinicians. A narrative

review by Shawky discussed the available experimental and clinical data on the increased cardio-metabolic risk in offspring of women with PCOS exploring the different theories supporting that risk and analyzing the sex differences. Clearly, the increased cardiometabolic risk in offspring of mothers with PCOS probably needs the interaction of environmental (not only prenatal but also postnatal) and genetic factors over the lifespan. A sex interaction cannot be excluded as mediator of cardiometabolic outcomes. Gao et al. firstly performed a systematic review with meta-analysis, including 18 studies (case-control and cohort studies) for a total of 1265 PCOS patients and 894 controls. These studies aimed to evaluate the potential role of apelin and chemerin, two newly identified adipokines widely expressed in different organs. The data synthesis demonstrates that only chemerin, but not apelin, is significantly higher in PCOS patients suggesting that chemerin may be a therapeutic target to reduce the cardiovascular risk in patients with PCOS. Acupuncture is more and more used as a complementary and alternative treatment for infertility with controversial data regarding its efficacy (7). A comprehensive systematic review by Ye et al. on the use of acupuncture in women with PCOS underlined that all available meta-analyses agree that clinical evidence about the effectiveness of acupuncture for improving fertility is scarce in PCOS patients. Therefore, robust and well-designed clinical trials are needed to confirm or refute these hypotheses of efficacy. In the current Research Topic, the effect of acupuncture in PCOS patients under metformin administration was tested by Chen et al. in a systematic review with meta-analysis of 9 RCTs including a total of 1159 women. Even if acupuncture improved the ovulation and pregnancy rate by about 30% in PCOS patients with a potential action on the insulin resistance, the risk of bias was high for many studies and the quality of the evidence was defined as low to very low (8). Another interesting RCT by Zhang et al. assessed the effect of canagliflozin, a sodium-glucose cotransporter-2 (SGLT-2) inhibitor, a novel class of hypoglycemic oral drugs that enhance the renal glucose loss in 51 women with PCOS. Three months of canagliflozin administration induced only a significant, but clinically modest, beneficial effect on total testosterone and glucose concentrations.

Surprisingly, only one paper was published in this Research Topic about evidence-based treatment for infertile PCOS patients, such as letrozole administration (9, 10). The retrospective analysis by Shi et al. on a total of 539 infertile patients with PCOS, showed that letrozole administered starting on day 5 following menstrual bleeding is associated with a cumulative conception rate about 10% higher in comparison with administration on day 3 due to a potentially better endometrial thickness.

During the last years, the follicular output rate (FORT) has acquired a progressively more important role for scientists and clinicians as a marker of good reproductive prognosis. FORT is the ratio of pre-ovulatory follicle count on the trigger day to the antral follicle count (AFC), and is considered an important criterion by which to quantify the follicular development potential (11). Jiang et al. retrospectively analyzed the effects of FORT on reproductive performance in 454 infertile patients with PCOS scheduled for IVF cycles and showed that FORT is directly related to the cumulative pregnancy and live birth rates when the FORT was less than 70%. After stratification analyses, each additional unit of FORT increased the cumulative live birth rate by 1.3% confirming that FORT may be used as a simple and non-invasive tool to assess the dynamic changes of follicular growth in response to exogenous gonadotropins in the clinical practice.

Finally, in the current Research Topic, Bibi et al. published for the first time a link between some specific mitochondrial DNA mutations and PCOS using next-generation sequencing (NGS). Six regions with common variations in all analyzed genomes were identified, even if individual variations were also reported. Interestingly, the score-based pathogenicity analysis of mitochondrial variants that demonstrated frameshift mutations in the ND2 gene was associated to the highest risk for PCOS, potentially opening the door to predisposition tests for PCOS.

In conclusion, the reading of the current Research Topic, beside the scientific value of the specific papers published, arouse in us two main considerations. First, very few conclusions may be drawn from systematic reviews after considering the suboptimal quality of many of the studies available. Second, there is great interest from Asian scientists in regard to PCOS and its management in conjunction with alternative and adjuvant treatments. We hope that this Research Topic may stimulate the design of well-structured and powered RCTs, also in Western countries, to confirm or refute these new, interesting, and potentially useful strategies of treatment for women with PCOS.

Author contributions

SP and DD conceptualized the study, acquired the main data, drafted the article, provided their final approval of the version to be published and agree to be accountable for all aspects of the work especially regarding its accuracy and integrity.

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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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Dendrobium officinale polysaccharide ameliorates polycystic ovary syndrome *via* regulating butyrate dependent gut–brain–ovary axis mechanism

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Research has shown that dendrobium officinale polysaccharide (DOP) can promote follicular development and inhibit the apoptosis of ovarian granular cells in PCOS rats. However, DOP cannot be absorbed directly by the stomach and small intestine but is degraded into short-chain fatty acids by gut microbiota in the large intestine and regulates the composition of gut microbiota. How DOP improved ovarian function in PCOS rats through the blood–brain barrier is unclear. In this study, we generated letrozole-induced PCOS rat models and studied the therapeutic effect and mechanism of DOP. 16S rRNA amplicon sequencing analysis, GC-MS short-chain fatty acid detection, and Gene Expression Omnibus database searching were conducted to screen the significantly changed pathways, and a series of experiments, such as enzyme-linked immunosorbent assay, RT-qPCR, Western blot, and immunohistochemistry, were performed. We found that DOP treatment could improve ovarian morphology and endocrine disorders, restore the normal estrus cycle, increase gut microbiota α diversity, and alter β diversity and enrichment of butyrate-producing bacterium in PCOS rats. In addition, compared with PCOS rats, those treated with DOP exhibited higher butyrate and polypeptide YY levels, possibly due to the regulation of G protein-coupled receptor 41 expression. These results indicated that DOP relieved the symptoms of PCOS rats which may be related to the mechanism of butyrate dependent gut–brain–ovary axis protection.

KEYWORDS

dendrobium officinale polysaccharide, polycystic ovary syndrome, ovary, gut microbiota, butyrate

Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine and metabolic disorder prevalent in women of reproductive age. It is characterized by ovulatory dysfunction, hyperandrogenism, and polycystic ovarian morphology (1) and is accompanied by various metabolic abnormalities, such as insulin resistance, hyperinsulinemia, and adiposity (2). It has been demonstrated that gut microbiota composition changes and dysbiosis occur in PCOS animal models and women with PCOS, which is closely related to hyperandrogenism (3–5). Gut bacteria-released metabolites have an effective role in weight control by stimulating gut satietogenic hormones, controlling lipid metabolism in adipose tissue, influencing insulin signaling, and improving gut barrier function (6).

Metformin (MET) is an insulin sensitizer, which can improve menstruation, hyperinsulinemia, hyperandrogenemia, and BMI in PCOS (7–10), but long-term use can lead to gastrointestinal distress and even lower pregnancy rates (11, 12). Therefore, traditional Chinese medicine, which has few side effects and a wide range of targets, is becoming an option for the treatment of PCOS.

Dendrobium officinale Kimura et Migo is a traditional and valuable Chinese medicine and is popularly consumed as a functional dietary supplement. Dendrobium officinale polysaccharide (DOP) is the main active component that has various biological functions including antioxidant (13), anti-angiogenesis (14), anti-fatigue (15), and anti-apoptosis (16), as well as improving gut health (17). DOP treatment in type 2 diabetic rats improved the liver metabolism disorder and protected the liver against oxidative stress and inflammatory injury (18). Moreover, DOP could also improve insulin resistance and abnormal lipid metabolism in obese mice (19). Those studies suggest that DOP has great potential in the treatment of diseases related to lipid metabolism abnormalities. Research has shown that DOP can promote follicular development and inhibit apoptosis of ovarian granular cells in PCOS rats (20), but DOP cannot be digested and absorbed by the stomach and small intestine (21), and how DOP acts on the ovary through the blood–brain barrier remains unclear.

Herein, this study was conducted to investigate the effects of DOP on estrus cycle, pathophysiology, endocrine hormone, gut microbial diversity, and metabolites in PCOS rats to clarify the mechanism of DOP improving PCOS.

Abbreviations: DOP, dendrobium officinale polysaccharide; PCOS, polycystic ovary syndrome; MET, metformin; CMC, carboxymethyl cellulose; NS, normal saline; LH, luteinizing hormone; FSH, follicle stimulating hormone; FBG, fasting blood glucose; T, testosterone; E_2, estradiol; PYY, peptide tyrosine-tyrosine; GPR41, G protein-coupled receptor 41; SCFAs, short-chain fatty acids; CYP17A1, 17-a hydroxylase.

Materials and methods

Materials

Dendrobium officinale polysaccharide (VTY24621, Clara Reagent Grade, 98%) was purchased from Dehang Wuzhou (Beijing, China). Primary antibodies PI3 Kinase p85 (Cat#4257), anti–Phospho-AKT (ser473) (Cat#4060), and anti–Phospho-mTOR (ser2448) (Cat# 5536) were purchased from Cell Signaling Technology (USA). Anti-GPR41 (Cat#AF9057) and anti-GAPDH (Cat#AF7021) were purchased from Affinity Biosciences Technology (Jiangsu, China). SYBR Premix EX Taq™ (RR047A) and SYBR Premix EX Taq™ Kit (RR820A) were purchased from TaKaRa (Dalian, China), and PCR primers were purchased from Gencreate (Wuhan, China). RIPA tissue/cell lysate was purchased from Solarbio Life Sciences (Beijing, China). The ELISA kits [testosterone (E05101m), insulin (E05070r), polypeptide YY (E13432r), and estradiol (E05110r)] were purchased from CUSABIO (Wuhan, China). Metformin hydrochloride (H11021518) was purchased from Beijing Jingfeng Pharmaceutical Group Co., Ltd. (Beijing, China).

Animals

Eight-week-old female Sprague-Dawley rats (180 ± 20g) were purchased from Changsha Tianqin Biotechnology Co., Ltd. (Hunan, China) [SCXK (Hunan) 2019-0014, No. 430726210100078487] and raised at the Laboratory Animal Center of Youjiang Medical University for Nationalities [SYXK (Guangxi): 2017-0003] (22 ± 2°C, relative humidity 55 ± 5%, 12-h light/dark cycle). All the experimental procedures were approved by the Animal Welfare and Ethics Committee of Youjiang Medical University for Nationalities (YY. No 2020032511).

Establishment of PCOS model and treatment

After 1 week of acclimatization, vaginal smears were taken for 5 consecutive days, and 30 rats with normal estrus cycles were selected for subsequent experiments. Then the rats were randomly divided into two groups: the normal saline group (NS group) and the model group. The model group was orally administered with letrozole [1 mg/kg/day, dissolved in 0.5% carboxymethyl cellulose (CMC)], and the normal saline group was orally administered with 0.5% CMC (1 ml/kg/day) for 28 days. Three rats were randomly selected from each group for model validation, and the successful PCOS model in rats was ensured by testing serum testosterone levels and ovarian histopathological observations. Then the model group was

randomly divided into dendrobium officinale polysaccharide group (DOP group), metformin group (MET group), and polycystic ovary syndrome group (PCOS group). For the PCOS group, the rats were orally administered with normal saline (1 ml/kg/day). For the DOP group, the rats were orally administered with DOP (200 mg/kg/day). For the MET group, the rats were orally administered with MET (300 mg/kg/day) and for the NS group, and the rats were orally administered with normal saline. During treatment, vaginal smears were performed for 10 consecutive days. After 28 days of treatment, all rats were sacrificed, then fasting blood glucose (FBG), serum, ovaries, colons, and colon feces were collected for further analysis. During the process and treatment, the rats were weighed weekly.

Serum collection and hormone level determination

After 28 days of treatment, all rats were anesthetized with isoflurane and blood was taken from the abdominal aorta, left at 4°C overnight, then the serum was isolated by centrifuging at 4,000 rpm for 10 min then stored at -80°C. FSH and LH levels were measured by the Beijing North Institute of Biotechnology Co., Ltd. (Beijing, China). The serum concentrations of hormones (testosterone, fasting insulin, polypeptide YY, and estradiol) were determined using ELISA kits.

Fecal sample collection and determination

Feces were collected from the colon of rats and frozen in liquid nitrogen for 5 min then stored at -80°C. The 16S rRNA amplicon sequencing and GC-MS short-chain fatty acids were detected respectively by OE Biotech Inc. (Shanghai, China) and Luming Biotech Inc. (Shanghai, China).

Ovary morphology analysis

The ovarian tissue samples were fixed in 4% paraformaldehyde solution at 4°C over 24 h, then embedded in paraffin and cut into 3~4-μm thickness for hematoxylin–eosin staining. Analysis was performed using a light microscope (Olympus, Japan).

Quantitative real-time PCR analysis

Total RNA was prepared from frozen rat ovarian tissues using the TRIzol method, and cDNA was synthesized using a PrimeScript RT Reagent Kit with gDNA Eraser. Quantitative real-time PCR was performed according to the SYBR Premix EX Taq^{TM} kit instruction and run on the LightCycler 96 system (Roche, USA). The housekeeping gene β-actin was used for normalization, and the relative expressions of each gene were calculated by the $2^{-\Delta\Delta CT}$ method. Gene primers are listed as follows: β-actin—forward 5′-CGATGGGAAGTGCTGGATAG-3′ and reverse 5′-CGGTTAGAGTAGGTGACGTTG-3′; PI3K—forward 5′-AATACACCTGGTGCTCGACAC-3′ and reverse 5′-CCTCTGATCTTGACCCTGAAC-3′; AKT—forward 5′-CACAGGTCGCTACATGCCA-3′ and reverse 5′-GTAAGGAAGGGATGCCTAGAG-3′; mTOR—forward 5′-TGCCAACTACCTTCGGAACC-3′ and reverse 5′-GCTCGCTTCACTTCAAACTCC-3′; CYP17A1—forward 5′-ATCCGAGAAGTGCTGCGTATC-3′ and reverse 5′-GGCATGAACTGATCTGGCTG-3′.

Western blot analysis

Appropriate amount of ovarian tissue was added into RIPA tissue/cell lysate solution to extract total protein and determine the concentration. Then, 4× protein loading buffer was added and boiled to denature. Thirty-microgram protein samples were separated with 8% SDS-PAGE gel at 120 V for 1 h. The isolated proteins were transferred onto PVDF membranes and incubated at 250 mA for 1 to 2.5 h. After incubation of the PVDF membranes with the blocking solution, the protein strips were mixed with primary antibody (diluted concentration, 1:1,000) and incubated overnight at 4°C. The blots were then washed with TBST buffer and incubated with goat anti-rabbit antibodies for 1 h at room temperature. The chemiluminescent assay kit and the protein expression levels were normalized to GAPDH. ImageJ software was used to analyze the results.

Immunohistochemical analysis

The procedure of immunohistochemical staining was carried out according to the instructions. To put it simply, paraffin sections of colon tissue are first dewaxed and hydrated, followed by an antigenic repair, followed by dripping of an endogenous peroxidase blocker, incubated at room temperature for 10 min, washed with PBS for 3 min × 3 times, and finally dropped with primary antibody at 4°C and incubated overnight. On the second day, they are washed with PBS buffer for 3 min × 3 times, incubated with reaction enhancement solution at 37°C for 20 min, and washed with PBS buffer for 3 min × 3 times. Goat anti-rabbit lg G polymer was dropped, followed by DAB display, tap water washing, hematoxylin staining solution incubation for 20 s, alcohol dehydration, xylene transparency, and neutral gum sealing. The staining results were observed under a light microscope.

Statistical analysis

Data are presented as the means ± standard deviation (SD). Data analysis and mapping were performed separately using SPSS 20.0 and GraphPad Prism 8.0 software. Integrated optical density was measured using Image Pro Plus 6.0 software. Differences among multiple groups were compared using one-way analysis of variance followed by LSD (L)-Dunnett's T3(3) analysis; $p < 0.05$ was considered to indicate a statistically significant difference.

Results

DOP treatment reduced the body weight in rats

Before the experiment starts, there was no significant difference in body weight between the NS group and model group (Supplement 1). After rats were orally administered with letrozole for 4 weeks, the body weight of rats was significantly higher than in the NS group (Figure 1A). After treatment for 4 weeks, the body weight of rats in the DOP group was significantly lower than in the PCOS group (Figure 1B), indicating that DOP has a certain effect on body weight loss.

DOP treatment could restore the estrus cycle in rats

The estrous cycle in rat averages 4~5 days and is generally divided into four stages: proestrus, estrus, metestrus, and diestrus. The stages of estrus are distinguished by identifying different cell types (22). Proestrus: nucleated epithelial cells are the majority, oval in shape, and occasionally there are a few white cells and irregular keratinized epithelial cells. Estrus: most of them are irregular-shaped keratinized epithelial cells, which gather together in piles and are shaped like leaves. Metestrus: keratinized epithelial cells, nucleated epithelial cells, and leukocytes are all visible in similar proportions. Diestrus: many neutrophils and a few nucleated epithelial cells are present. During the treatment, vaginal smears were examined in rats for 10 consecutive days (approximately two estrus cycles) at 8 to 9 a.m. from day 19 to assess the effect of treatment (Figure 2-1), and it was found that the NS rats still maintained regular estrous cycles (Figure 2-2A), while the PCOS rats remained in the diestrus (Figure 2-2B), but rats treated with DOP/MET had improved estrus cycles (Figures 2-2C-D). A normal estrus cycle should have four consecutive estrus stages; thus, we counted the estrus stages for each rat. A complete estrus cycle observed is defined as estrus regular and conversely as irregular. The results showed that rats in the NS group all had regular estrous cycles but not in the PCOS group. After DOP/MET treatment which can reverse to irregular in most of the rats, the recovery rate of estrus cycles was up to 66.7% and 83.3% (Figure 2-2E).

DOP treatment improved endocrine disorders but without affecting fasting glucose in rats

To evaluate the therapeutic effects of DOP, we collected serum for steroid hormone level detection. The testosterone (T), luteinizing hormone (LH), insulin, and estradiol (E_2) levels in the PCOS group were significantly higher than in the NS group (Figure 3A–C, 3E), and DOP/MET treatment was significantly reduced, while the serum FSH levels in the PCOS group were significantly lower than those in the NS group (Figure 3D), and DOP/MET treatment significantly increased the serum FSH level when compared to the PCOS group. There was no significant difference in fasting blood glucose among all groups (Figure 3F).

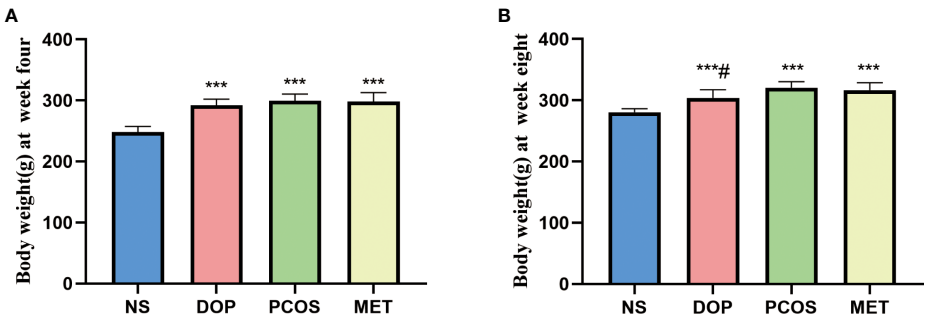

FIGURE 1
Effects of DOP treatment on body weight in rats, n = 6. Note: compared with the NS group, ***$p < 0.001$; compared with the PCOS group #$p < 0.05$.

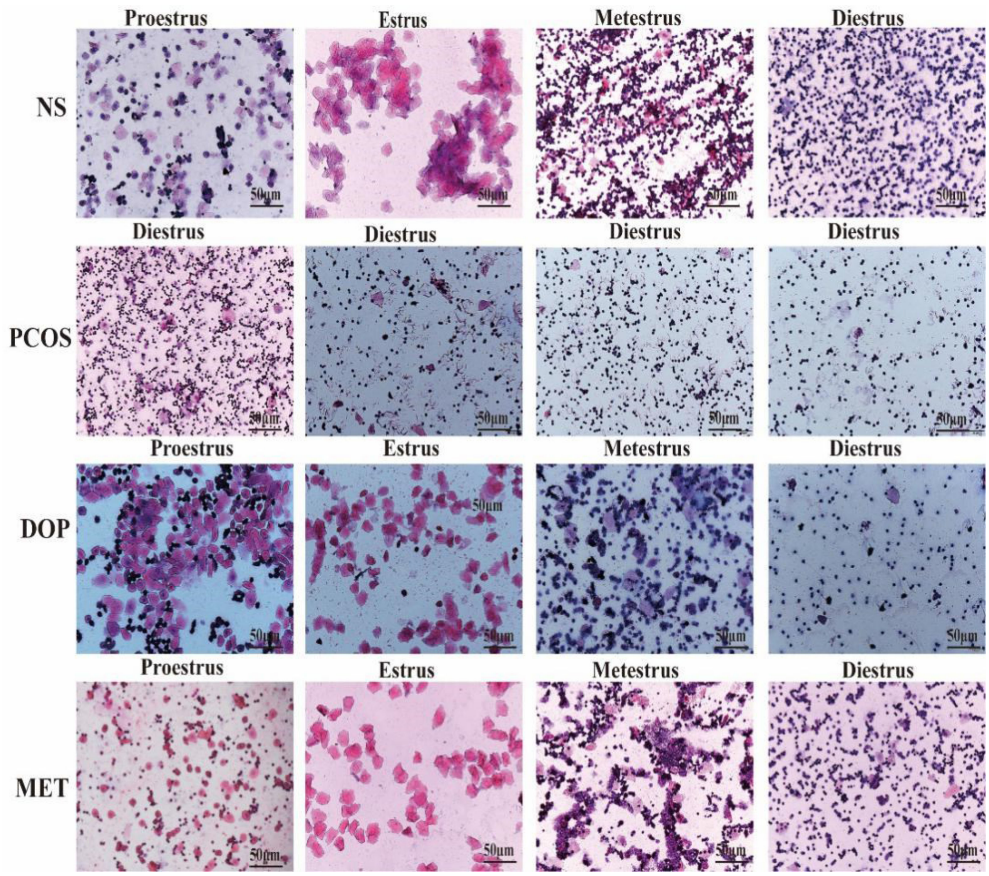

FIGURE 2-1
Vaginal smear identification of estrus stage in rats **(H&E)**. Note: scale bar = 50 μm.

DOP treatment can improve the morphology of polycystic ovary in rats

As shown in Figure 4, the ovarian morphology in the NS group was normal (Figures 4A–a). In contrast, the ovaries of letrozole-treated rats showed typical PCOS characteristics, including increased cystic follicles, fewer corpora lutea, and thinning of granulosa cell layers (Figures 4D–d). After DOP/MET treatment, we found that polycystic ovary morphology was markedly attenuated and showed more corpora lutea (Figures 4B–b, C–c).

DOP treatment regulated the PI3K–AKT–mTOR pathway in rats

In order to study the molecular mechanism of DOP treatment to improve the phenotype of PCOS rats, we extracted data set GSE98595 from the GEO database and predicted the differently expressed genes. Metascape was used for gene enrichment analysis (Supplement 2). As shown in Figure 5A, the first three gene sets with the most significant enrichment were hsa:04144, hsa:04010, and hsa:04151. It is noteworthy that the PI3K-Akt signaling pathway regulates the activation of primordial follicles (23). The results showed that PI3K, AKT, mTOR, and CYP17A1mRNA expression in the PCOS group was significantly higher than that in the NS group (Figures 5B–E). After DOP/MET treatment, PI3K, AKT, mTOR, and CYP17A1mRNA expression was significantly lower than in the PCOS group. Western blot results further confirmed that compared with the PCOS group, the expression of PI3K, p-AKT, and p-mTOR proteins in the DOP group was significantly reduced (Figures 5F–J).

The diversity of the gut microbiota was altered by DOP treatment in rats

As mentioned above, our study has proved that DOP can improve endocrine disorders and follicle development in PCOS

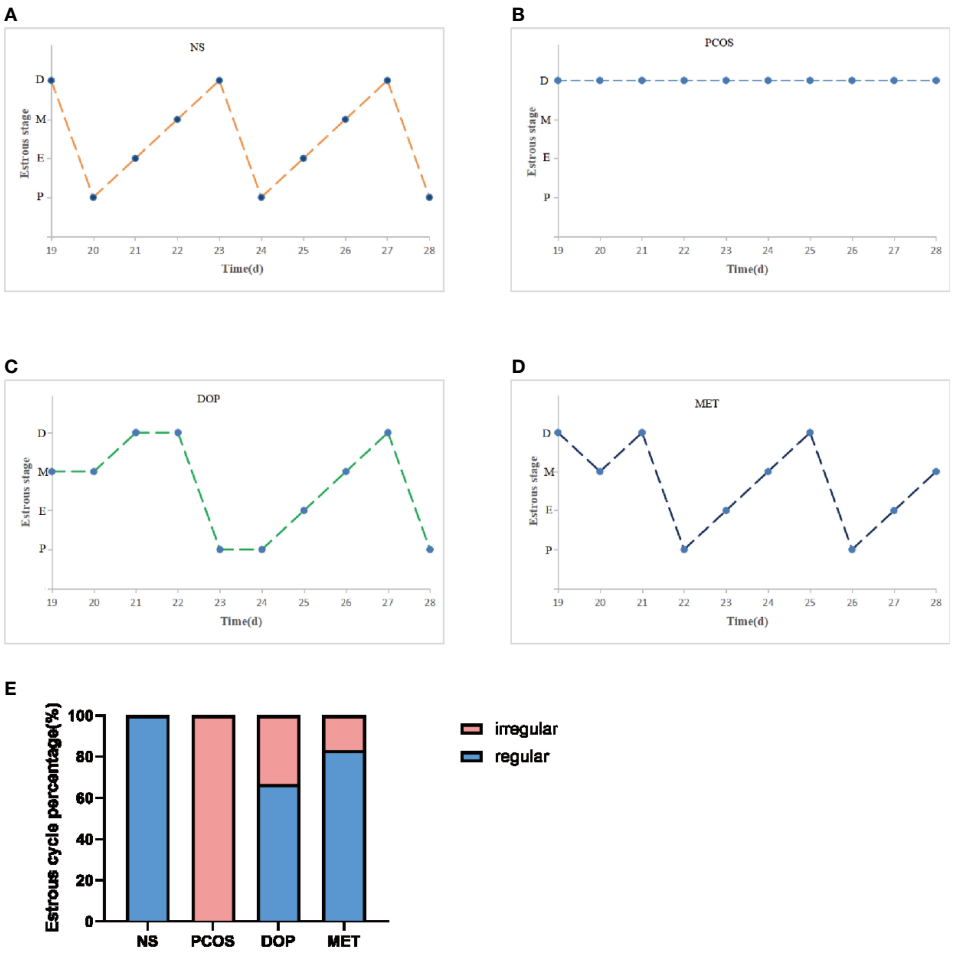

FIGURE 2-2
Effects of DOP treatment on estrus cycle in rats, n = 6. **(A–D)** Rats were examined at the estrus stage for 10 consecutive days. D: diestrus, M: metestrus, E: estrus, P: proestrus. **(E)** The proportions of regular and irregular estrus cycles in each group of rats.

rats, but DOP cannot be directly absorbed by the stomach and small intestine, which finally degraded into SCFAs by gut microbiota in the large intestine, and the composition of gut microbiota was modulated (21). How DOP affects endocrine and ovarian function in PCOS rats *via* the blood–brain barrier is unclear. Due to these reasons, the V3–V4 regions of the 16S rRNA gene were sequenced in 24 fecal samples to analyze. PD_whole_tree index and Chao1 index were used to compare the gut microbiota α diversity among the four groups. It was found that the gut microbiota α diversity in the DOP group was significantly increased compared with the NS group (Figure 6A), but based on Chao1 index analysis, there was no significant difference in α diversity (Figure 6B). The overall distribution of gut microbes was assessed based on β diversity, which was calculated using PCoA (Figure 6C and Supplement 3). A one-way analysis of similarities (ANOSIM) test was used to evaluate the similarities between groups. The results showed that there were significant differences in gut microbiota composition among all groups (ANOSIM $R = 0.586$, $p = 0.001$).

DOP treatment ameliorated the gut dysbiosis in rats

Microbial community structure and its metabolites affect animal health. At the phylum level, there were no taxonomic differences observed between all groups and more than 97% of gut bacteria are *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* (Figure 7A). At the genera level, the 30 most abundant gut bacteria are shown in Figure 7B. To further study the different microbial compositions in the feces of rats in different groups, we constructed a cladogram to show its evolutionary characteristics (Figure 7C), and LefSe analysis was performed to distinguish microbial biomarkers (Figure 7D). Compared

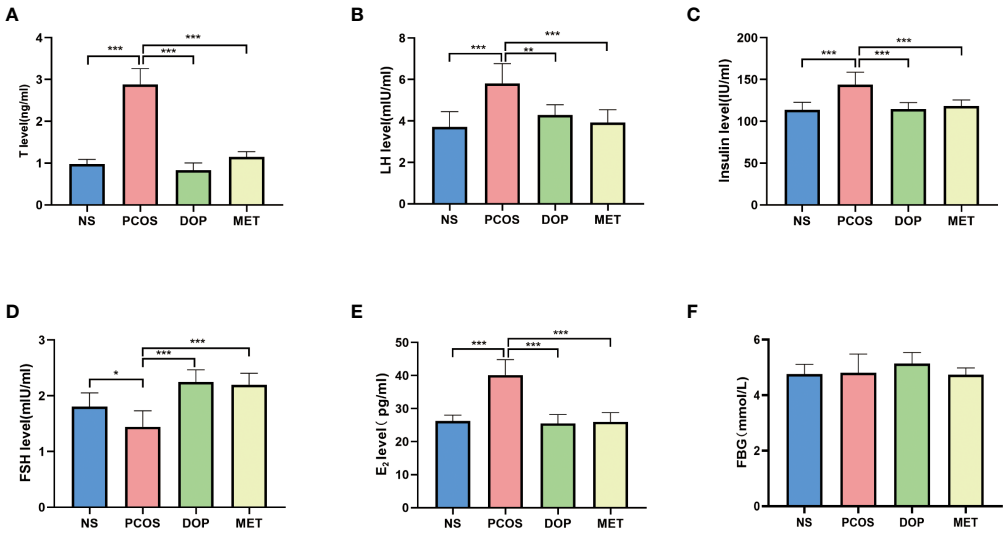

FIGURE 3
Effects of DOP treatment on sex hormone levels and fasting blood-glucose (FBG) in rats, n = 5–6. **(A)** T, **(B)** LH, **(C)** insulin, **(D)** FSH, **(E)** E_2, and **(F)** FBG. Note: compared with the PCOS group, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

with the NS group, the PCOS group showed an increase in the prevalence of *Actinobacteria*, *Prevotellaceae_UCG_001*, and *Alloprevotella* (Figures 7E, G, I). Notably, bacteria from the *Blautia* and *Lachospiraceae_ND3007_group* genera were most significantly enriched in the DOP group (Figures 7H, J). Compared with the PCOS group, *Deferribacteres* were more abundant in the MET group (Figure 7F). The results showed that there were more harmful bacteria enriched in the intestinal of PCOS rats, such as *Prevotellaceae_UCG_001*, *Ruminococcus*, and *Clostridiales*, and DOP/MET treatment increased the abundance of short-chain fatty acid bacteria, such as *Blautia*, *Lachospiraceae_ND3007_group*, and *Deferribacteres*.

DOP treatment elevated butyrate levels in feces in rats

As the abundances of SCFA producers (such as *Blautia*, *Lachospiraceae_ND3007_group*, and *Desulfovibrionaceae*) in the gut microbiota were enriched, the fecal levels of SCFAs were

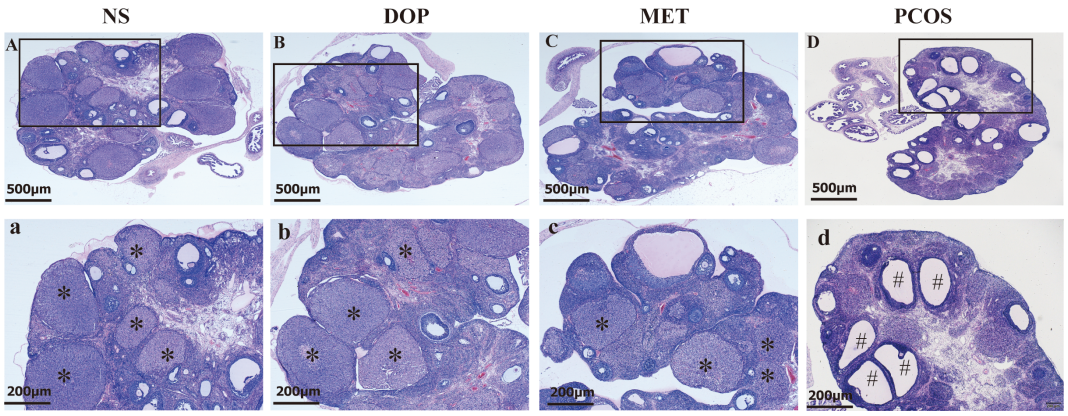

FIGURE 4
Effect of DOP on the morphology of ovarian pathological tissue in rats. Note: * for corpus luteum, # for cystic follicle, scale bar: **(A–D)** = 500 μm, a-d = 200 μm.

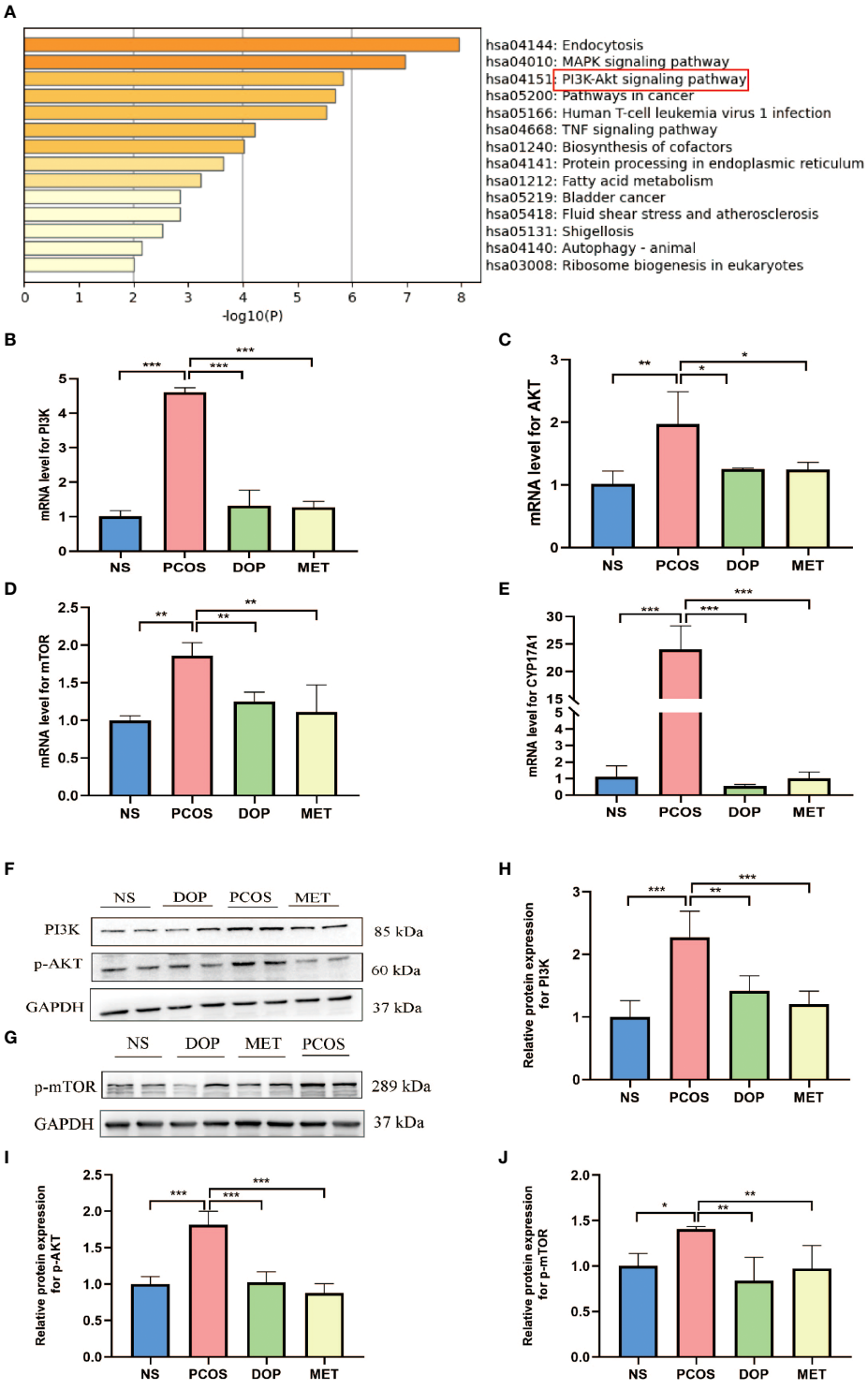

FIGURE 5
Effects of DOP treatment on the PI3K–Akt–mTOR signaling pathway in rats by qPCR and Western blot. **(A)** Top 14 clusters from Metascape pathway enrichment analysis differently expressed associated genes. Significance is indicated by the –log10 (P) value. **(B–E)** Ovarian gene PI3K, Akt, mTOR, and CYP17A1 expression levels, n = 3. **(F–J)** Expressions of PI3K, p-Akt, and p-mTOR protein are expressed as the fold change in the optical density of a target protein, and GAPDH expression served as the control, n = 2. Note: compared with the PCOS group, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

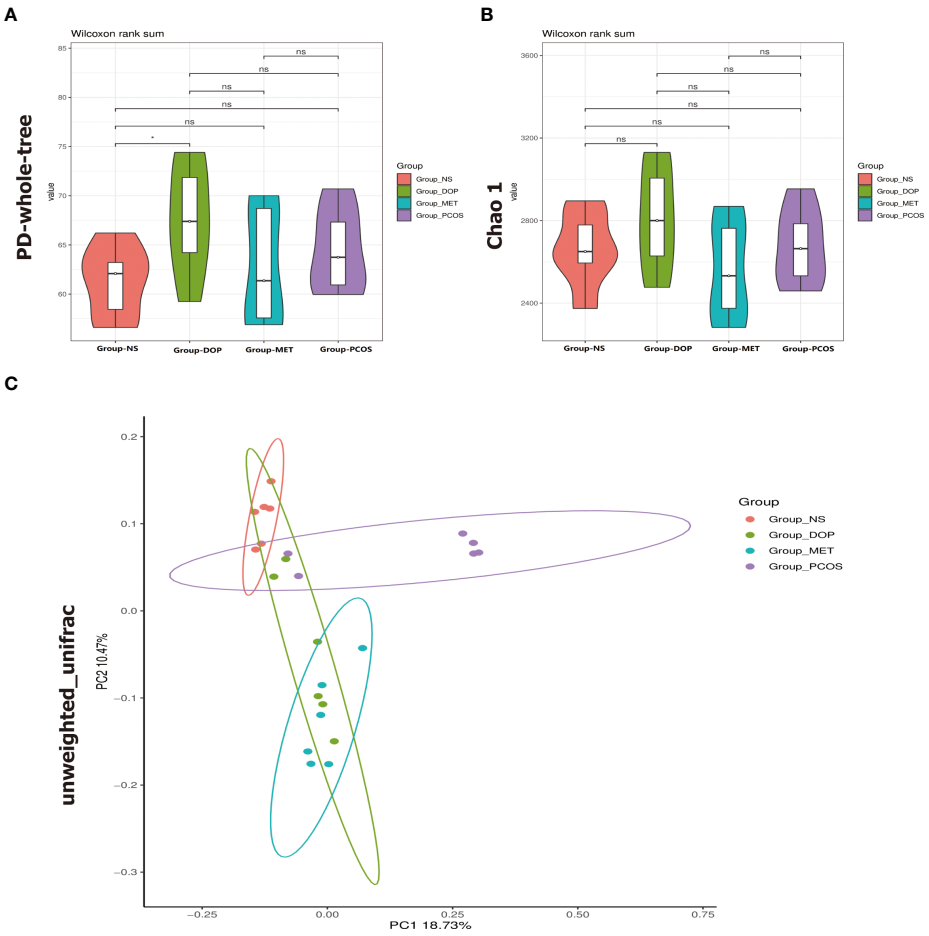

FIGURE 6
Effects of DOP treatment on gut microbial diversity in rats, n = 6. **(A)** Phylogenic diversity whole tree index (PD_whole_tree index). **(B)** Chao1 index. **(C)** Principal coordinate analysis (PCoA) using the unweighted_unifrac_distance. Note: compared with the NS group, ns $p > 0.05$, * $p < 0.05$.

monitored. Compared with the PCOS group, the levels of fecal butyric acid in the DOP and MET groups were significantly increased (Figure 8A), while propionic acid levels were significantly increased only in the MET group (Figure 8B). There was no significant difference in the fecal levels of acetic acid among all groups (Figure 8C).

DOP treatment attenuated PCOS through a butyrate dependent gut–brain mechanism

As previously mentioned, the levels of butyric acid in rat feces were higher after DOP/MET treatment. To determine whether butyric acid mitigates PCOS phenotypes through the gut–brain axis, we assessed the levels of brain–gut regulators peptide tyrosine-tyrosine (PYY) and G protein-coupled receptor 41 (GPR41) in rats. Compared with the PCOS group, the levels of serum PYY in the NS, DOP, and MET groups were all significantly increased (Figure 9A); the colonic GPR41 expression and integrated optical density were also significantly increased in the NS, DOP, and MET groups (Figures 9B, C). The results showed that the improvement mechanism of DOP on PCOS rats was closely related to the regulation of PYY and GPR41.

Discussion

Polycystic ovary syndrome is one of the leading causes of anovulatory infertility in women. It has been suggested that the main causes of PCOS are genetic and environmental interactions. For example, an unhealthy lifestyle, diet, or any infectious agent can increase the risk of developing PCOS.

Letrozole is an effective aromatase inhibitor that can block the transformation of androgen to estrogen *in vivo* and is widely

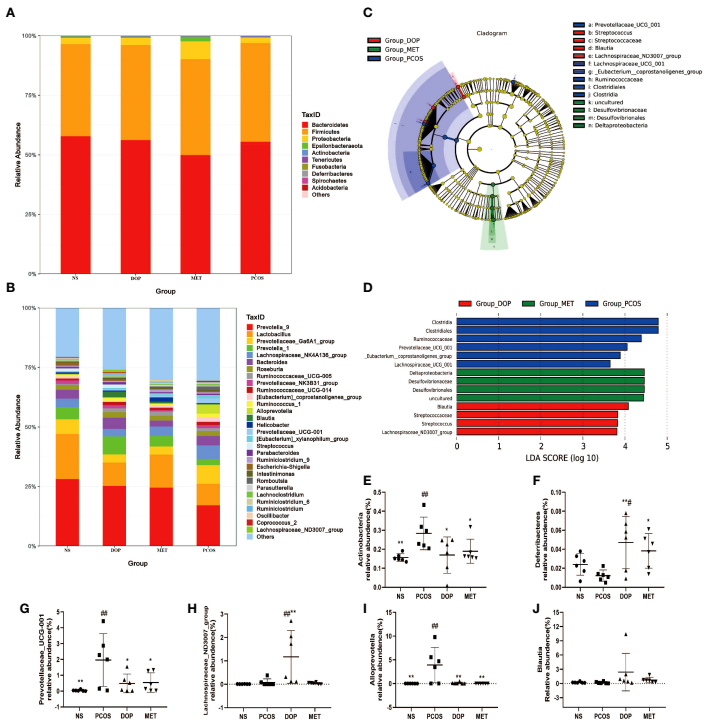

FIGURE 7
Effect of DOP treatment on gut microbial composition, n = 6. **(A)** Microbial distribution at the phylum level. **(B)** Microbial distribution of the top 30-genus level. **(C)** Cladograms representing the linear discriminant analysis effect size (LEfSe) results. **(D)** Linear discriminant analysis (LDA) results between different experimental groups. LDA scores above 3.00 and $p < 0.05$ are shown. **(E-J)** Relative abundance of *Actinobacteria*, *Deferribacteres*, *Prevotellaceae_UCG_001*, *Lachospiraceae_ND3007_group*, *Alloprevotella*, and *Blautia* between all groups. Note: compared with the PCOS group: * $p < 0.05$, ** $p < 0.01$; compared with the NS group: # $p < 0.05$, ## $p < 0.01$.

used in the construction of PCOS rat models, which can produce endocrine characteristics and ovarian morphological changes similar to clinical PCOS, including hyperandrogenemia, ovulation disorder, and obesity (24). In our study, the body weight and testosterone levels were significantly higher in PCOS rats; after DOP treatment, the body weight and testosterone significantly decreased, which was consistent with previous findings (20, 25). Studies have shown that androgen has strong assimilative activity, promoting food intake, energy absorption, and fat storage. Body weight loss in DOP rats may be closely related to decreased testosterone levels.

Hyperandrogenemia is one of the main features of polycystic ovary syndrome. High androgen and E_2 levels in serum are not conducive to the growth of dominant follicles and inhibit ovulation (26). In our study, more cystic follicles were observed in the ovaries of rats in the PCOS group, and the normal estrus cycle was lost. The compensatory hyperinsulinemia is thought to be a major driver of

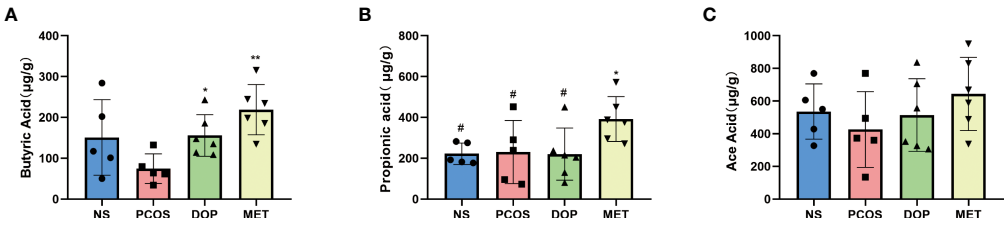

FIGURE 8
Effect of DOP treatment on fecal short-chain fatty acid (SCFA) levels, n = 5–6. Note: compared with the PCOS group *$p < 0.05$, **$p < 0.01$; compared with the MET group #$p < 0.05$.

hyperandrogenemia in polycystic ovary syndrome which amplifies luteinizing hormone-mediated androgen synthesis. Therefore, metformin is often used to improve insulin sensitivity in patients with polycystic ovary syndrome. Our results showed that MET treatment decreased serum insulin, testosterone, E_2, and LH levels and promoted the secretion of FSH in PCOS rats, while DOP treatment showed similar effects to MET, indicating that DOP has the effect of improving endocrine disorder in PCOS rats.

The PI3K/AKT/mTOR pathway is closely related to follicular development (23). In PCOS mouse models, it was observed that excessive androgen upregulation of mTORC1 resulted in dominant follicular selection disorder and follicular dysplasia (27), while rapamycin blocked the central mTOR signaling, leading to inhibition of the gonadal hormone axis and significant reduction in LH and estradiol levels in puberty rats (28). Previous studies have shown that metformin can promote the expression of glucose transporter 4, inhibit the expression of the androgen receptor, block the insulin receptor/PI3K/AKT/mTOR signaling pathway, and improve the abnormal metabolism in PCOS (29). 17-A hydroxylase (CYP17A1) plays a key role in steroid synthesis by converting the progesterone to androgen and thereby increasing the level of androgen. Clinical studies suggest that enhanced CYP17A1 enzyme activity and expression may account for hyperandrogenism in PCOS (30). LH stimulates CYP17A1 mRNA expression and androgen production in theca cells *via* activation of the PI3K/Akt pathway (31). Therefore, it was observed in this study that the high secretion of LH may be the cause of the high expression of CYP17A1 mRNA and the elevated testosterone level. In the present study, we observed increased CYP17A1 gene expression and activation of the PI3K/AKT/mTOR signaling pathway and that multiple cystic follicles appear in the PCOS group; after DOP and MET treatment, CYP17A1 and PI3K/AKT/mTOR pathway were downregulated, and the morphology of the polycystic ovary was improved.

PCOS is a complex endocrine and metabolic disease, and there has been increased interest in the interplay with gut dysbiosis. Although our study has proved that DOP can improve endocrine disorders and follicle development in PCOS rats, DOP cannot be digested by enzymes encoded by the human genome. How DOP affects endocrine and ovarian function in PCOS rats *via* the blood–brain barrier is worthy of further study. As a dietary fiber, DOP can be degraded into SCFAs by gut microbiota in the large intestine and modulate the composition of gut microbiota (21). A high-fiber diet can remodel the gut microbiota and relieve chronic metabolic inflammation, reproductive function, and brain–gut

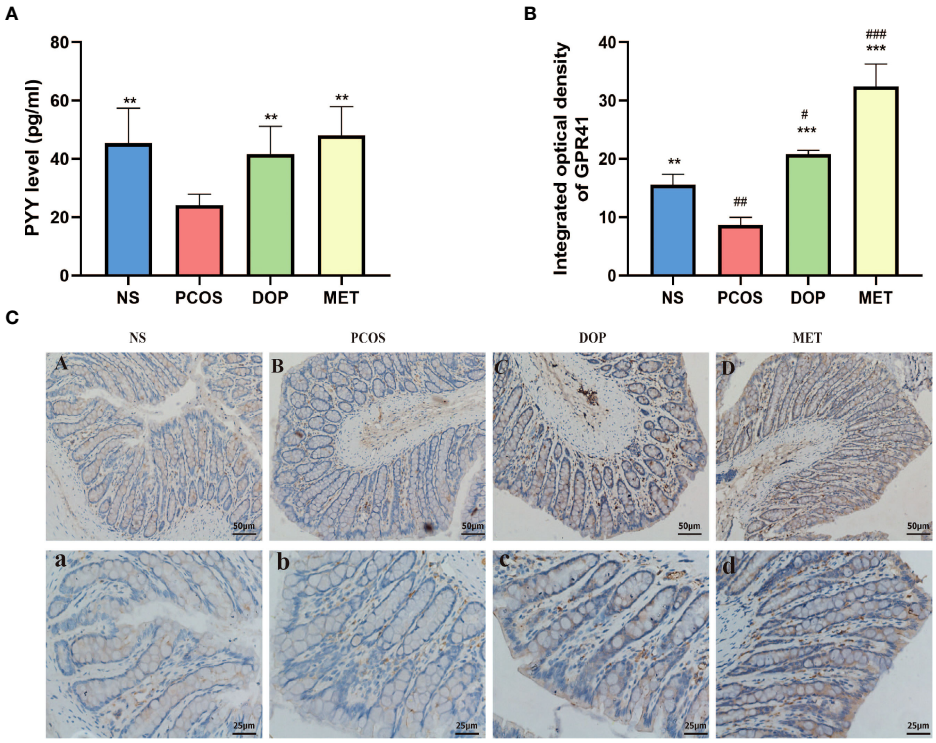

FIGURE 9
Effects of DOP treatment on gut–brain mediators. **(A)** Serum levels of PYY, n = 5. **(B-C)** Colonic GPR41 expression, location, and integrated optical density, n = 3, scale bar = 50 and 25 μm. Note: compared with the PCOS group, $^{**}p < 0.01$, $^{***}p < 0.001$; compared with the NS group, $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$.

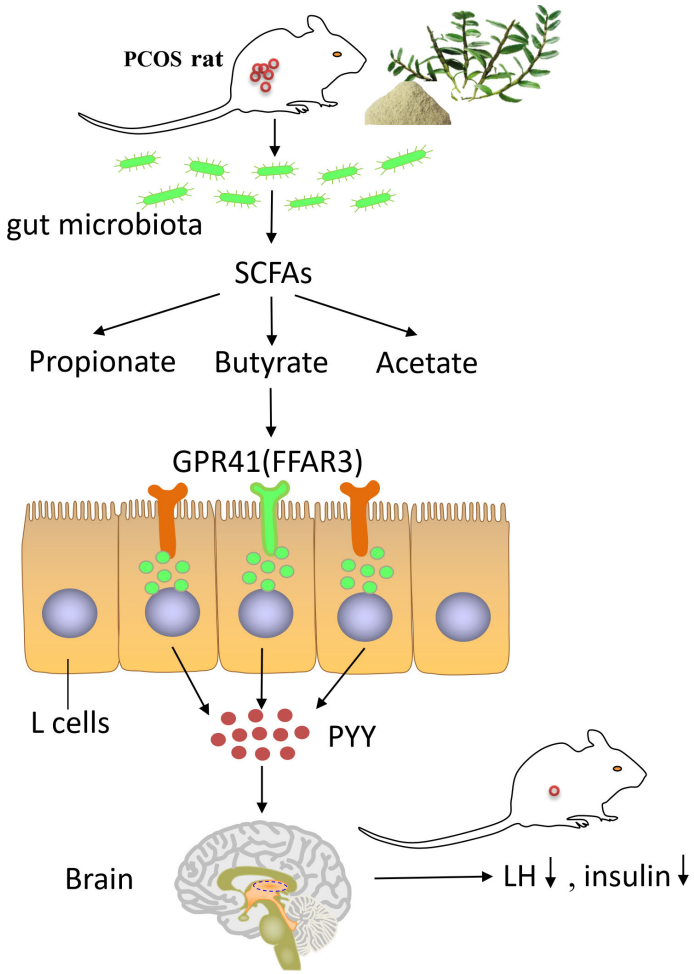

FIGURE 10
The summary of how DOP alleviates PCOS through gut–brain–ovary regulation. Dendrobium officinale polysaccharide can promote the remodeling of the gut microbiota and the production of butyrate bacterium in PCOS rats. High levels of butyrate through G protein-coupled receptor 41 to stimulate the secretion of PYY by entero-endocrine L-cells. PYY regulates the levels of steroid hormones through the gut–brain axis and ameliorates the polycystic ovary phenotype in PCOS rats.

polypeptide secretion in PCOS patients (32). Thus, we collected rat feces for 16S rRNA and SCFAS detection, trying to find the detailed mechanism of DOP improving PCOS.

In Figure 6, compared with the NS group, there is no significant difference in the PCOS group while DOP treatment can significantly increase the α diversity. PCoA analysis found that β diversity of the gut microbiota has a significant difference between fecal levels from the NS group and PCOS group which could be partially restored by DOP/MET treatment. These data indicated that DOP/MET treatment could reshape the gut microbiota community structure of PCOS rats. Studies have reported that women with PCOS had decreased α diversity and altered β diversity and gut microbiota composition (33, 34). Dysbiosis of the gut microbiota in the host can activate the immune system and interfere with the function of insulin receptors, causing hyperinsulinemia, increasing the production of androgen in the ovary, and preventing the development of normal follicles (35).

LEfSe analysis found that the abundance of beneficial bacteria such as *Blautia* and *Lachospiraceae_ND3007_group* was higher in the DOP group. *Blautia*, a bacterial group belonging to the family *Lachnospiraceae* of phylum Firmicutes, could ferment carbohydrates and produce acetate and butyrate which has been reported to be negatively correlated with obesity and T2D (36, 37). Decaffeinated green and black tea polyphenols reduced body weight in diet-induced obese mice which was strongly associated with *Blautia* enrichment in the gut (38). *Lachospiraceae_ND3007_group* has been reported to be negatively correlated with testosterone (39). These could reasonably explain why body weight and testosterone levels in

PCOS rats decreased and butyric acid levels increased after DOP treatment. In the PCOS group, the abundance of *Prevotellaceae UCG-001*, *Ruminococcus*, and *Clostridiales* was higher. According to reports, *Prevotellaceae UCG-001* is thought to be associated with impaired glucose tolerance (40). *Prevotella copri* is considered to be the main species driving the link between branched-chain amino acid biosynthesis and insulin resistance, exacerbating glucose tolerance and increasing circulating levels of branched-chain amino acids (41). *Ruminococcus* is a kind of Gram-positive anaerobic bacteria, which can secrete β-glucuronidase, destroy colonic mucosa, and participate in the invasion and metastasis of tumors (42). Moreover, *Clostridiales* which are significantly enriched in T2D women (43) can metabolize peptones and amino acids to produce lactic acid as the major product (44). In our study, the rats in the PCOS group were observed to exhibit higher T, insulin, and lower butyric acid levels. The intestine is an important organ for metformin's pharmacological effects, promoting glucose uptake and lactic acid production by influencing the composition of gut microbiota (45). Therefore, the possibility exists that the metabolic benefits linked to metformin treatment may in part depend upon its action in the gut. In the intestines, metformin does not only improve the glucose uptake in T2D individuals, reshape the human microbiota, promote the growth of beneficial bacterial species, and counteract the expansion of detrimental bacterial species but also promotes the short-chain fatty acid (SCFA) production, protects the intestinal barrier, and regulates the secretion of gut peptides (46, 47). In this study, *Desulfovibrionaceae* and *Desulfovibrionales* in the MET group are the dominant genera. Interestingly, *Desulfovibrionaceae* and *Desulfovibrionales* are sulfate-reducing bacteria that can break down lactic acid produced by the microbial community into propionic and butyric acids, which are known to contain mechanisms for combating oxidative stress (48). This may explain why higher levels of propionic and butyric acid were detected in the MET group.

In this study, we observed that levels of butyrate were lower in the PCOS group while higher in the DOP group and MET group. Interestingly, lower butyrate levels were also detected in PCOS patients (49), suggesting a correlation between altered butyrate levels and PCOS (50). In addition, reduced butyric acid levels in diet-induced obesity are an important cause of reduced insulin resistance (51). Butyrate has been shown to act on G protein-coupled receptors (GPR41 and GPR43), resulting in GLP-1 and PYY secretion and thereby regulating sex hormone levels to ameliorate PCOS (52, 53). PYY is secreted by the entero-endocrine L-cells of the distal ileum and colon which may affect sex hormone levels by crossing the blood–brain barrier and binding to neuropeptide Y receptors (54). Studies have reported that PCOS patients have lower PYY, which is negatively correlated with LH and insulin (33, 49). In this study, the levels of serum PYY were lower, but LH and insulin were higher in PCOS rats. Notably, DOP/MET treatment reversed these hormone levels.

This evidence suggests that the ameliorative effect of DOP on PCOS rats is mainly due to promoting the release of butyrate by gut microbiota, which affects the secretion of sex hormones through the butyrate-GPR41-PYY mechanism, rather than DOP itself.

Conclusions

The present study highlighted that DOP could reduce body weight, reverse endocrine disorder, restore the normal estrus cycle, improve polycystic ovary morphology, remodel the gut microbiota, and promote butyrate production in PCOS rats. The ameliorative mechanism is to improve the steroid hormone disorder, promote follicular development, and improve the symptoms of polycystic ovary through the butyrate-dependent gut–brain (GRP41-PYY) axis (Figure 10). These results provide theoretical references for DOP as a potential supplement for PCOS treatment.

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 below: NCBI PRJNA855132, available at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA855132.

Ethics statement

The animal study was reviewed and approved by Animal Welfare and Ethics Committee of Youjiang Medical University for Nationalities.

Author contributions

Project administration, supervision, and writing—review and editing: QJ and YH; investigation, methodology, and writing—original draft: XF; methodology and data curation: DW; conceptualization: LH; software: HL; validation: BL. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.962775/full#supplementary-material

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Acupuncture combined with metformin versus metformin alone to improve pregnancy rate in polycystic ovary syndrome: A systematic review and meta-analysis

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Objective: The aim of this study was to evaluate the comparison between acupuncture combined with metformin versus metformin alone in improving the pregnancy rate of people with polycystic ovary syndrome (PCOS).

Methods: A literature search of eight databases resulted in nine randomized controlled trials (RCTs) that assessed the effect of acupuncture combined with metformin on pregnancy rate in PCOS patients compared with metformin alone. Subsequently, data extraction and analysis were conducted to evaluate the quality and risk of bias of the methodological design of the study, and meta-analysis was conducted on the RCT data.

Results: Nine RCTs and 1,159 women were included. Acupuncture can improve pregnancy rate. It was analyzed according to the diagnostic criteria of PCOS [Z = 2.72, p = 0.007, relative risk (RR) 1.31, 95% CI 1.08 to 1.60, p = 0.15, I^2 = 41%]. Analysis was performed according to different diagnostic criteria of pregnancy (Z = 3.22, p = 0.001, RR 1.35, 95% CI 1.13 to 1.63, p = 0.12, I^2 = 42%). Acupuncture can improve ovulation rate. Subgroup analysis was performed according to the number of ovulation patients (Z = 2.67, p = 0.008, RR 1.31, 95% CI 1.07 to 1.59, p = 0.04, I^2 = 63%) and ovulation cycle (Z = 3.57; p = 0.0004, RR 1.18, 95% CI 1.08 to 1.29, p = 0.57, I^2 = 0%). Statistical analysis also showed that acupuncture combined with metformin could improve homeostatic model assessment of insulin resistance (HOMA-IR) [mean difference (MD) −0.68, 95% CI −1.01 to −0.35, p = 0.003, I^2 = 83%].

Conclusions: Based on the results of this study, compared with metformin alone, acupuncture combined with metformin has a positive effect on pregnancy rate, ovulation rate, and insulin resistance in PCOS. However, due to the limitations regarding the number and quality of the included studies, the above conclusions need to be verified by further high-quality studies.

Systematic Review Registration: https://www.crd.york.ac.uk/PROSPERO/#myprospero.

KEYWORDS

acupuncture, metformin, polycystic ovary syndrome, pregnancy rate, insulin resistance, systematic review, meta-analysis

Introduction

Polycystic ovary syndrome (PCOS) is the most common hormonal disorder in women and is also one of the most common factors that cause infertility (1). With the opening of the three-child policy in China, the reproductive needs of women of childbearing age are increasingly urgent, but the incidence of PCOS in these women is as high as 9% to 18% (2), which has a great impact on pregnancy. Studies have shown that insulin resistance (IR) is a key feature of the pathophysiology of PCOS (3), with 85% of patients being affected by IR. IR disrupts the follicular environment (4) by leading to hyperandrogenemia (5), affecting follicular development and ovulation, which is not conducive to pregnancy. In addition, people with obesity account for 35%–60% (6) of the population with PCOS, which is closely related to IR and the pathological mechanism of PCOS (7). Weight gain has been shown to further aggravate IR (8, 9). The above factors affect women's health.

Acupuncture has become more and more popular in the world as a complementary and alternative therapy for infertility. In 2010, a study in the United States showed that 29% of patients used complementary and alternative drugs with the aim to treat infertility, of whom 22% chose acupuncture (10). In China, traditional medicine is even more popular. A large number of clinical and animal experiments have shown that acupuncture has significant effects in the treatment of infertility and anovulation caused by PCOS, including improving clinical pregnancy rate, ovulation rate, live birth rate, insulin resistance, menstruation, hormone levels, follicular development, and hyperandrogenaemia, and regulating the secretory function of hypothalamic pituitary ovarian axis (HPOA) (11–17) but it may also cause subcutaneous bleeding or pain and other mild adverse reactions. Studies have shown that metformin is one of the most important drugs for reducing insulin resistance in PCOS patients (18). Such drugs have been shown to improve clinical pregnancy rate and ovulation rate, and have positive effects on hyperinsulinemia and ovarian androgen hypersecretion.

There are no detailed and systematic methodological evaluations and data consolidations between acupuncture combined with metformin versus metformin alone. The main objective of this study was to conduct a systematic review and meta-analysis comparing whether acupuncture combined with metformin can further improve pregnancy rates compared to metformin alone, thus providing a more effective treatment for this population.

Methods

This study was conducted according to Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) (19). This study was listed on the Prospective Register of Systematic Reviews (PROSPERO) on 15 February 2022, with registration number: CRD42022302940.

Data sources

In this study, the following eight databases [PubMed, Embase, Cochrane Central Register of Controlled Trials (CENTRAL), Web of Science (SCI), Chinese Biomedical Database (CBM), Chinese National Knowledge Infrastructure (CNKI), Wan Fang Data Knowledge Service Platform, and VIP Journal Integration Platform (VIP)] were searched from the database construction to 14 April 2022, including four English databases and four Chinese databases. At the same time, to retrieve Chinese Clinical Trial Register, Clinical Trials.gov, relevant articles in the reference lists were also collected. The retrieval was carried out by the combination of medical subject headings (MeSH). Keywords searched in PubMed include

"Acupuncture" and "Metformin" and "Polycystic Ovary Syndrome" and "Infertility" and "Randomized Controlled Trial". See appendix for detailed search strategies.

Study selection and data extraction

Two researchers (YL and HYL) independently screened the retrieved articles, read the titles and abstracts, and then excluded the repeated studies and irrelevant articles. According to the inclusion criteria and exclusion criteria, eligible studies were identified, data were extracted and cross-checked, and any ambiguity was resolved through discussion and consensus. If no consensus was reached, a third researcher (QLX) was asked to adjudicate. Any literature that is removed will be recorded. The experimental groups received acupuncture (acupuncture, moxibustion, electroacupuncture, acupoint embedding therapy, acupoint injection, acupuncture ear, warm needling, fire-needle, or floating needle) combined with metformin; the control groups were treated with metformin alone. Inclusion criteria were as follows: (a) subjects were diagnosed with PCOS; (b) the treatment group used acupuncture combined with metformin, while the control group used only metformin; and (c) the study type was a randomized controlled trial. Exclusion criteria were as follows: (a) subjects were treated with drugs other than metformin; (b) the use of traditional Chinese medicine; (c) the study was conducted on animals; and (d) the study was not reported in Chinese or English. This study was divided into two groups: the acupuncture combined with metformin group and the metformin alone group. Data were extracted independently by two researchers (YL and HYL), and checked by another researcher (XC) to extract information that might be related to the research results, as follows: First author, year of publication, number of participants, age, infertility duration, treatment duration, interventions, diagnostic criteria, outcome indicators, side effects and adverse events, and other information. The results were recorded in an Excel spreadsheet (Table 1).

Risk of bias assessment

Cochrane RoB2.0 was used to evaluate risk of bias in the individual studies (20). The following six items were extracted from each of the RCTs for evaluation: (a) randomization process; (b) deviations from intended interventions; (c) missing outcome data; (d) measurement of the outcome; (e) selection of the reported result; and (f) overall. When the appropriate method is used and described appropriately and clearly, the study was considered to be low risk; otherwise, it was rated as high risk, or some concerns if the method could not be accurately judged. Two researchers (YL and HYL) independently assessed these factors and, if necessary, a third researcher (QLX) was consulted to resolve disagreements (Figures 1, 2).

Outcomes

Main outcome measures: pregnancy rate: positive morning urine beta human chorionic gonadotropin (β-hCG) or blood β-hCG, basal body temperature (BBT) for more than 3 weeks, pregnancy sac detected by color ultrasonography, or fetal bud and heartbeat detected by colour ultrasonography at 7 weeks of gestation.

Statistical analysis

RevMan5.3 software was used for statistical analysis. Relative risk (RR) and its 95% confidence interval (CI) were used for dichotomous variables, MD or standardized mean difference (SMD) were used for continuity variables, and 95% CI was given. $p < 0.05$ was considered as a statistically significant difference. According to the study of the object of study, observation group intervention, and control group, judging whether the concrete application of similar clinical outcome index, according to the result of the I^2 test to determine the statistical heterogeneity. I^2>50% is considered as high heterogeneity. The methods of subgroup analysis and sensitivity analysis were used, and the study adopts a random-effects model.

Results

Studies retrieved

In total, 330 related literatures were screened initially, and nine studies (21–29) ultimately met our inclusion criteria after the different rounds of screening. A total of 1,159 patients with PCOS who received acupuncture or acupuncture combined with metformin were identified from nine randomized controlled trials (Figure 3).

Quality of the evidence: Summary of findings table

We used the GRADE method to present a "Summary of Findings" table. The quality of evidence for outcome measures (pregnancy rate, ovulation rate, and HOMA-IR) was evaluated for a subject review comparison (acupuncture combined with metformin vs. metformin alone). We used the GRADE criteria to assess the quality of evidence, study limitations, inconsistencies, inaccuracies, and publication bias. The two researchers (ZL and QLX) independently judged the quality of the evidence (high, moderate, low, or very low) and resolved differences through discussion Table 2.

TABLE 1 Characteristics of the studies included in this systematic review (acupuncture + metformin vs. metformin).

No.	Study, publication year (country)	No. of patients (O/A)	Age: mean ±SD or range (years)	Duration of infertility: mean ±SD or range (years)	Intervention	Control	Period of treatment	Diagnostic criteria	Side effects and adverse events	Type of outcomes
1	Li YC,2021 (China)21	I:57/57 C:57/57	I:31.42 ±3.22 (20-40) C:31.48 ±3.25 (21-39)	NR	Acupuncture +Metformin	Metformin	3 months	2018 "Guidelines for Diagnosis and Treatment of Polycystic Ovarian Syndrome" in China	NR	pregnancy, ovulation, TCM symptom score, FPG,FINS,HOMA-IR,Blood fat.
2	Wang JY,2020 (China) 22	I:30/30 C:30/30	I:26.43 ±3.52 (22-38) C:26.23 ±3.48 (21-38)	NR	Acupuncture +Metformin	Metformin	3 months	NR	NR	pregnancy,Sex hormone level, FPG,HOMA-IR, BMI,etc.
3	Li L,2014 (China) 23	I:53/53 C:51/51	I:27.10 ±2.50 (22-40) C:25.20 ±1.80 (21-38)	I:3.6(2.0-7.5) C:3.3(2.0-7.2)	Acupuncture +Metformin	Metformin	6 months	Rotterdam	Gastrointestinal reaction	Sex hormone level, BBT,B-scan ultrasonography, BMI,WHR, Ferriman-Gallway, HOMA-IR,FPG, FINS,OGTT,ST, FBG,etc.
4	Zhai ZY,2017 (China) 24	I:40/40 C:40/40	I:23.70 ±2.20 (20-29) C:23.10 ±1.90 (20-28)	I:4.40±1.20 (2-7) C:4.10 ±1.40(2-7)	Acupuncture +Metformin	Metformin	3 months	Rotterdam	Gastrointestinal reaction, Menstrual abnormalities	ovulation,BMI, WHR,Sex hormone level,FPG,FINS, APN.
5	Peng XY,2020 (China) 25	I:30/30 C:30/30	I:28.42 ±1.32 (22-32) C:28.32 ±1.35 (22-33)	I:2.02±0.12 (0.6-4) C:2.06 ± 0.11(0.6-4)	Acupuncture +Metformin	Metformin	6 months	2018 "Guidelines for Diagnosis and Treatment of Polycystic Ovarian Syndrome" in China	NR	pregnancy, ovulation,Sex hormone level, B-scan ultrasonography.
6	Li SS,2015 (China) 26	I:75/75 C:75/75	I:25.10 ±2.30 (23-34) C:24.10 ±2.20 (21-33)	I:3.2(2-4) C:3.3(2-5)	Acupuncture +Metformin	Metformin	6 months	NR	NR	pregnancy, ovulation,Sex hormone level, B-scan ultrasonography, HOMA-IR,BMI, Ferriman-Gallway, OGTT,ST.
7	Tang J,2017 (China) 27	I:52/52 C:52/52	I:28.53± 6.62(22-42) C:28.26 ±6.70 (21-40)	I:3.72±1.38 (1-7) C:3.41 ±1.52(1-8)	Acupuncture +Metformin	Metformin	I:3 menstrual cycles C:3 months	Rotterdam	NR	pregnancy, ovulation,Total effects,BMI,FPG, Sex hormone level, FINS.
8	Zhang ZL,2016 (China) 28	I:50/50 C:50/50	I:26.30± 4.40(22-38)	I:3.2±0.6(2-7) C:3.1± 0.5(2-6)	Acupuncture +Metformin	Metformin	6 months	Rotterdam	NR	BMI,WHR,B-scan ultrasonography,

(Continued)

TABLE 1 Continued

No.	Study, publication year (country)	No. of patients (O/A)	Age: mean ±SD or range (years)	Duration of infertility: mean ±SD or range (years)	Intervention	Control	Period of treatment	Diagnostic criteria	Side effects and adverse events	Type of outcomes
			C:27.2 ±4.1(23-37)							Sex hormone level, Ferriman-Gallway
9	Liu YE, 2018 (China)29	I:138/138 C:140/140	I:30.00 ±7.00 (23-37) C:31.20 ±8.10 (23-39)	I:2.35±1.65 (0.7-4.0) C:2.51± 1.83 (0.7-4.3)	Acupuncture +Metformin	Metformin	2 months	Rotterdam	NR	pregnancy, ovulation,Sex hormone level, AMH,BMI, menstrual cycle,etc.

AMH, anti-Müllerian hormone; APN, Adiponectin;BBT, basal body temperature; BMI,Body Mass Index; C, control; FINS,Fasting insulin; FPG, fasting plasma glucose; HOMA-IR, Homeostatic model assessment of insulin resistance; I, intervention; NR, not reported; OGTT, oral glucose tolerance test; ST, insulin release test; TCM, Traditional Chinese Medicine; WHR, Waist-to-Hip Ratio.

Main results

Pregnancy

Six studies of acupuncture combined with metformin reported pregnancy rates (22, 23, 25–27, 29). In terms of pregnancy rates, the results were statistically significantly different between the two groups (Z = 3.22, p = 0.001, RR 1.35, 95% CI 1.13 to 1.63, p = 0.12, I^2 = 42%), indicating that acupuncture combined with metformin was superior to metformin alone in improving pregnancy. We found that different diagnostic criteria were adopted in clinical studies on pregnancy rate; thus, we conducted subgroup analysis and used B-ultrasound for diagnosis (Z = 2.39, p = 0.02, RR 1.49, 95% CI 1.07 to 2.07, p = 0.03, I^2 = 66%). B-ultrasound was not used for the diagnostic group (Z = 2.14, p = 0.03, RR 1.30, 95% CI 1.02 to 1.65, p = 0.64, I^2 = 0%), indicating that the heterogeneity of the test was mainly derived from the criteria of pregnancy (Figure 4). Additionally, there are different diagnostic criteria for PCOS; when summarizing the characteristics, we found that there are two different sets of diagnostic criteria. The first is the Rotterdam standard, and the second is a set of PCOS diagnosis and treatment guidelines in China. We also performed subgroup analysis of the above two kinds of diagnostic criteria, finding that heterogeneity when using the Rotterdam criteria (I^2 = 25%) was significantly lower than the overall heterogeneity (I^2 = 41%) (Figure 5).

Ovulation

Seven studies of acupuncture combined with metformin reported ovulation rates, calculated by number of ovulations in four studies (21, 24, 25, 29) and ovulation cycles in three studies (23, 26, 27). We analyzed these studies separately according to their different calculation methods.

Calculated by the number of ovulation: 532 participants in these studies, 224 women in the trial group and 180 women in the control group ovulated. The results were statistically significant (Z = 2.67, p = 0.008, RR 1.31, 95% CI 1.07 to 1.59,

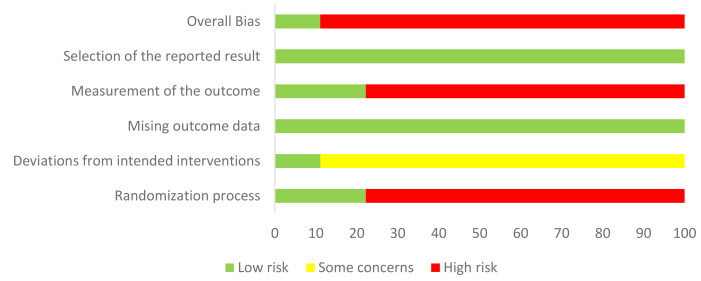

FIGURE 1
Risk of bias summary.

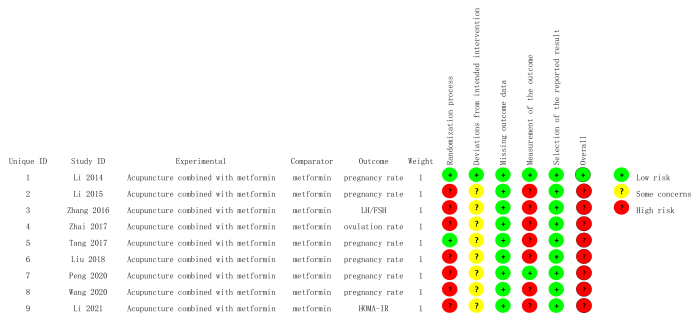

FIGURE 2
Risk of bias graph.

$p = 0.04$, $I^2 = 63\%$). Calculated according to ovulation cycle: 683 participants in these studies, cycle ovulation rates were recorded for 278 women in the trial group and 226 women in the control group. The results had low heterogeneity, and indicated that acupuncture combined with metformin had a positive effect on improving ovulation rate compared with metformin alone ($Z = 3.57$; $p = 0.0004$, RR 1.18, 95% CI 1.08 to 1.29, $p = 0.57$, $I^2 = 0\%$) (Figure 6).

Homa-Ir

HOMA-IR was reported in three studies of acupuncture combined with metformin (21–23) with a total of 282 participants, 144 in the experimental group and 138 in the control group. The results showed that the difference in HOMA-IR between the two groups was statistically significant ($Z = 4.02$; $p < 0.0001$), with high heterogeneity (MD −0.68, 95% CI −1.01 to −0.35, $p = 0.003$, $I^2 = 83\%$). After sensitivity analysis ($p = 0.34$, $I^2 = 0\%$), heterogeneity was derived from a research (22), and it was found that the sample size of the study was only 30 cases. Therefore, sample size may be the main source of heterogeneity (Figure 7).

Side effects and adverse events

The incidence of side effects and adverse events was reported in two trials. A total of 21 patients treated with acupuncture combined with metformin had gastrointestinal reactions and 2 had menstrual abnormalities. After metformin alone treatment, a total of 23 patients had gastrointestinal reactions and 6 patients had menstrual abnormalities.

Discussion

In this systematic review and meta-analysis, acupuncture combined with metformin was suggested to have a positive effect on pregnancy rate, ovulation rate, and HOMA-IR in patients

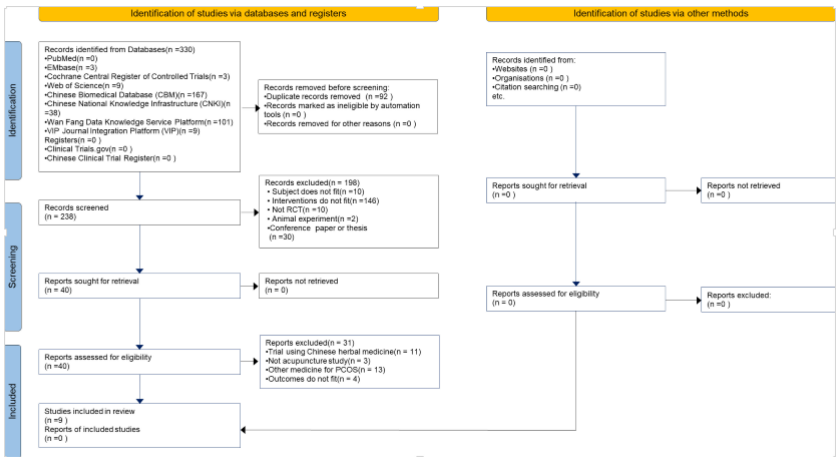

FIGURE 3
Flowchart of the study selection process.

TABLE 2 Summary of findings table.

Acupuncture+ Metformin compared to Metformin for PCOS

Patient or population: patients with PCOS **Settings:** outpatients **Intervention:** Acupuncture+ Metformin **Comparison:** Metformin

Outcomes	Illustrative comparative risks* (95% CI)		Relative effect (95% CI)	No of Participants (studies)	Quality of the evidence (GRADE)	Comments
	Assumed risk	Corresponding risk				
	Metformin	**Acupuncture+ Metformin**				
Pregnancy- B-ultrasound	**Study population**		**RR 1.49** (1.07 to 2.07)	502 (4 studies)	⊕⊕⊖⊖ **low**[1,2]	
	563 per 1000	**840 per 1000** (603 to 1000)				
	Moderate					
	404 per 1000	**602 per 1000** (432 to 836)				
Pregnancy- Not B-ultrasound	**Study population**		**RR 1.3** (1.02 to 1.65)	254 (2 studies)	⊕⊕⊖⊖ **low**[1]	
	429 per 1000	**557 per 1000** (437 to 707)				
	Moderate					
	407 per 1000	**529 per 1000** (415 to 672)				
Pregnancy- Rotterdam	**Study population**		**RR 1.24** (1.06 to 1.45)	636 (4 studies)	⊕⊕⊖⊖ **low**[1]	
	547 per 1000	**678 per 1000** (580 to 793)				
	Moderate					
	414 per 1000	**513 per 1000** (439 to 600)				
Pregnancy- China	**Study population**		**RR 2.14** (1.02 to 4.49)	60 (1 study)	⊕⊕⊖⊖ **low**[1,3]	
	233 per 1000	**499 per 1000** (238 to 1000)				
	Moderate					
	233 per 1000	**499 per 1000** (238 to 1000)				
Ovulation - number of ovulations	**Study population**		**RR 1.31** (1.07 to 1.59)	532 (4 studies)	⊕⊕⊖⊖ **low**[1,2]	
	674 per 1000	**883 per 1000** (721 to 1000)				
	Moderate					
	649 per 1000	**850 per 1000** (694 to 1000)				
Ovulation - ovulation cycles	**Study population**		**RR 1.18** (1.08 to 1.29)	683 (3 studies)	⊕⊕⊖⊖ **low**[1]	
	677 per 1000	**798 per 1000** (731 to 873)				
	Moderate					
	674 per 1000	**795 per 1000** (728 to 869)				
HOMA-IR	The mean homa-ir in the intervention groups was **0.68 lower** (1.01 to 0.35 lower)			282 (3 studies)	⊕⊕⊖⊖ **low**[1,3,4]	

*The basis for the assumed risk (e.g. the median control group risk across studies) is provided in footnotes. The corresponding risk (and its 95% confidence interval) is based on the assumed risk in the comparison group and the relative effect of the intervention (and its 95% CI). CI, Confidence interval; RR, Risk ratio;
GRADE Working Group grades of evidence High quality, Further research is very unlikely to change our confidence in the estimate of effect. Moderate quality, Further research is likely to have an important impact on our confidence in the estimate of effect and may change the estimate. Low quality, Further research is very likely to have an important impact on our confidence in the estimate of effect and is likely to change the estimate. Very low quality, We are very uncertain about the estimate.
[1]Evidence downgraded by two levels for serious risk of bias, the majority of the RCTs have unclear or high risk of bias. [2]Evidence downgraded by one level for serious inconsistency 50% <I2<75%. [3]Evidence downgraded by one level for serious imprecision, low number of events (total number of events < 300). [4]Evidence downgraded by two levels for serious inconsistency I2≥75%.

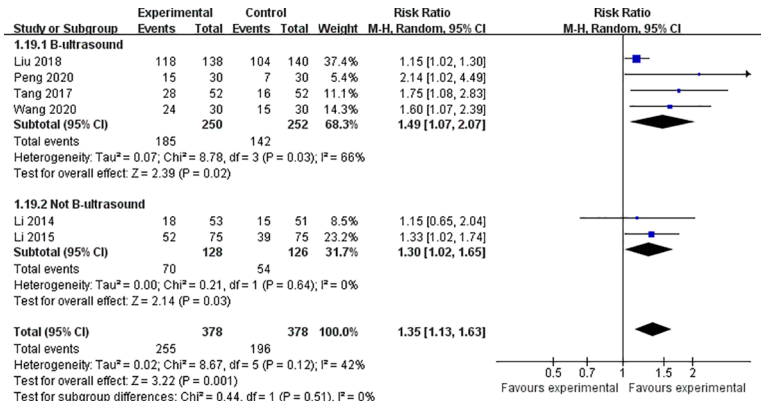

FIGURE 4
Forest plot of effects of acupuncture combined with metformin versus metformin alone on pregnancy rate (diagnostic criteria of pregnancy).

with PCOS compared to metformin alone. Subgroup analysis showed that the causes of heterogeneity were related to diagnostic criteria and random methods.

These findings are consistent with previous systematic evaluation and RCT results. Acupuncture alone or in combination with Western medicine in treating PCOS infertility can improve pregnancy rate, ovulation rate, hormone level, ovarian function, insulin resistance, and obesity (15, 30–33). However, the research results of Wu et al. were contrary to this (34). This study reported that acupuncture was not effective at treating infertility in PCOS patients. From the perspective of trial design, this study broke the traditional "step-by-step" evidence-based medicine research mode; that is, literature studies, observational studies, and small RCTs were not performed before performing a large-sample, multicenter RCT (35). This differs from the studies we have included in this review. Among the specific therapeutic methods, the choice of needles, acupoints, stimulation intensity, qi generation, treatment frequency, and course of treatment all have an impact on the curative effect. Therefore, the different results of acupuncture efficacy may be related to the lack of uniformity and objectivity of the current relevant clinical research standards.

Secondly, through meta-analysis, it was found that different diagnostic criteria of PCOS can cause differences in heterogeneity, and the heterogeneity of Rotterdam diagnostic criteria (I^2 = 25%) was significantly lower than the overall heterogeneity (I^2 = 41%). Currently, the Rotterdam standard is generally accepted and internationally recognized. In this study, five articles (23, 24, 27–29) adopted this standard. Fifteen years later, based on the disease characteristics of Han women in China, epidemiological investigation and research on the Chinese PCOS population was conducted. The 2018 "Guidelines for Diagnosis and Treatment of Polycystic Ovarian Syndrome" in China were formulated; two of the included studies were based on this standard (21, 25). When comparing the two criteria, the Rotterdam diagnostic criteria have a

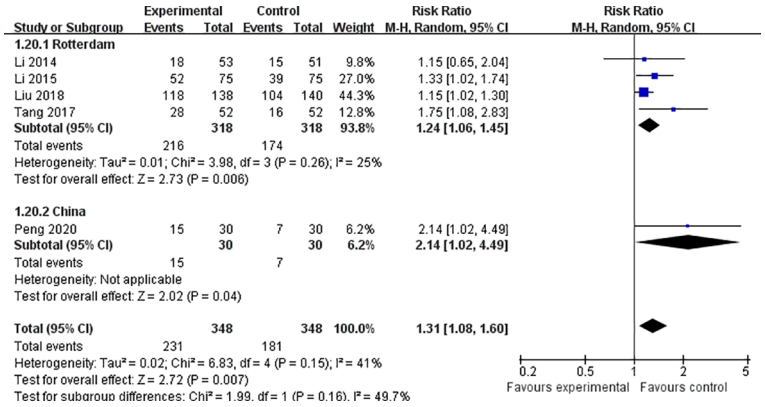

FIGURE 5
Forest plot of effects of acupuncture combined with metformin versus metformin alone on pregnancy rate (diagnostic criteria of PCOS).

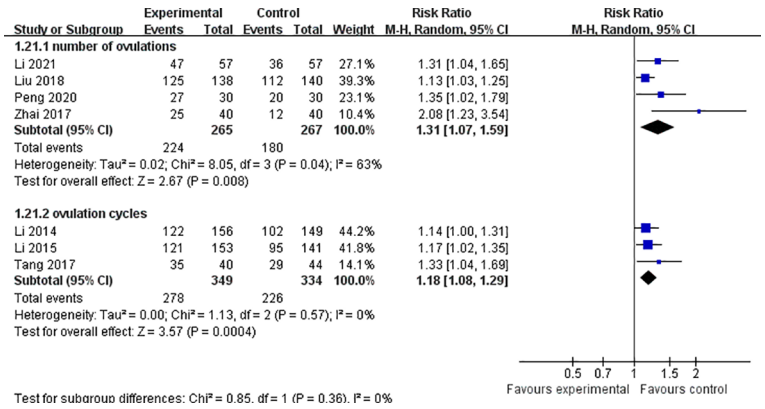

FIGURE 6
Forest plot of effects of acupuncture combined with metformin versus metformin alone on ovulation rate.

wider range than China's, but China's criteria are more detailed. Chinese standards put forward the concept of "suspected PCOS" as the first step of diagnosis, with the second step being to confirm PCOS. As a result, the clinical studies using the Chinese standard included both patients with early suspected status and confirmed status. However, as the original data did not separate these two groups of patients, there was heterogeneity in the analysis. However, this improvement to the guidelines raises the bar for future health risk assessment, long-term clinical management, and pregnancy assistance strategies in patients who have not been fully diagnosed in the early stages of the condition.

Through different diagnostic criteria for pregnancy, our subgroup analysis found that the heterogeneity of pregnancy diagnosis by ultrasound was lower than that by laboratory indicators (hCG detection only in serum or urine). We think more about the diagnosis of pregnancy. Based on the last 3 years of studies retrieved from Clinical Trials.gov, the Chinese Clinical Trial Register and Prosper, in studies of PCOS and *in vitro* fertilization/intra-cytoplasmic sperm injection (IVF/ICSI) (36–39), ultrasound was widely used as the diagnostic standard for pregnancy. In the subgroup analysis, it was found that clinical pregnancy criteria were generally adopted relatively recently, indicating that Chinese clinical studies were closer to international studies.

Quality of the evidence

In this review, we included only nine RCTs, most of which had small sample sizes. The quality of the evidence was low or very low. The main problems are risk of bias, imprecision, and inconsistency of the research results (Table 2).

Limitations

The limitations of this systematic evaluation are as follows (1): the included intervention measures, such as acupuncture forms, acupoint selection, treatment frequency, and course of treatment, vary greatly, and further subgroup analysis cannot be carried out due to the limited number of studies, affecting the accuracy of the results; and (2) due to incomplete information about the authors of most of the studies, we were only able to contact four of the authors by email, and did not receive any replies. It can be seen that the author of the original study is not very positive about the return visit of the author of the systematic evaluation. It may be because the author doesnot commonly use this contact method, or the recognition and research significance of the systematic evaluation are not high within the industry, and the author is not confident in his own scheme.

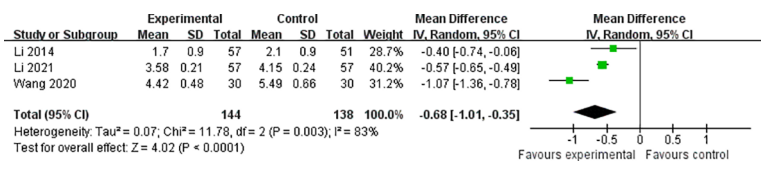

FIGURE 7
Forest plot of effects of acupuncture combined with metformin versus metformin alone on HOMA-IR.

Conclusion

Implications for practice

We cannot exclude clinically relevant differences in pregnancy rate, ovulation rate, LH/FSH, HOMA-IR, and FPG for acupuncture combined with metformin versus metformin. The pregnancy rate, ovulation rate and HOMA-IR of participants receiving acupuncture combined with metformin may have improved compared to metformin alone. Due to differences in pregnancy diagnostic criteria, we are not sure if this is effective in studies without a definitive B-ultrasound diagnosis. Due to the low quality of evidence and the limited number of RCTs available in this area, our ability to determine whether acupuncture combined with metformin is more effective at treating PCOS than metformin alone is limited.

Implications for research

It is hoped that acupuncture combined with metformin will improve the pregnancy rate of PCOS women.Further well-designed and well-performed randomized controlled trials are needed to definitively answer this question. Under uniform diagnostic criteria, a standard set of acupuncture points and stimulation methods should be considered, and the control group should receive the same metformin regimen as the acupuncture group.

Data availability statement

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

Author contributions

YL and HYL conducted literature searches, evaluated study inclusion, and extracted data. XC and JLY analyzed data and drafted the manuscript. QLX's assessment was incorporated into the study and cross-checked with XC. YL revised the language and the article. JW, XYZ, YMZ, CYL, MJW, and ZL conceived the study and revised the manuscript. All authors read and approved the final version of 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.

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Canagliflozin combined with metformin versus metformin monotherapy for endocrine and metabolic profiles in overweight and obese women with polycystic ovary syndrome: A single-center, open-labeled prospective randomized controlled trial

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Objectives: Canagliflozin (CANA), a kind of sodium-glucose cotransporter-2 (SGLT-2) inhibition, study in which the role of CANA monotherapy in polycystic ovary syndrome (PCOS) has been investigated, and it could become a novel option in the PCOS treatment. Nevertheless, trials focused on SGLT-2 combination therapy's efficacy, and safety in PCOS patients are limited. This randomized controlled trial compared the efficacy and safety of CANA and metformin (MET) combination therapy and MET monotherapy in endocrine and metabolic profiles of overweight and obese women with polycystic ovary syndrome (PCOS).

Methods: Fifty-one overweight or obese non-diabetic PCOS women between 18 and 40 years old were enrolled. Patients were randomly allocated to receive either CANA/MET or MET treatment. The CANA/MET group received CANA 100 mg once daily plus MET 1000 mg twice daily, while the MET group received MET 1000 mg twice daily for three months. Changes in menstrual pattern, anthropometric parameters, gonadal parameters, glucose and lipid homeostasis, and adverse events (AEs) were evaluated.

Results: Compared with the MET group, women have a significantly lower level of total testosterone (TT), area under the curve for glucose (AUCGlu), and area under the curve for insulin (AUCIns) to AUCGlu ratio in the combination group. There were no significant differences in menstrual frequency, body weight, body mass index, follicle-stimulating hormone, luteinizing hormone, free androgen index, sex hormone-binding globulin, androstenedione, fasting

blood glucose, fasting insulin, AUCIns, homeostasis model assessment-insulin resistance (HOMA-IR), triglycerides, total cholesterol, low-density lipoprotein cholesterol, apolipoprotein A1 (Apo A1), apolipoprotein B (Apo B), and APO B/A1 ratio. AEs were seen in 57.70% (15/26) and 68.00% (17/25) of patients in the CANA/MET and MET groups, respectively.

Conclusions: In overweight and obese women with PCOS, CANA and MET combination therapy may be similar to MET monotherapy in improving menstrual frequency, weight control, hyperandrogenemia, and relieving insulin resistance. CANA/MET may have more benefits in reducing TT, AUCGlu, and the AUCIns/AUCGlu ratio within three months than MET monotherapy.

Trial registration: ClinicalTrials.gov, NCT04973891.

KEYWORDS

sodium-glucose co-transporter 2 inhibitors, canagliflozin, metformin, weight-loss, polycystic ovary syndrome

Introduction

According to different diagnostic criteria, polycystic ovary syndrome (PCOS) is one of the most prevalent reproductive endocrine disorders. It affects 4–21% of women of reproductive age (1, 2). On ultrasonography, hyperandrogenism (HA), ovulatory dysfunction, and polycystic ovaries are features of this syndrome (3, 4). In addition to these diagnostic features, obesity and insulin resistance (IR) are common abnormalities associated with PCOS (5). Approximately 50% of women with PCOS are overweight or obese (6), which could significantly amplify and worsen metabolic and reproductive outcomes regardless of PCOS phenotypes (7, 8). Hyperinsulinemia caused by IR is believed to promote HA in PCOS because insulin may augment luteinizing hormone (LH)-induced androgen production and reduce the liver's sex hormone-binding globulin (SHBG) synthesis (9, 10). Excess androgen in PCOS women could aggravate IR and lead to compensatory hyperinsulinism, further enhancing ovarian theca cell androgen secretion (11–14). Overall, obesity, IR, and HA may interact with and influence one another, contributing to PCOS development. Besides, regardless of ovulatory status, women with PCOS still risk their fertility potential being reduced (15). This may be caused by, for example, pregnancy complications (16) and the alternations in oocyte competence (17) and in endometrial competence (18). Furthermore, it is suggested that these disorders may be exacerbated by obesity, HA, and IR *via* various mechanisms, such as the affection of the physiological microenvironment in the follicular fluid, inflammation, and oxidative damage (17, 18). Currently, there is no specific remedy or cure for PCOS (19), and the therapy for PCOS administration has typically focused on the control of symptoms (20).

Metformin (MET), the most extensively used insulin-lowering drug in PCOS (21), reduces hepatic glucose production, inhibits gluconeogenesis and lipogenesis, and enhances insulin sensitivity in peripheral tissues (22). Various MET functions in PCOS have been proposed, such as weight reduction, decreased serum testosterone levels, lipid metabolism disorder amelioration, and endothelial function improvement (23). It is well documented that obesity treatment is essential for PCOS management. A mere 5% reduction (24) in body weight could reduce IR, hyperinsulinemia, and HA; increase SHBG production, and improve abnormal reproductive measures (24, 25). For weight loss, it is found that MET monotherapy can achieve sound effects but is not perfect in morbidly obese PCOS women (26).

Sodium-glucose cotransporter-2 (SGLT-2) inhibitors, novel hypoglycemic oral drugs that promote renal glucose loss (27),

Abbreviations: AND, androstenedione; Apo A1, apolipoprotein A1; Apo B, apolipoprotein B; AUCGlu, area under the glucose curve; AUCIns, area under the insulin curve; BMI, body mass index; CANA, canagliflozin; FAI, free androgen index; FBG, fasting blood glucose; FINS, fasting insulin; FSH, follicle-stimulating hormone; HOMA-IR, homeostasis model assessment-insulin resistance; LDL-C, low-density lipoprotein cholesterol; LH, luteinizing hormone; MET, metformin; N/A, not applicable; PCOS, polycystic ovary syndrome; SGLT-2, Sodium-glucose cotransporter-2; SHBG, sex hormone-binding globulin; TG, triglycerides; TC, total cholesterol; TT, total testosterone.

are widely used clinically in patients with diabetes. Many studies have shown that SGLT-2 inhibitors can reduce fat mass (28), and blood pressure (28), ameliorate glucose homeostasis (29), alleviate oxidative damage and inflammation (30), and protect the cardiovascular system (31). In addition, several studies have substantiated the view that SGLT-2 inhibitors can significantly reduce weight in non-diabetic overweight and obese individuals with few adverse events (AEs) (32–36). Based on the advantages of both anthropometric and metabolic profiles, the emergence of SGLT-2 inhibitors for PCOS treatment has aroused general interest (27, 37). Cai et al. found that canagliflozin (CANA) was not inferior to MET in improving weight loss and IR, and its supplementation in PCOS patients should be considered (38). However, few trials have focused on SGLT-2 combination therapy's efficacy and safety in PCOS patients.

Therefore, this randomized controlled trial (RCT) explored the difference in anthropometric indices, menstrual frequency, gonadal parameters, glucose and lipid homeostasis, and AEs between CANA/MET combination therapy and MET monotherapy in women with PCOS over three months. The present study aimed to provide additional options for PCOS treatment.

Methods

Participants

Patients in this open-label RCT were selected from the outpatient clinics of Shengjing Hospital of China Medical University Endocrinology Department, Shenyang, Liaoning, China, from April 2021 to March 2022.

Ethics

This single-center, open-label, 1:1 RCT was examined and approved by the Scientific Research and New Technology Ethical Committee of the Shengjing Hospital of China Medical University (No.2021PS555K) and pre-registered at ClinicalTrials. gov (NCT04973891). All participants read and signed a written informed consent form before testing.

Inclusion and exclusion criteria

Inclusion criteria: (i) 18-40 years (ii) Body mass index (BMI) ≥ 24 kg/m^2 (iii) PCOS diagnosis fulfills the Rotterdam 2003 criteria phenotype B with HA and oligo-/anovulation (4) (iv) A negative serum pregnancy test before enrollment.

Exclusion criteria: (i) Patients who were pregnant, intended to become pregnant, were breastfeeding or did not agree to birth control. (ii) Medication history in the recent three months included oral contraceptive pills, SGLT-2 inhibitors, glucagon-like peptide-1 receptor agonists, thiazolidinediones, MET, and Chinese herbs. (iii) Comorbidities (diabetes, abnormal thyroid function-hyperthyroidism or hypothyroidism, 21-hydroxylase deficiency, hyperprolactinemia, androgen-secreting tumors, congenital adrenal hyperplasia, and Cushing syndrome), (all based on patient' s medical records) (iv) Severe hepatic (alanine aminotransferase, aspartate aminotransferase > 3 times the normal value) or renal function (eGFR < 60 ml/min per 1.73 m2) damage. (v) Current or past (last three months) involvement in other interventional studies. (vi) 17α-dihydroxy-progesterone > 2ng/ml, (vii) Women with persistent or recurrent symptomatic urinary tract infection (UTI), gastrointestinal (GI) problems, or any other conditions that could endanger the patient's safety.

Study process

Eligible PCOS patients who provided consent were recruited and randomly allocated to either the CANA/MET group or the MET group. Randomization was performed using a computer-generated random number sequence. CANA and MET tablets were provided by Janssen Ortho, LLC, and Bristol-Myers Squibb Company, respectively. For CANA, subjects were required to take 100 mg once daily before breakfast; for MET, subjects were asked to take 1000 mg/day (500 mg twice daily with meals) for one week, with the dose increased to 2000 mg/day (1000 mg twice daily with meals), if tolerable. The management was for three months. All eligible patients were instructed to maintain their habitual diet, exercise level, and contraceptive use throughout the study period. They were also required to abstain from any drug with possible endocrine or metabolic effects.

Each participant completed assessments at two-time points: baseline and 12 weeks post-randomization. All PCOS subjects had to fast when measurements were taken. At the beginning of the study, data on body composition, glucose and lipid homeostasis, and sex steroid hormone concentration were measured and recorded. At the end of the study, the subjects underwent repeat assessments identical to the initial visit. We frequently contacted PCOS patients through weekly phone calls or communication tools, asking about their menstrual cycle and medication AEs, reminding them to take supplements daily, and arranging a convenient time for the next visit.

Assessment of anthropometric indexes

Each subject's weight and height were measured and recorded by a nurse to calculate body mass index [(BMI);

weight (kg)/height (m2)] wearing light indoor clothing without shoes. We acknowledged that according to the WHO, overweight/obesity was defined as a BMI ≧ of 25 since the individuals included were all Chinese, and a BMI of 24 and 28 were cutoffs for overweight and obesity for both males and females over 18 years of age (39, 40). Height was measured using a standardized wall-mounted radiometer (± 0.1 cm) (Seca 71; Hamburg, Germany), and body weight was measured using a multi-frequency bioelectrical impedance analyzer (InBody 770 scanner; In-body Bldg; Seoul, Korea). Anthropometric indices were assessed at baseline and 12 weeks post-randomization.

Assessment of menstruation

Due to all included PCOS patients meeting the Rotterdam 2003 criteria phenotype B (4), with ovulatory dysfunction (oligo-/anovulatory), irregular menstruation involved oligomenorrhea and amenorrhea. Oligomenorrhea refers to women with less than six menstrual periods within 12 months, while amenorrhea refers to individuals who have stopped menstruating for more than six months. Each bleeding counts as one menstrual cycle. Menstrual frequency recovery was defined as the recurrence of regular menstrual cycles in patients and was recorded at 12 weeks post-randomization.

Assessment of biochemical parameters

Follicle-stimulating hormone (FSH) (mIU/mL) and LH (mIU/mL) levels were tested by chemiluminescent immunoassay. Total testosterone (TT) (ng/ml) was determined using an electrochemiluminescent immunoassay (ECLLA). HA was defined as TT was higher than 0.5ng/mL (17). Sex hormone-binding globulin (SHBG) (nmol/L) was tested using immunochemiluminescence (Unicel Dxl 800; Beckman Coulter, USA). Free androgen index (FAI) (%) was calculated as TT (nmol/L) × 100/SHBG (nmol/L) ratio, and TT (ng/mL) was converted to TT (nmol/L) divided by 3.467 (nmol/L). Androstenedione (AND) (ng/ml) was tested using luminescence. LH, FSH, TT, SHBG, FAI, and AND assessment was performed at baseline and 12 weeks post-randomization.

Glucose tolerance and insulin sensitivity were assessed at baseline and 12 weeks post-randomization using the oral glucose tolerance test (OGTT). Blood samples were taken at 0, 60, and 120 min after a sugar meal and analyzed for blood glucose (mmol/L) using the hexokinase-6 phosphate dehydrogenase method or the chemiluminescence (double-antibody sandwich) for blood insulin (μU/mL) (Abbott Architect ci 16200; Abbott, USA). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as fasting insulin (FINS) (μU/mL) × fasting blood glucose (FBG) (mmol/L)/22.5. The area under the glucose curve (AUCGlu) (mmol/L · min) and insulin (AUCIns) (mU/L · min) were obtained by calculating the sum of the trapezoidal areas between 0, 60, and 120 min.

The deionization and enzyme method was used to evaluate triglyceride (TG) (mmol/L) concentration. Total cholesterol (TC) (mmol/L) was measured using the cholesterol oxidase method, and low-density lipoprotein cholesterol (LDL-C) (mmol/L) was measured using the chemically modified enzyme method. Apolipoprotein A1 (Apo A1) (g/L) and apolipoprotein B (Apo B) (g/L) were detected by immunoturbidimetry, and the Apo B/A1 ratio was also calculated (Abbott Architect ci 16200; Abbott, USA). The TG, TC, LDL-C, Apo A1, Apo B, and Apo B/A1 ratio assessment was performed at baseline and 12 weeks post-randomization. The AE severity was recorded and rated as mild, moderate, or severe.

Sample size estimation

No evidence of PCOS in women treated with CANA/MET or MET combination has been reported. The sample size of the pilot study should be calculated based on its original purpose, comprehensive consideration, calculation, and analysis, so as to get the sample size. Based on the above characteristics, the explanation is as follows: The primary outcome was the three months change in body weight. The strategy of sample size calculation was based on the assumptions that the mean reduction of body weight (−2.10 ± 2.35) for the MET group, and the expectation of two more times reduction in the CANA/MET group (mean=−4.20), we required 21 subjects for each group. By considering α=0.05, power=80%, and an approximately 20% dropout rate, 50 patients were examined and equally assigned to each group (n=25).

Statistical analysis

Continuous data were presented as mean, median, standard deviation (SD), and interquartile spacing; categorical data were presented as frequencies or percentages. AEs were calculated based on intention-to-treat principles, yet the treatment efficacy was measured using per-protocol analysis. First, normality was assessed using the D'Agostino and Pearson omnibus/Shapiro-Wilk test. The paired t-test or Wilcoxon signed-rank test was used for intragroup comparisons for continuous data. In contrast, an independent sample t-test or Mann-Whitney U test was performed for intergroup comparisons. For categorical variables, the chi-square test was used. Statistical significance was defined as a P-value < 0.05 (2-tailed). Results were obtained using GraphPad Prism Version 7.0 (GraphPad Software, Chicago, IL, USA) and SPSS (version 23.0; SPSS Inc., Chicago, IL, USA).

Results

Participants

A total of 66 patients with PCOS based on the Rotterdam 2003 criteria were recruited from an outpatient endocrinology department. During the screening process, fifteen patients were excluded for definite reasons: four patients had a strong desire for pregnancy during the test; eight patients declined to participate; two patients had a history of taking multiple drugs (one with oral contraceptives and another with orlistat); one patient combined with other diseases (suffered from diabetes). Then, fifty-one PCOS patients who met the inclusion criteria were enrolled in the study, including 26 in the CANA/MET group and 25 in the MET group. Five patients dropped out in the CANA/MET group (2 patients with unintended pregnancy; 3 patients were affected by the COVID-19 quarantine). Five patients withdrew from the trial in the MET group (1 patient with unintended pregnancy; 2 were affected by the COVID-19 quarantine; 1 was lost to follow-up, and 1 had severe vaginal bleeding). Finally, 21 participants in the CANA/MET group and 20 in the MET group who completed the trial were included in the final analysis. Follow-up rates were 80.76% (21/26) and 80.00% (20/25), respectively (Figure 1).

Baseline information

The two groups did not differ significantly in age, weight, or BMI (P=0.6118, P =0.2365, and P =0.1024, respectively). No statistically significant differences were found in interest outcomes according to baseline information. All baseline data were presented in Table 1.

Assessment of anthropometric parameters

No significant differences were found in body weight [CANA/MET: -6.66 ± 4.24 vs. MET: -5.85 ± 3.32; (P=0.5386)] and BMI [CANA/MET: -2.49 ± 1.55 vs. MET: -2.20 ± 1.30; (P=0.5441)] between the two groups. Within-group comparisons showed a significant decrease in body weight and BMI in the CANA/MET group (P < 0.0001 and P < 0.0001, respectively) and the MET group (P < 0.0001 and P < 0.0001, respectively) (Table 2).

Assessment of menstruation and gonadal parameters

After 12 weeks of treatment, an improvement in menstrual cycle irregularity was detected in CANA/MET group (80.95%, 17/21) and MET (80.00%; 16/20). There was no significant difference between the two interventions (P=0.6228). There was a clinically significant decrease in TT in the CANA/MET group compared to MET [CANA/MET: -2.49 ± 1.55 vs. MET: -2.20 ± 1.30; (P=0.0233)]. No differences were noted in FSH [CANA/MET: -0.75 ± 2.51 vs. MET: -0.68 ± 2.17; (P=0.9309)], LH [CANA/MET: -1.91(-7.40 to 2.49) vs. MET:

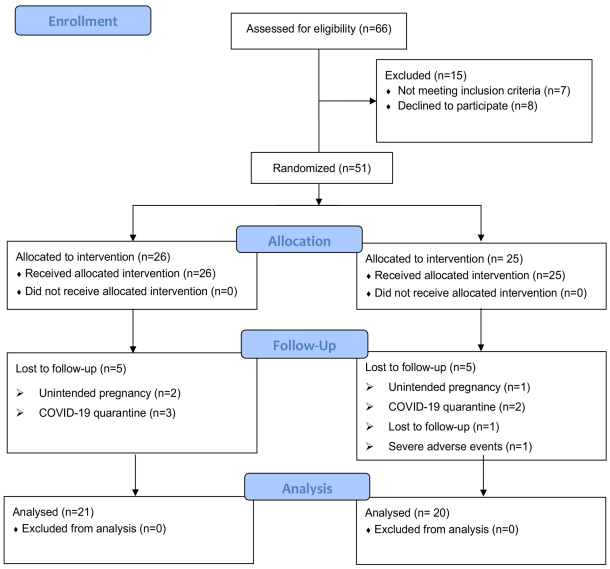

FIGURE 1
Patient selection flow diagram.

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TABLE 1 Demographic data and baseline characteristics of patients.

	CANA/MET (N = 21)	MET (N = 20)	*P* value
Age (years)	26.38 ± 5.89	5255 ± 4.36	0.6118
Height (m)	1.62 ± 0.04	1.63 ± 0.05	0.4495
Body weight (kg)	81.23 ± 9.83	74.78 ± 8.91	0.2365
BMI (kg/m^2)	31.11 ± 3.02	29.33 ± 3.19	0.1024
FSH (mIU/mL)	6.58 ± 1.54	6.05 ± 1.60	0.2800
LH (mIU/mL)	10.85 (6.36-16.22)	11.63 (9.69-16.87)	0.4304
TT (ng/mL)	0.95 (0.78-1.08)	0.89 (0.74-1.09)	0.7616
FAI (%)	28.62 ± 16.4	19.26 ± 9.46	0.0738
SHBG (nmol/L)	13.60 (8.55-20.15)	18.45 (13.13-21.98)	0.1626
AND (ng/ml)	3.57 ± 1.29	4.48 ± 1.42	0.0715
FBG (mmol/L)	5.70 (5.27-6.02)	5.30 (5.16-5.80)	0.1625
FINS (mU/L)	21.5 (14.35-24.20)	16.70 (14.58-24.33)	0.5919
AUCGlu (mmol/L*min)	1086 ± 208.7	985.3 ± 160.7	0.0915
AUCIns (mU/L*min)	14808 ± 6668	13867 ± 7201	0.6664
AUCIns/AUCGlu	13.97 ± 6.83	13.90 ± 6.55	0.9728
HOMA-IR	5.70 (3.38-6.08)	4.25 (3.26-6.44)	0.6515
TG (mmol/L)	1.54 (1.09-2.01)	1.49 (1.07-1.74)	0.6668
TC (mmol/L)	4.90 ± 0.93	4.74 ± 0.63	0.5353
LDL-C (mmol/L)	3.06 ± 0.97	3.01 ± 0.54	0.8401
Apo A1 (g/L)	1.16 ± 0.15	1.25 ± 0.20	0.1516
Apo B (g/L)	0.98 ± 0.26	0.88 ± 0.18	0.2032
Apo B/A1	0.85 ± 0.23	0.72 ± 0.18	0.0732

CANA, canagliflozin; MET, metformin; BMI, body mass index; FSH, follicle stimulating hormone; LH, luteinizing hormone; TT, total testosterone; FAI, free androgen index; SHBG, sex hormone-binding globulin; AND, androstenedione; FBG, fasting blood glucose; FINS, fasting insulin; AUCGlu, area under the glucose curve; AUCIns, area under the insulin curve; HOMA-IR, homeostasis model assessment-insulin resistance; TG, triglycerides; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; Apo A1, Apolipoprotein A1; Apo B, Apolipoprotein B.

0.42(-7.10 to 4.19); (*P*=0.1990)], FAI [CANA/MET: -9.47 ± 11.65 vs. MET: -5.11 ± 7.40; (*P*=0.1631)], SHBG [CANA/MET: 0.10(-3.45 to 5.30) vs. MET: 2.95(-2.15-10.30); (*P*=0.4579)], and AND [CANA/MET: -0.36 ± 1.17 vs. MET: -0.39 ± 1.58; (*P*=0.9555)]. Within-group comparisons showed that both groups had significantly lower TT levels ($P < 0.0001$ and $P = 0.0343$, respectively). In the CANA/MET group, the FAI ($P = 0.0457$) at 12 weeks decreased significantly compared to baseline, but no changes were observed in the MET group. In the MET group, SHBG (*P*=0.0303) at 12 weeks increased significantly compared to that at baseline, but no changes were observed in the CANA/MET group. Both groups showed no significant changes in FSH, LH, or AND levels after treatment (Table 2).

Glucose homeostasis assessment

Participants in the CANA/MET group had a significant decrease in AUCGlu [CANA/MET: -158 ± 225.4 vs. MET: 2.63 ± 180.7; (*P*=0.0182)] and the AUCIns/AUCGlu ratio [CANA/MET: -2.86 ± 5.71 vs. MET: 0.51 ± 0.61; (*P*=0.0164)] compared with MET. There were no significant differences in FBG [CANA/MET: -0.33(-0.95 to -0.05) vs. MET: -0.11(-0.49 to 0.1); (*P*=0.1711)], FINS [CANA/MET: -7(-10.4 to -2) vs. MET: -4.2 (-9.8 to -0.7); (*P*=0.4565)], AUCIns [CANA/MET: -4264 ± 5627 vs. MET: -2640 ± 6108; (*P*=0.3869)], and HOMA-IR [CANA/MET: -1.83(-3.01 to -0.96) vs. MET: -1.29(-2.9 to -0.05); (*P*=0.4015)]. Within-group comparisons revealed that both groups had significantly lower FINS levels (*P*=0.0003 and *P*=0.0041, respectively), the AUCIns/AUCGlu ratio (*P*=0.0327, and $P = 0.0255$, respectively), and HOMA-IR ($P = 0.0002$ and $P = 0.0028$, respectively). Decreased FBG, AUCGlu, and AUCIns levels were observed in the CANA/MET group (*P*=0.0007, $P = 0.0044$, and $P = 0.0024$, respectively). However, these differences were not observed in the MET group (Table 2).

Assessment of lipid homeostasis

No significant differences were found in all lipid parameters: TG [CANA/MET: -0.27 ± 0.51 vs. MET: -0.05 ± 0.59; (*P*=0.2011)], TC [CANA/MET: -0.22 ± 0.43 vs. MET: -0.27 ± 0.48; (*P*=0.7954)], LDL-C [CANA/MET: -0.12 ± 0.49 vs. MET: -0.19 ± 0.50; (*P*=0.6894)], Apo A1 [CANA/MET: -0.02 ± 0.33 vs. MET: -0.02 ± 0.16; (*P*=0.9465)], Apo B [CANA/MET: -0.05 ±

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TABLE 2 Information of 12-weeks post treatment and changes in endocrine and metabolic profile.

	CANA/MET (N = 21)		MET (N = 20)		*P* value (Change)
	12 weeks	Change from baseline	12 weeks	Change from baseline	
Anthropometric characteristics					
Body weight (kg)	75.40 ± 8.68[d]	-6.66 ± 4.24	72.49 ± 9.97[d]	-5.85 ± 3.32	0.5386
BMI (kg/m^2)	28.62 ± 2.91[d]	-2.49 ± 1.55	27.14 ± 3.50[d]	-2.20 ± 1.30	0.5441
Gonadal hormones					
FSH (mIU/mL)	5.84 ± 2.24	-0.75 ± 2.51	5.36 ± 1.94	-0.68 ± 2.17	0.9309
LH (mIU/mL)	8.59 (3.96-12.16)	-1.91 (-7.40 to 2.49)	10.27 (8.22-13.61)	0.42 (-7.10 to 4.19)	0.1990
TT (ng/mL)	0.53 (0.45-0.84)[d]	-0.33 ± 0.23	0.71 (0.55-0.91)[a]	-0.18 ± 0.18	***0.0233***
FAI (%)	19.15 ± 13.19[a]	-9.47 ± 11.65	14.14 ± 12.57	-5.11 ± 7.40	0.1631
SHBG (nmol/L)	13.6 (9.55-24.10)	0.10(-3.45 to 5.30)	22.35(14.78-26.70)[a]	2.95 (-2.15-10.30)	0.4579
AND (ng/ml)	3.22 ± 1.35	-0.36 ± 1.17	3.79 ± 2.21	-0.39 ± 1.58	0.9555
Glucose and lipid-related parameters					
FBG (mmol/L)	5.20 (4.88-5.35)[c]	-0.33 (-0.95 to -0.05)	5.30 (4.96-5.42)	-0.11(-0.49 to 0.1)	0.1711
FINS (mU/L)	12.0 (8.20-20.15)[c]	-7 (-10.4 to -2)	14.70 (10.80-20.40)[b]	-4.2 (-9.8 to -0.7)	0.4565
AUCGlu (mmol/L*min)	928.3 ± 124.5[b]	-158 ± 225.4	988.5 ± 129.0	2.63 ± 180.7	***0.0182***
AUCIns (mU/L*min)	10543 ± 6888[b]	-4264 ± 5627	11691 ± 5212	-2640 ± 6108	0.3869
AUCIns/AUCGlu	11.12 ± 7.12[a]	-2.86 ± 5.71	11.76 ± 4.64[a]	0.51 ± 0.61	***0.0164***
HOMA-IR	3.14 (1.91-4.71)[c]	-1.83 (-3.01 to -0.96)	3.51 (2.36-4.71)[b]	-1.29 (-2.9 to -0.05)	0.4015
TG (mmol/L)	1.20 (0.84-1.63)[a]	-0.27 ± 0.51	1.43 (1.03-2.06)	-0.05 ± 0.59	0.2011
TC (mmol/L)	4.54 ± 0.80[a]	-0.22 ± 0.43	4.54 ± 0.52	-0.27 ± 0.48	0.7954
LDL-C (mmol/L)	2.83 ± 0.70	-0.12 ± 0.49	2.83 ± 0.49	-0.19 ± 0.50	0.6894
Apo A1 (g/L)	1.20 ± 0.21	-0.02 ± 0.33	1.25 ± 0.26	-0.02 ± 0.16	0.9465
Apo B (g/L)	0.92 ± 0.26	-0.05 ± 0.13	0.90 ± 0.13	0.01 ± 0.17	0.2887
Apo B/A1	0.78 ± 0.23[a]	-0.08 ± 0.14	0.74 ± 0.16	0.02 ± 0.22	0.1450

PCOS, polycystic ovary syndrome; CANA, canagliflozin; MET, metformin; BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone; TT, total testosterone; FAI, free androgen index; SHBG sex hormone-binding globulin; AND, androstenedione; FBG, fasting blood glucose; FINS, fasting insulin; AUCGlu, area under the glucose curve; AUCIns, area under the insulin curve; HOMA-IR, homeostasis model assessment-insulin resistance; TG, triglycerides; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; Apo A1, apolipoprotein A1; apo B, apolipoprotein B; N/A, not applicable.

Bold and italic fonts indicate statistically significant between the two groups.

[a] P < 0.05, vs. baseline and 12-week visits.

[b] P < 0.01, vs. baseline and 12-week visits.

[c] P < 0.001, vs. baseline and 12-week visits.

[d] P < 0.0001, vs. baseline and 12-week visits.

0.13 vs. MET: 0.01 ± 0.17; (*P*=0.2887)], and the Apo B/A1 ratio [CANA/MET: -0.08 ± 0.14 vs. MET: 0.02 ± 0.22; (*P*=0.1450)]. The TG and TC levels and the Apo B/A1 ratio declined from baseline only in the CANA/MET group (*P* =0.0314, *P*=0.0396, and *P* =0.0377, respectively). Both groups showed no significant changes in LDL-C, Apo A1, and ApoB levels after treatment (Table 2).

AE assessment

AEs were seen in 57.70% (15/26) and 68.00% (17/25) patients in the CANA/MET and MET groups, respectively. Only one subject in the MET group had to withdraw due to severe vaginal bleeding. The details are summarized in Table 3.

Discussion

To our knowledge, this is the first 3-month randomized clinical trial comparing the efficacy and safety of CANA (100 mg once daily)/MET (1000 mg twice daily) and MET (1000 mg twice daily) in overweight and obese women with PCOS. Our results supported MET as conventional therapy for PCOS, given its amelioration of menstrual frequency, body weight, BMI, TT, FINS, HOMA-IR, and AUCIns/AUCGlu either combined with CANA or as monotherapy. In addition, we found that CANA/MET might be more beneficial in reducing TT, AUCGlu, and the AUCIns/AUCGlu ratio than MET monotherapy within three months. CANA/MET supplementation may be similar to MET monotherapy in PCOS administration to improve menstrual pattern, weight control, and HOMA-IR in overweight and obese PCOS women.

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TABLE 3 AEs of two treatment groups.

	CANA/MET (N = 26)	MET (N = 25)
Patients with AEs		
Severe AEs		
Vaginal bleeding	0	1
Mild and moderate AEs		
Nausea	11	14
Abdominal discomfort	1	4
Abdominal pain	2	1
Bloating	0	1
Diarrhea	4	8
Loss of appetite	2	4
Anorexia	1	0
Acid reflux	1	1
Headache	1	0
Dizziness	3	0
Asthenia	1	0
Bitter mouth	0	1

CANA, canagliflozin; MET, metformin; AEs, adverse events.

Weight loss is considered essential in PCOS management. In our study, participants in the CANA/MET group had a mean weight loss of 5.83 kg (7% of their body weight), and those in MET had 2.29 kg (3% of their body weight). However, the difference between the two groups did not reach statistical significance. This result agrees with an RCT that found that the effects of dapagliflozin (DAPA) (10 mg once daily)/MET (2000 mg once daily) combination therapy may be similar to those of MET (2000 mg once daily) monotherapy in promoting weight loss in PCOS patients (41). Furthermore, a similar non-significant difference in weight loss was reported in two RCTs that administered SGLT-2 inhibitors (empagliflozin 25 mg daily; canagliflozin 100 mg daily) monotherapy compared with MET in women with PCOS (38, 42). So far, the efficacy of SGLT-2 inhibition in weight control compared to MET in PCOS women has been rarely reported in the previous literature, with only above mentioned three RCTs. Interestingly, a meta-analysis involving seven studies with 2297 participants indicated that SGLT-2 inhibitor plus MET was superior to MET for weight control in people with type 2 diabetes for no more than 52 weeks (43). Another meta-analysis of eight studies with 750 individuals found that SGLT-2 inhibitor monotherapy for 12 weeks or more could lead to modest weight loss in non-diabetic overweight and obese individuals (44). This discordance might be due to the relatively short duration of the intervention or the different diseases and populations. Evidence related to the SGLT-2 combination strategy for PCOS treatment is rare. Further studies on the appropriate dosage and duration of SGLT-2 inhibitors are urgently required.

There was an improvement in menstrual cycle frequency in the CANA/MET and MET groups, with no significant difference. This result is in line with that of Cai et al. (38). For gonadal hormones, we found that the significant change in TT and FAI with both treatments from baseline was consistent with previous study findings, showing that DAPA/MET or MET could reduce TT and FAI (41). However, the results were inconsistent with some studies on SGLT-2 inhibitor monotherapy in PCOS (38) (42, 45). For instance, Tan et al. illustrated that supplementation with licogliflozin (50 mg three times daily) had no significant effect on reducing TT and FAI within two weeks compared to placebo (45). After 12 weeks of empagliflozin (EMPA) (25 mg once daily) intake, Javed et al. reported no significant decrease in TT and FAI (42). A recent study by Cai et al. also suggested that CANA (100mg once daily) had no significant effect on the reduction of TT and FAI at 12 weeks (38). Therefore, though in our trial we found that CANA/MET may be superior to MET in the reduction of TT in women with PCOS. The significant difference in FAI, an indicator that better discriminated PCOS than TT (46), did not exist between the two interventions. Therefore, we should be cautious not to draw definite conclusions due to the small sample size.

Metformin corrects endocrine and metabolic abnormalities in women with PCOS; it counteracts IR by inhibiting hepatic glucose production (22). By reducing the maximum kidney's glucose reabsorptive capacity and glucosuria threshold, SGLT2 inhibitors enhance glucose excretion, reducing fasting and postprandial plasma glucose levels and improving insulin secretion and insulin sensitivity (47) Our study found that CANA/MET and MET could reduce FINS and HOMA-IR, with no significant difference among the treatment groups. Consistent with a recent randomized study by Tan et al., FINS and HOMA-IR levels declined significantly after two weeks of licogliflozin treatment (50 mg three times daily) in women with PCOS (45). Cai et al. also indicated that CANA was not inferior to MET regarding FINS and HOMA-IR reduction after 12 weeks of intervention (38). Javed et al. demonstrated similar results based on treatment with EMPA (25 mg once daily) for 12 weeks (42). Furthermore, a comparative 24-week study of patients with PCOS found that 10 mg DAPA daily or 10 mg of DAPA with 2,000 mg of MET daily significantly reduced patients' HOMA-IR; however, the difference between the two interventions was not statistically significant (41). In our study, AUCGlu, AUCIns, and AUCIns/AUCGlu ratio decreased prominently in the CANA/MET group after 12 weeks; only AUCGlu and the AUCIns/AUCGlu ratio showed a statistically significant difference between the two comparisons. The AUCIns/AUCGlu ratio is relevant to pancreatic beta-cell dysfunction and the incidence of diabetes (48). Currently, there is only one related study examining AUCGlu and AUCIns between licogliflozin and placebo in PCOS (45), which found that

licogliflozin (50 mg three times daily) could result in significant reductions in AUCGlu and AUCIns levels after a 2-week trial. No relevant studies have focused on the AUCIns/AUCGlu ratio.

In our study, the serum TG levels decreased significantly in the CANA/MET group. No significant differences were found in TG and LDL-C levels between the two treatments. SGLT-2 inhibition was found to affect the plasma lipid profile of diabetic patients by decreasing TG levels and increasing LDL-C levels (49). The LDL-C levels in the CANA/MET group were not altered from baseline, which is consistent with Cai et al. reports (38). Literature suggests that an increased Apo B/A1 ratio may worsen endocrine and metabolic profiles in women with PCOS. Also, it might be a valuable tool to screen PCOS intensity by evaluating IR and metabolic syndrome (50). We found that the Apo B/A1 ratio declined after CANA and MET combination therapy. Nevertheless, due to the small sample size and limited duration, trials with larger sample sizes are needed.

In our trial, CANA and MET were well tolerated by most patients. Only one patient withdrew owing to serious AEs and vaginal bleeding, which was considered unrelated to the study drug. Surprisingly, no UTI problems were observed in the CANA/MET therapy group. We speculate on possible reasons for the results. On the one hand, before starting medications, each participant was told to drink more water throughout the entire intervention period, also for the MET group. On the other hand, this may be because patients under this treatment had a small dose of CANA (100 mg q.d.) rather than CANA (300 mg q.d.). Nevertheless, we should still focus on the AEs after SGLT-2 inhibition supplementation, due to the small sample size. Most of the patients had mild or moderate GI problems in both groups, consistent with Cai et al. reports (38). Moreover, most AEs appeared in the initial stage of the experiment, especially after 1–3 weeks.

Our study has several strengths. Firstly, this is the first time assessing the differences between CANA/MET combination therapy and MET monotherapy in PCOS management. Until now, four clinical studies focusing on SGLT-2 inhibitors in PCOS management, and only one study compared the difference between DAPA/MET and MET monotherapy (41). The reason for selecting CANA as an intervention is that the primary outcome was set to be body weight, and CANA has displayed a better function of glucose excretion than DAPA. The second advantage is that the AUCIns/AUCGlu ratio after SGLT-2 inhibition supplementation in PCOS women has been assessed for the first time. AUCGlu and the AUCIns/AUCGlu ratio significantly lowered after combination therapy, and glucose metabolism amelioration is essential to PCOS management and its long-term complications. In our trial, we found that CANA/MET may be more beneficial in improving TT. It is suggested that SGLT-2 inhibition could lower total fat mass in PCOS rats with HA (28). Interestingly, though we did not focus on the change of fat mass, there was a significantly lower level of TG in the CANA/MET group compared with the baseline. In the MET group, no significant decrease in TG levels was seen. We speculate that SGLT-2 inhibition may reduce the effect of lipotoxicity, thereby ameliorating the metabolism of androgens in PCOS. Further basic and clinical studies are needed to investigate this issue. The present study has several limitations. First, this study was a single-center, open-label, lack-of-a-placebo controlled clinical design such that physicians and PCOS patients were not blinded to the medication. In the process of study design, we told all the included patients about their specific therapy strategies. The absence of blinding could make a possible subjective bias inevitable; this weakness, nevertheless, may be offset by the benefits of communicating with patients regularly, showing concern for patients, and responding to the questions as quickly as possible and thereby achieving better patient adherence as well as improving therapeutic relationships. Most of our patients were lost to follow-up for objective reasons like the COVID-19 pandemic. This could have impacted the final results' reliability despite the rate being just 20%. Second, residual confounding factors, such as individual life modifications (dietary pattern and/or exercise), may affect the outcomes despite considering many potential confounders. Actually, though all eligible patients were instructed to maintain their habitual diet, exercise level, and contraceptive use throughout the study period, this was not monitored to ensure compliance. If the included patients did alter their habitual diet, exercise level, and contraceptive use, the results we achieved might not be due to interventions alone. There is a need for a specific diet, exercise, and contraceptive use monitoring in future studies. In addition, this trial had a relatively small sample size, and its period was too short to evaluate SGLT-2 inhibitor long-term effects in combination with MET in PCOS management. Finally, we focused only on gonadal and metabolic parameters between the two groups. Other indicators associated with body composition, blood pressure, and oxidative stress were not assessed.

SGLT-2 inhibitors may be promising new therapeutic drugs for PCOS. However, whether used alone or in combination with other agents, its efficacy still needs to be explored. It is essential to understand the mechanisms underlying SGLT-2 inhibition in PCOS and its long-term complications. The sample size should be expanded in further clinical trials, and a meta-analysis should be conducted to obtain high-quality conclusions. More studies are needed to be carried out to determine more efficient PCOS therapies.

Conclusion

In overweight and obese women with PCOS, CANA and MET combination therapy may be similar to MET monotherapy in improving menstrual frequency, weight control, HA, and

relieving IR. Compared with MET monotherapy, CANA/MET may have more benefits in reducing TT, AUCGlu, and AUCIns/AUCGlu ratio within three months. Additional trials are necessary to assess the SGLT-2 inhibitor supplementation's long-term effects in patients with PCOS.

Data availability statement

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

Ethics statement

Ethical approval was obtained from the Scientific Research and New Technology Ethical Committee of the Shengjing Hospital of China Medical University (No.2021PS555K). Written informed consent was obtained from all participants prior to inclusion in the study. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

JZ and BH conceived, designed, and performed the experiments; JZ, XC, and CX analyzed the data; JZ wrote the paper. BH reviewed and edited the final manuscript. All authors read and approved the final 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

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Underlying mechanisms of acupuncture therapy on polycystic ovary syndrome: Evidences from animal and clinical studies

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Polycystic ovary syndrome (PCOS) is a common endocrine and metabolic disorder among women of reproductive age. Current standard treatment includes lifestyle change, oral pharmacological agents, and surgical modalities. However, the efficacy of current therapies is less than satisfactory. Clinical evidence has shown that acupuncture is effective for regulating hormone levels, promoting ovulation, and attenuating insulin resistance in patients with PCOS. Acupuncture may affect the production of β-endorphin, which may lead to gonadotropin-releasing hormone secretion and then affect ovulation, menstrual cycle, and fertility. The mechanism of acupuncture for patients with PCOS has not been comprehensively reviewed so far. Better understanding of the mechanisms of acupuncture would help popularize the use of acupuncture therapy for patients with PCOS. In this narrative review, we aimed to overview the potential mechanisms and evidence-based data of acupuncture on PCOS, and analyze the most frequently used acupoints based on animal and clinical studies. The results of this study will contribute to a better understanding of the current situation in this field.

KEYWORDS

acupuncture, animal studies, clinical studies, mechanism, polycystic ovary syndrome, review

Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine-metabolic disorder in reproductive-aged women, affecting up to 15% of reproductive age women (1), and has become the leading cause of menstrual disorders and anovulatory infertility in women (2, 3). The major clinical manifestations of PCOS consist of ovulatory dysfunction, hyperandrogenism, and polycystic ovaries, along with insulin resistance, obesity, and metabolic dysfunction (4, 5). In addition, PCOS is also linked with other complications,

such as type 2 diabetes, endometrial dysfunction and cancer, cardiovascular disease, depression, and pregnancy complications (6–10). The current standard treatment for PCOS includes lifestyle change, pharmacological therapy, and surgical modalities, but the effect is less than satisfactory (11, 12). Lifestyle change is the first-line therapy for PCOS patients, especially for overweight and obese women. However, this is a very difficult task for many people (13). The main pharmacological therapy for PCOS patients is the oral selective estrogen receptor modulator like clomiphene citrate (CC). CC is ineffective in 40% of PCOS patients and is associated with significant side effects, such as headaches, bloating, mood swings, and breast tenderness (14, 15). Letrozole is considered the first-line treatment to induce ovulation, with the ability to improve clinical pregnancy rates and reduce time-to-pregnancy in women with PCOS (15). However, it is associated with higher risks of hot flashes, arthralgias, fatigue, and myalgias (16). Metformin, an insulin sensitizer, has been widely used for the treatment of PCOS patients (17). It increases insulin sensitivity, but associated with unsatisfactory weight loss and increased risk of hypoglycemia (18, 19). Gonadotropin has been proven to be effective in ovulation induction. But it may lead to the development of multiple follicles and increase the risk of ovarian hyperstimulation syndrome (20). Therefore, there is a need for a new therapy, which should be inexpensive, easily administered, and free of serious side effects.

Acupuncture has been used as a medical means in China for thousands of years (21). The use of acupuncture in the reproductive endocrinology and infertility of PCOS has recently gained increased popularity worldwide. Several clinical trials have shown that acupuncture may have beneficial effects on ovulatory dysfunction and infertility in patients with PCOS. Acupuncture has also been reported to improve insulin sensitivity and decrease testosterone in patients and animals with PCOS (22). However, there is an insufficient amount of research evidence to support the clinical efficacy of acupuncture treatment for PCOS women, and the mechanisms of acupuncture are unclear, previous clinical and experimental studies indicate that acupuncture influences PCOS-like symptoms *via* various mechanisms. Recent reviews have demonstrated that acupuncture adjusts hormone levels by regulating hypothalamic-pituitary-ovarian (HPO) axis or regulating levels of anti-Müllerian hormone (AMH) and P450arom. A recent systematic review on effect of acupuncture on PCOS in animal models summarized that acupuncture could improve insulin resistance by upregulating the insulin receptor substrate-1 (IRS-1)/PI3K/glucose transporter 4 (GLUT4) pathway, or inhibiting the PI3K/AKT/mTOR pathway, or activating the adenosine 5'-monophosphate activated protein kinase (AMPK) pathway in PCOS animals (23).

Until now, no review has been published to summarize the effects of acupuncture both in PCOS patients and animal models. Therefore, the present study aims to review the potential mechanism and evidence-based data of acupuncture on PCOS, and analyze the most commonly used acupoints. The results of this narrative review may provide directions for future research in this area.

Searching methods

Search strategy

A comprehensive literature search was performed by two independent investigators in PubMed, Web of Science, and Scopus databases from establishment to July 2022 to identify related publications. The advanced search option was used to select relevant keywords and identify Medical Subject Headings (MeSH) terms [i.e., (Acupuncture* OR Electroacupuncture*) AND (Polycystic Ovary Syndrome* OR Polycystic Ovarian Syndrome* OR PCOS*)].

Eligibility criteria

All human and animal studies concerning the effect of acupuncture on PCOS treatment were included in this review. There was no country restriction, but the language was limited to English. Moreover, no restrictions were imposed involving publishing date, type of subjects or type of reported outcomes. Researches were excluded if the type of intervention was moxibustion or transcutaneous electrical acupoint stimulation or acupressure.

Study selection

The following assignments were independently accomplished by two investigators. We identified 150 articles on PubMed, 246 articles on Web of Science, and 158 articles on Scopus. After duplicates were removed, 263 articles conformed to our search criteria. The titles and abstracts of the identified articles were reviewed, and irrelevant search results were deleted. After that step, the full text of the remaining literature was assessed. A total of 62 publications were finally included in this review (Table 1). Among them, 28 publications were animal studies, and 34 publications were clinical studies. The flow chart of the screening process is shown in Figure 1.

The etiology of PCOS

The exact etiology of PCOS is still unclear because of the complicated pathophysiological processes. Mounting evidence suggests that PCOS may be related to hyperandrogenism, insulin resistance, genetic factors, and negative emotions. Hyperandrogenemia plays a vital role in the pathogenesis of PCOS (70). Androgen stimulation may increase the release of

TABLE 1 Characteristics of acupuncture in the treatment of PCOS from main studies included.

Year	Author	Subject	Intervention type	Acupoints	Frequency and Duration	Mechanisms
2000	Stener-Victorin (24)	PCOS rats	EA	NR	every second or third day up to 12 times	↓ovarian NGF concentrations
2003	Stener-Victorin (25)	PCOS rats	EA	NR	12times	↓ NGF, endothelin 1
2004	Bai (26)	PCOS rats	EA	SP6, E128	2 per wk for 8 wks	reversed the NGF abundance
2005	Manni (27)	PCOS rats	EA	NR	every second day	↓beta2-ARs mRNA
2008	Manneras (28)	PCOS rats	EA	ST29, SP6	every second weekday for 4 –5 wks	↑insulin sensitivity
2009	Feng (29)	PCOS rats	EA	ST29, SP6	5 per wk for 4-5 wks	↓hypothalamic GnRH and AR expression levels
2009	Manneras (30)	PCOS rats	EA	NR	every second weekday for 4–5 wks	↓expression of genes encoding markers of sympathetic activity
2009	Stener-Victorin (31)	PCOS women	EA	CV3, CV6, ST29, SP6, SP9, LI4, PC6	2 per week for 2 wks, 1 per week for 6 wks, 1 every second wk for 8 wks	↓muscle sympathetic nerve activity
2010	Johansson (32)	PCOS rats	EA	NR	5 per wk for 4-5 wks	↑GLUT4, ↓high-density lipoprotein/low-density lipoprotein cholesterol
2011	Jedel (33)	PCOS women	EA	CV3, CV6, ST29, SP6, SP9	2 per wk for 2 wks, 1 per wk for 6 wks, 1 every other wk for 8 wks	improve hyperandrogenism and menstrual frequency
2011	Pastore (34)	PCOS women	EA/MA	UB23, UB28, SP6, SP9, PC6, TE5, GV20	2 per wk for 4 wks, 1 per wk for 4 wks	↓ LH/FSH
2012	Billhult (13)	PCOS women	EA/MA	CV3, CV6, ST29, SP6, SP9	2 per wk for 2 wks, 1 per wk for 6 wks, 1 every other week for 8 wks	hope, get result, feel of responsibility, skepticism and proof of effect, feel normal
2012	Feng (35)	PCOS rats	EA/MA	NR	5 per wk for 4–5 wks	restored disturbed oestrous cyclicity
2012	Franasiak (36)	PCOS women	EA/MA	UB23, UB28, SP6, SP9	2 per wk for 4 wks, 1 per wk for 4 wks	not change AMH concentrations
2013	Stener-Victorin (37)	PCOS women	EA	CV3, CV6, ST9, SP6, and SP9	2 per wk for 2 wks, 1 per wk for 6 wks, 1 every other wk for 8 wks	↓MADRS-S and BSA-S, ↑SF-36, PCOSQ
2013	Johansson (38)	PCOS rats	EA/MA	ST29, SP6	5 per wk for 4-5 wks	↓weight of the subcutaneous fat depot
2013	Rashidi (39)	PCOS women	acupuncture	NR	NR	↑embryo quality
2013	Yu (40)	PCOS women	EA	ST25, CV12, CV6, SP6, BL17, BL32	3 per wk	↓BW, BMI, WHR, FINS, ↑ISI and APN
2013	Johansson (41)	PCOS women	EA/MA	CV3, CV6, ST25, ST29, SP6, SP9, LI4, GV20, LR3, PC6, GV20	2 per wk for 10 –13 wks	↑ovulation frequency
2013	Sun (42)	PCOS rats	EA	CV3, CV4	once daily for 14 consecutive days	↑P450arom, ↓P450c17α
2013	Zheng (43)	PCOS women	acupuncture	NR	once a day for 6 months	↓BMI, WHR, ovarian volume, luteotrophic hormone, LDL-C, T, ↓ LH/FSH, ↑Menstrual frequency and HDL-C
2016	Stener-Victorin (44)	PCOS women	EA	CV4, CV12, ST29, ST34, ST32, SP6, ST36, LI4, CV6, CV10, ST27, SP10, SP6, LR3, PC6	3 per wk over 5 wks	↓HbA1c, ↓circulating and adipose tissue androgens

(Continued)

TABLE 1 Continued

Year	Author	Subject	Intervention type	Acupoints	Frequency and Duration	Mechanisms
2016	Ramadoss (45)	PCOS rats	EA	biceps femoris and erector spinae muscle	alternate days for 4-5 wks	↓ sympathetic tone
2017	Benrick (46)	PCOS women	EA	ST27, ST28, ST29, SP6, SP9	NR	↑whole-body glucose uptake, ↑sympathetic/partly
2017	Maliqueo (47)	PCOS rats	EA	NR	5 per wk for 5-6 wks	↑low-density lipoprotein-cholesterol
2018	Kokosar (48)	PCOS women	EA	CV3, CV12, ST29, ST32, ST34, ST36, SP6, LI4	NR	↑sympathetic nervous system ↑whole body glucose uptake
2018	Ma (49)	PCOS rats	EA	ST29, SP6	5 per wk for 4 wks	↑angiogenesis in the antral follicles
2018	Cui (50)	PCOS rats	EA	SP6, ST29, GV20	3 wks	↓global DNA methylation and Dnmt3b expression, ↓LH/FSH, ↑Menstrual frequency and HDL-C
2019	Shi (51)	PCOS rats	EA	CV3, CV4	for 14 consecutive days	↓AMH
2019	Wang (52)	PCOS women	EA	CV3, CV6, ST29, SP6, SP9, LI4, GV20, ST25, LR3, PC6	2 per wk for 16 wks	↑serum NE, ↓5-HT, ↓GABA
2019	Budihastuti (53)	PCOS women	EA	CV3, CV6, ST29, SP6, LI4, ST36	2 per wk for 6 wks	↑oocytes' growth
2019	Rouhani (54)	PCOS women	EA	ST21, ST25, ST28, ST29, REN12, REN6, REN4, SP9, SP6, ST40	20 times	↓body fat and BMI, WHR, fasting insulin, ↑insulin sensitivity
2020	Benrick (55)	PCOS women	EA	CV3, CV12, ST29, ST 32 ST34, ST36, SP6, LI4	NR	normalize gene expression in skeletal muscle
2020	Peng (56)	PCOS rats	EA	ST29, SP6	5 wks	↓sterol regulatory element binding protein 1
2020	Li (57)	PCOS women	EA/MA	ST29, CV3, CV12, ST34, ST33, SP6, ST36, ST27, CV6, CV10, SP10, SP6, LR3	3 per wk for 6 months	↓homeostatic model assessment for insulin resistance
2020	Peng (12)	PCOS rats	EA	ST29, SP6	5 wks	↑autophagy
2020	Tong (58)	PCOS rats	EA	ST29, SP6	5 per wk for 4 wks	regulating ovarian innervation
2020	Xu (59)	PCOS rats	EA	CV3	14 consecutive days	↓T, FAI, LH, LH/FSH, AMH, INHB, FINS, ↑E2, FSH, and SHBG
2021	Budihastuti (60)	PCOS women	EA	CV3, CV6, ST29, SP6, LI4, ST36	2 per wk for 6 wks	↑folliculogenesis/endometrial thickness
2021	Chen (61)	PCOS rats	acupuncture	CV4, RN3, CV6, SP6, EX-CA1	each day over 11 days	↓LncMEG3, PI3K/AKT/mTOR pathway, granulosa cell autophagy
2021	Dong (62)	PCOS women	EA	CV3, CV6, ST29, SP6, SP9, LI4, GV20, ST25, LR3, PC6	16 wks	↓weight, BMI, hipline, homeostatic model assessment of insulin sensitivity, ↓visfatin, HDL-C, WHR, fasting glucose, ↑resistin and IL-6
2021	Wang (63)	PCOS rats	EA	CV6, SP6, ST36	5 per wk for 2 wks	modulate the kisspeptin system
2021	Xiang (64)	PCOS women	EA	RN12, ST25, SP15, GB26, CV6, CV4, SP10, ST40, ST36, SP9	2 per wk until the day of oocyte collection	↑ IRS-1/PI3K/GLUT4 signaling pathway
2021	Wu (65)	PCOS women	acupuncture	ST36, CV4	2 per wk for 3 months	↓miR-32-3p, ↑PLA2G4A
2021	Zhao (66)	PCOS women	EA	NR	3 per wk for 12 wks	↓LH, AMH, T, ↑P450arom, E2
2022	Dong (67)	PCOS women	EA	CV3, CV6, GV20, ST29, ST25, SP6, SP9, LI4, LR3, PC6	2 per wk with a maximum of 32 treatments	↓DHEA secretion and the acne score

(Continued)

TABLE 1 Continued

Year	Author	Subject	Intervention type	Acupoints	Frequency and Duration	Mechanisms
2022	Pan (68)	PCOS women	acupuncture	RN4, EX-CA1, ST29, ST36, SP6, RN6, RN12, DU20, ST25, KI3, KI6, LR3, SP10, PC6	2 per wk for three menstrual cycles	↑pregnancy and ovulation rate
2022	Zhang (69)	PCOS rats	EA	ST29, SP6, LR3, PC6	NR	↓white adipose tissue, ↑brown adipose tissue

AMH, anti-Müllerian hormone; APN, adiponectin; AR, androgen receptor; BMI, body mass index; BSA-S, Brief Scale for Anxiety; BW, body weight; DHEA, Dehydroepiandrosterone; EA, electro-acupuncture; E2, estradiol; FAI, free androgen index; FINS, fasting serum insulin; FSH, follicle-stimulating hormone; GLUT4, Glucose transporter type 4; GnRH, gonadotropin releasing hormone; IL, Interleukin; INHB, inhibin B; ISN, insulin sensitivity index; LDL-C, LH, luteinizing hormone; MA, manual acupuncture; MADRS-S, Montgomery Åsberg Depression Rating Scale; NGF, nerve growth factor; NR, not reported; P450arom, P-450 aromatase; PCOSQ, PCOS Questionnaire; SHBG, sex hormone-binding globulin; SF-36, Swedish Short-Form 36; T, testosterone; WHR, waist-hip ratio; wk, week; wks, weeks.

gonadotropin-releasing hormone (GnRH), which may lead to an increase in the frequency and amplitude of luteinizing hormone (LH) pulses. Excessive LH release, in turn, may cause excessive production of androgens (71). Moreover, the low levels of follicle-stimulating hormone (FSH) and inadequate conversion of androgen to estradiol prevent the recruitment of dominant follicles, leading to anovulation (71–73). Insulin resistance is another important factor in the pathogenesis of PCOS (74). An abnormal insulin signaling pathway was found in the ovarian tissues of PCOS patients. This may be because prolonged hyperinsulinemia activates the mTOR/S6 kinase pathway, which enhances the serine phosphorylation of IRS-1 and eventually induces insulin resistance in the hypothalamus (75, 76). In addition, insulin levels are also positively correlated with androgen levels in PCOS patients (77). The secretion of androgens increased in PCOS patients under insulin stimulation, which enhanced the activity of cytochrome P450c17α hydroxylase and then increased androgen production. Adiponectin, an adipocyte-specific protein that regulates insulin sensitivity and glucose catabolism, have been found decreased in patients with PCOS (78, 79). Furthermore, high insulin levels in PCOS patients may also accelerate the pulse of LH secretion and stimulate the synthesis of androgens by follicular membrane cells, resulting in hyperandrogenemia and anovulation (80). The incidence of PCOS is often clustered in families, and first-degree relatives are at higher risk (81). Genes such as the CYP17 gene, androgen receptor gene, and SHBG gene have been confirmed to be involved in androgen metabolism (82–84). Recent studies have shown that insulin receptor genes (IRS1 and IRS2) are associated with the incidence of PCOS (85). Chronic negative emotions such

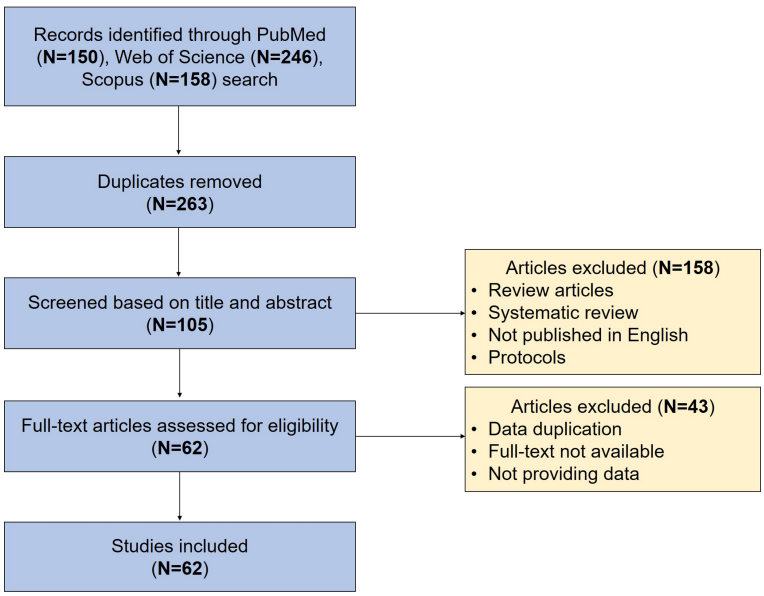

FIGURE 1
Flow chart of the study selection process.

as depression and low self-esteem could make the body in a state of stress. Such emotions can directly inhibit the hypothalamic-pituitary-adrenal axis, leading to obstacles in the HPO axis regulation mechanism and ovarian dysfunction, which induce PCOS (86).

Acupuncture-A possible treatment for PCOS

Acupuncture, a representative of traditional Chinese medicine, has been widely used for treating diseases in China for at least 2,000 years. Currently, acupuncture is increasingly accepted as a complementary therapy for many disorders worldwide (87). The effectiveness of acupuncture for diseases such as chronic prostatitis, chronic musculoskeletal pain, and chronic severe functional constipation, etc. have been confirmed by many high-quality randomized controlled trials (88–90). Electroacupuncture (EA) is a new form of acupuncture treatment in which acupuncture is combined with electrical stimulation. Multiple clinical trials have shown that manual acupuncture and EA are both effective for treating PCOS (48, 57, 64, 65, 68). The effects of acupuncture for PCOS involved improvement in ovulation rate, pregnancy rate, insulin resistance, negative emotion, sexual hormone disturbance, and lipid metabolism dysfunction (Figure 2) (91).

Acupuncture therapy has been used as a complementary and alternative treatment for oligo/anovulatory women with PCOS (91). Studies have revealed that acupuncture might reduce cortisol concentrations and regulate central and peripheral β-endorphin production and secretion (92). Considering that acupuncture has a potential effect on β-endorphin, which can impact GnRH secretion and levels, it is postulated that acupuncture may play an important role in improving ovulation induction and fertility. In 2016, a systematic review including five randomized controlled trials (RCTs) with 413 women reported insufficient evidence to support the use of acupuncture for the treatment of ovulation disorders in women with PCOS (93). Further in 2019, the updated review added three other new RCTs with a total of 1546 women covering the uncertainty of the effect of acupuncture on the live birth rate, multiple pregnancy rate and ovulation rate compared to sham acupuncture (91). However, acupuncture may ameliorate the restoration of regular menstrual periods. In recent years, accumulating scientific studies have investigated the acupuncture meridians and the neuroendocrinological aspects of the meridians, considering that acupuncture may have a role in normalizing the HPO axis, which in turn influences the menstruation cycle pattern (91, 94). In addition, the evolving omics techniques and emerging analysis tools of biological information may facilitate acupuncture research and help to reveal the mechanisms of acupuncture action on PCOS.

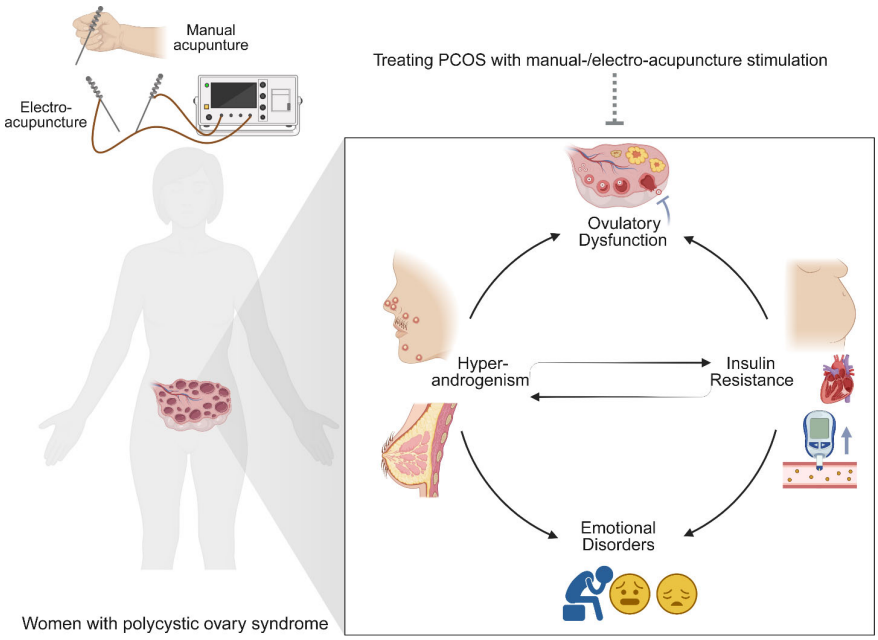

FIGURE 2
Acupuncture treatment attenuates the major clinical manifestations of polycystic ovary syndrome (PCOS). PCOS is featured by hyperandrogenism (hirsutism, acne, alopecia, etc.), ovulatory dysfunction, polycystic ovaries, obesity, insulin resistance, metabolic dysfunction, and emotional disorders. Manual-/electro-acupuncture improves the PCOS-related symptoms through different biological mechanisms.

Evidence-based study of acupuncture for PCOS

A lot of systematic reviews and meta-analyses have been performed to provide evidence-based information in this field. A Cochrane systematic review have been conducted by Lim et al. in 2011, and updated in 2016 and 2019 (91, 93, 95). These studies assessed the effectiveness of acupuncture treatment for oligo/anovulatory women with PCOS for both fertility and symptom control. They concluded that the efficacy of acupuncture on pregnancy outcomes in PCOS patients was uncertain due to the limited number of RCTs and the low quality of evidence (91). A systematic review in 2017 revealed that acupuncture is likely to improve ovulation rate and menstruation rate, but the level of evidence was low (96). In 2020, Wu et al. believed that there was no sufficient evidence supporting the effectiveness of acupuncture to promote live birth, pregnancy, and ovulation in PCOS patients (22). Interestingly, this systematic review suggested that acupuncture could promote the recovery of menstrual cycles as well as downregulate the levels of LH and testosterone in PCOS patients. A recent systematic review showed that acupuncture combined with metformin improved pregnancy rate, ovulation rate, and insulin resistance in PCOS patients compared to using metformin alone (97). Another study found that acupuncture combined with moxibustion improved pregnancy, ovulation, and miscarriage rates, as well as the levels of some sex hormones and metabolic indicators (98). The effectiveness of acupuncture on PCOS patients undergoing *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) was also evaluated by a systematic review (99). The results showed that acupuncture may increase the clinical pregnancy rate and ongoing pregnancy rate and decrease the risk of ovarian hyperstimulation syndrome in patients with PCOS undergoing IVF or ICSI. A systematic review by Zheng et al. found that acupuncture was relatively effective in improving glucose metabolism and insulin sensitivity in patients with PCOS (100). Furthermore, a systematic review assessed the efficacy of acupuncture on animal models with PCOS (23). They found that a definite conclusion was difficult to draw because the methodology was weak and heterogeneity was high. The methodological and reporting quality of systematic reviews on acupuncture treatment for patients with PCOS was also evaluated by a systematic review. This study demonstrated poor methodological and reporting quality of systematic reviews assessing acupuncture in patients with PCOS (101). Information of these systematic reviews and meta-analyses is shown in Table 2.

Potential mechanisms of acupuncture affecting pcos-related symptoms

Ovulatory dysfunction

Ovulatory dysfunction is one of the most sovereign characteristics of PCOS (102). Oocyte quality has been proved important for reproductive potential in women with PCOS (103). EA was proved to be effective in improving oocyte quality and embryonic development potential in infertile patients with PCOS (64). Upregulation of the IRS-1/PI3K/GLUT4 signaling pathway appears to be involved in the effect of EA. A similar study demonstrated that EA improved abnormal follicular development in PCOS patients by inhibiting the overexpression of AMH and increasing the expression of P450arom (66). The protective effect of EA on follicle growth in patients with PCOS was further confirmed by another clinical study (53, 60). In addition, acupuncture at an early stage of oocyte recruitment improved embryo quality in PCOS patients undergoing *in vitro* fertilization (39). A recent animal study revealed that acupuncture improved ovulation disorder by downregulating LncMEG3 expression, inhibiting the PI3K/AKT/mTOR pathway, and reducing granulosa cell autophagy (61). EA was also reported to improve follicular arrest in PCOS rats by decreasing the overexpression of AMH to normalize FSH and AMH imbalance in granulosa cells (51). Follicular maturation may be affected by endogenous ovarian angiogenesis, which may be another mechanism underlying EA in the treatment of PCOS (49). Interestingly, another animal study demonstrated that EA upregulates the numbers of preovulatory follicles and corpora lutea by increasing innervation of blood vessels near the hilum (58).

PCOS is a multi-symptom disorder linked with a range of reproductive hormonal disturbances (104). A recent clinical study showed that acupuncture improved the pregnancy rate and ovulation rate in infertile women with PCOS, and the effect may be related to the modulation of acupuncture on sex hormones disturbance (68). In another study, acupuncture induced a higher ovulation frequency in lean/overweight PCOS women (41). Meanwhile, acupuncture also reduced the serum levels of ovarian and adrenal sex steroid. In an animal study, EA improved the disturbed estrous cycles and upregulated the number of corpora lutea and area of the ovary in a pubertal rat model of PCOS (63). The increased LH and decreased estradiol and GnRH were all normalized by EA in this study. Furthermore, EA attenuated the upregulation of kisspeptin protein level in the arcuate nucleus, which might explain the efficacy of EA (63).

Hyperandrogenism

Evidence suggests that hyperandrogenism is an important clinical feature and mechanism of PCOS (105). Many clinical studies have shown that acupuncture can lower the serum level of testosterone in PCOS women (33, 41, 68, 106). In a study, the circulating and adipose tissue androgen levels in PCOS patients were decreased by EA (44). The effect of EA may be associated with decreased level of hemoglobin A1C. Another study showed that EA improved hyperandrogenism in PCOS patients, and regulation of AMH and P450arom may be involved in the potential mechanism of EA (66). In an animal study, acupuncture inhibited excessive androgen secretion in a rat model of PCOS. The efficacy of acupuncture may be related to the inhibitory effect on overexpression of androgen receptor and connexin 43 (59). Another animal study revealed that EA improved the local ovarian hyperandrogenic environment, probably through increasing P450arom level and decreasing P450C17a level (42). In addition, research showed that EA decreased the overexpression of AMH and regulated FSH and AMH imbalance in granulosa cells, improving hyperandrogenism in a rat model of PCOS (51). Low-frequency EA also decreased serum testosterone in rats with PCOS, and the efficacy may be mediated by central opioid receptors such as *Oprk1* and *Oprm1* in the hypothalamic arcuate nucleus (35).

Insulin resistance and obesity

There is general agreement that PCOS patients are insulin resistant, especially obese PCOS patients (107). Insulin resistance and related hyperinsulinemia may induce both the endocrine and reproductive traits of PCOS (108). The efficiency of acupuncture on insulin resistance in PCOS patients has been confirmed by many clinical studies (43, 57, 64). A recent study demonstrated that EA improved the insulin resistance score compared with the control group in PCOS patients, and the protective effect of EA might be through an upregulation of the IRS-1/PI3K/GLUT4 signaling pathway (64). Abdominal acupuncture also improved insulin resistance in patients with obesity-type PCOS, which may be related to the efficacy of acupuncture treatment on body–mass index, waist-to-hip ratio (WHR), and lipid metabolism dysfunctions (43). Consistent

TABLE 2 Characteristics of systematic reviews and meta-analyses assessing acupuncture for PCOS.

Year	Author	Country	Subject	Comparison	Indicator
2011	Lim (95)	Australia	RCTs	NA	NA
2016	Lim (93)	Australia	RCTs	acupuncture VS sham acupuncture, electroacupuncture VS physical exercise, electroacupuncture VS no intervention, acupuncture VS relaxation, acupuncture VS clomiphene	live birth rate, ovulation rate, clinical pregnancy rate, restoration of menstruation, multiple pregnancy, miscarriage, and adverse events
2017	Jo (99)	South Korea	RCTs	acupuncture VS sham acupuncture, acupuncture VS no treatment, acupuncture VS other treatments	clinical pregnancy rate, live birth rate, ongoing pregnancy rate, incidence of OHSS, adverse events
2017	Jo (96)	South Korea	RCTs	acupuncture VS sham acupuncture, acupuncture VS medication, acupuncture VS no treatment	ovulation rate, menstruation rate, LH, LH/FSH ratio, testosterone, fasting insulin, and pregnancy rate
2018	Luo (101)	China	systematic reviews	NA	methodological and reporting quality
2019	Lim (91)	Australia	RCTs	acupuncture VS sham acupuncture, acupuncture VS relaxation, acupuncture VS clomiphene; low-frequency electroacupuncture VS physical exercise or no intervention, acupuncture VS Diane-35	live birth rate, multiple pregnancy rate, ovulation rate, clinical pregnancy rate, restored regular menstruation period, miscarriage rate, and adverse events
2020	Wu (22)	China	RCTs	acupuncture VS sham acupuncture, acupuncture VS clomiphene citrate, acupuncture VS letrozole, acupuncture VS metformin, acupuncture VS Daine-35, acupuncture VS Chinese medicine, acupuncture VS treatment	live birth rate, pregnancy, ovulation, recovery of menstrual period and hormone levels
2021	Zheng (100)	China	RCTs	acupuncture VS no acupuncture	body mass index, waist-to-hip ratio, fasting plasma glucose, insulin resistance, triglycerides
2021	Li (23)	China	Animal studies	acupuncture plus PCOS animals VS PCOS animals	insulin resistance, testosterone, LH, LH/FSH ratio, fasting blood sample, fasting insulin, and body weight
2022	Li (98)	China	RCTs	acupuncture combined with moxibustion plus basic treatment VS basic treatment	pregnancy, ovulation, miscarriage, sex hormones, and metabolic disorders
2022	Chen (97)	China	RCTs	acupuncture plus metformin VS metformin	pregnancy rate, ovulation rate, insulin resistance

FSH, follicular stimulating hormone; LH, luteinizing hormone; NA, not available; OHSS, ovarian hyperstimulation syndrome; RCTs, randomized controlled trials; VS, versus.

with this study, EA was found to be effective in improving insulin resistance, as well as decreasing WHR and the levels of total cholesterol and low-density lipoprotein (LDL) cholesterol (54, 62). EA was also reported to attenuate insulin resistance by inactivating the mTOR/4E-BP1 signaling pathway in a rat model of PCOS (12). Simultaneously, EA ameliorated mitochondrial dysfunction and endoplasmic reticulum stress by enhancing autophagy. EA improved insulin sensitivity in PCOS models, and this efficacy may be associated with increased plasma insulin-like growth factor-I, increased expression of leptin and interleukin-6 (IL-6) and decreased expression of uncoupling protein 2 in visceral adipose tissue (28). Sterol regulatory element-binding protein-1 (SREBP-1) is an important transcription factor that regulates the expression of genes involved in lipogenesis and glycolysis (109). A study found that EA induced the activation of the AMPK pathway to suppress SREBP-1 expression and finally inhibited insulin resistance, mitochondrial dysfunction and oxidative stress in a PCOS rat model (56). A study investigated whether EA and manual acupuncture have different effects on insulin sensitivity in PCOS rats. They found that EA improved insulin sensitivity in soleus muscle and mesenteric adipose tissue, while manual had a greater effect on glucose tolerance (Figure 3) (38).

Glucose and lipid metabolism dysfunctions are found in most obese PCOS patients (110). In a clinical study, acupuncture treatment decreased miR-32-3p levels and increased the expression of PLA2G4A, leading to improvement in PCOS patients with diabetes (65). Gene expression and methylation were analyzed to reveal the mechanism of EA on glucose metabolism dysfunctions in PCOS patients. The results showed that EA regulated gene expression (such as *MSX1* and *SRNX1*) in skeletal muscle in insulin-resistant overweight/obese PCOS women (55). Interestingly, EA increased LDL cholesterol without affecting insulin sensitivity or adipose tissue function in a rat model of PCOS, which might suggest that a balance of sex hormones is necessary to restore metabolic function (47). The same research team also found that EA improved insulin sensitivity and decreased total high-density lipoprotein and LDL cholesterol in the same PCOS models (32). The protein expression of GLUT4 was found to be increased in skeletal muscle, which may be involved in the mechanism of EA on insulin sensitivity. In addition, the gut microbiota is known to be causal in the development of obesity/insulin resistance (111). A recent study showed that EA intervention decreased body weight, probably through regulating gut microbiota in PCOS rats (112). This study also demonstrated that EA can normalize visceral and subcutaneous fat content, brown adipose tissue weight, and glucose tolerance in the PCOS model.

Emotional disorders

An increased risk of depression and anxiety has been found in patients with PCOS (113, 114). It has been reported that

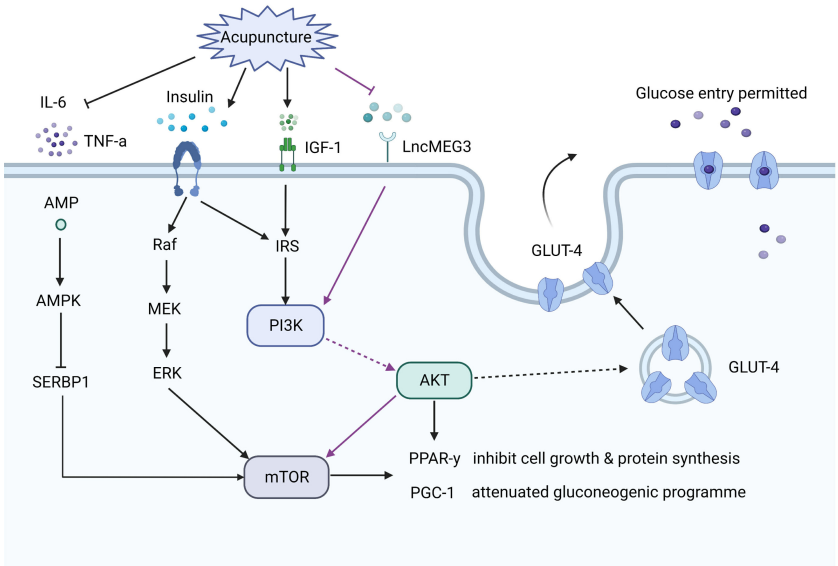

FIGURE 3
The effect of acupuncture on the insulin pathways. Acupuncture increases glucose transporter 4 (GLUT4) expression *via* upregulating the insulin receptor substrates-1/PI3K/GLUT4 pathway, or inhibiting the PI3K/AKT/mTOR pathway, or activating the adenosine monophosphate activated protein kinase (AMPK) pathway. Acupuncture also decreases the levels of linterleukin-6 and tumor necrosis factor-α, which are involved in insulin resistance.

acupuncture can improve depression and anxiety scores in women with PCOS (37). Interestingly, a recent study revealed that EA appears to improve symptoms of anxiety and depression and regulate the serum levels of norepinephrine (NE) and serotonin (5-HT) in unmarried PCOS patients (52). Anxiety and depression are associated with an autonomic nervous system imbalance (115). Hyperactivation of the sympathetic nervous system is involved in many psychological disorders, such as anxiety and depression (116). Chronic sympathetic overactivity also plays a critical role in the pathogenesis of PCOS (117). A previous clinical study showed that low-frequency EA decreased high muscle sympathetic nerve activity in PCOS patients (31). In addition, another study showed that the efficiency of EA was associated with activation of the sympathetic nervous system (48). In a PCOS rat model, low-frequency EA and physical exercise restored the ovarian expression of markers of sympathetic nervous system activity (30). EA intervention inhibited hyperactivity of the sympathetic nervous system in PCOS rats, which may be related to the inhibitory effects of EA on nerve growth factor (NGF) concentrations in ovaries (24). In addition, the p75 neurotrophin receptor ($p75^{NTR}$) plays a vital role in patterning the sympathetic nervous system during development (118). EA prevented the increase in $p75^{NTR}$ expression, probably by normalizing the sympathetic ovarian response to NGF action (27). Interestingly, the effect of EA on NGF abundance was only found in the ovaries of PCOS rats, but not in the brain (Figure 4) (26).

Acupoint selection

The conception of the acupoint is introduced in Traditional Chinese Medicine (TCM) as the matter that acupuncture acts on the body physiology and relieves symptoms. Increasing evidence has suggested that acupoints are mostly collagen fiber-rich regions, such as intermuscular connective tissue, peri-neurovascular connective tissue, and organ portal and peri-neural connective tissue (26). Moreover, acupoints on different meridians have different effects.

Acupoints SP6, ST29, CV6, LI4, CV3, ST36, SP9, and CV4 have been frequently used in these scientific studies (Figures 5, 6). In clinical applications, the most widely used acupoint was SP6, as it had been selected in sixteen researches. According to the theory

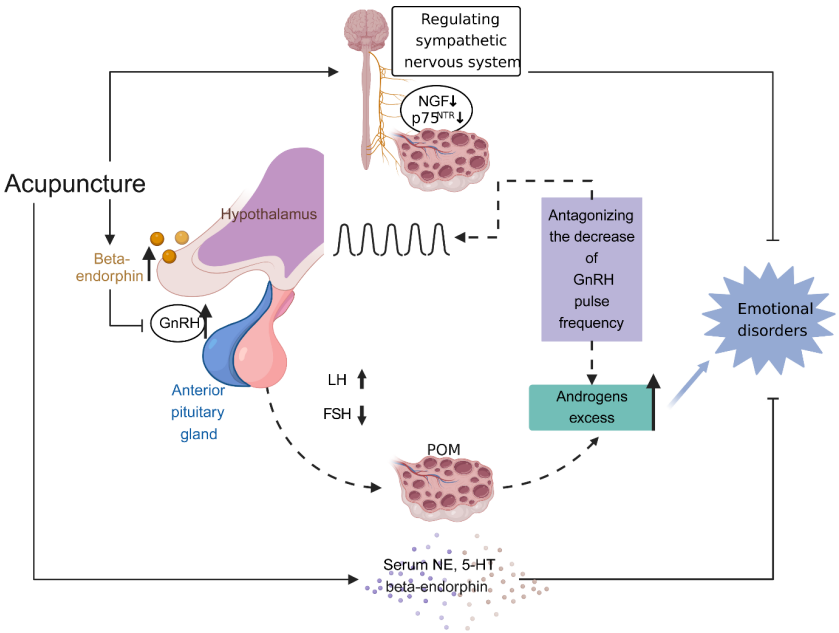

FIGURE 4
The effect of acupuncture on restoration of the hypothalamic-pituitary-ovarian (HPO) axis and amelioration of emotional disorders. Increased activity and secretion of gonadotropin-releasing hormone (GnRH) with persistently high pulse frequency causes elevated levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), contributing to the polycystic ovarian pathology (including impaired follicular development and excess androgen production). Elevated ovarian androgen antagonizes the ability of the progesterone to descend GnRH pulse frequency, leading to a proposed vicious cycle in the HPO axis in polycystic ovary syndrome (PCOS). Meanwhile, androgen excess has an effect on emotion disorders of women with PCOS. Acupuncture modulates central and peripheral β-endorphin production and secretion, influencing the release of GnRH, then normalising the ratio of LH and FSH, and eventually normalising the HPO axis. Moreover, acupuncture ameliorates emotion disorders of women with PCOS through regulating serum norepinephrine, 5-hydroxytryptamine and β-endorphin levels, balancing autonomic nervous system, and inhibiting the concentrations of nerve growth factor and the expression of p75 neurotrophin receptor in ovaries.

of TCM, SP6 (Sanyinjiao) is mainly characterized by the ability to nourish organs, activate blood, soothe the liver and regulate Qi, which can contribute to addressing gynecological problems. The second most involved acupoint was ST29 (Guilai), which was mentioned in fifteen studies. It promotes circulation to remove stasis, regulate menstruation and relieve pain. In addition, CV6, LI4, CV3, ST36, SP9, and CV4 were used in at least eight studies for treating women with PCOS.

Compared with clinical trials, fewer acupoints were stimulated in animal researches. EA was often applied for androgen excess-induced PCOS rat/mouse models. Among the acupoints selected for treatment, SP6 acupoint was the most commonly chosen, either alone or in association with other acupoints, which was consistent with the findings of clinical studies. Additionally, ST29 was more frequently used to mitigate hyperandrogenism and ovulatory dysfunction and to modulate the menstrual cycle. CV4 and ST36 were also frequently used to treat animals with PCOS. Acupoint ST36, in particular, has the ability to tonify Qi and circulation. It was demonstrated that acupuncture at ST36 could lower the levels of IL-6 and tumor necrosis factor (TNF-α) in PCOS animal serum, which might be associated with the ability of acupuncture to inhibit inflammation and oxidative stress (119).

Discussion

PCOS is a common but heterogeneous disease with symptoms that vary from age to age in patients, typically featuring chronic oligo-anovulation, hyperandrogenism, and/or metabolic disturbance (120). The routine administration after recommending lifestyle modification and some adscititious tips is symptomatic therapy with various agents (121, 122). Patients with PCOS often suffer from a high symptom burden but low tolerance and compliance to pharmacotherapy. Additional regimens need to be explored. With the building availability of acupuncture all over the world, patients with PCOS are increasingly seeking and accepting acupuncture to maintain reproductive health (91). Our previous research manifested that acupuncture can effectively relieve anxiety and depression in patients with PCOS, and its mechanism may be related to the regulation of the levels of serum β-endorphin and androgen (123). In addition, our multinational study protocol on acupuncture or metformin to improve insulin resistance in women with PCOS has been published (124). In recent years, there have been an increasing number of systematic reviews and/or meta-analyses on the effect of acupuncture on PCOS in both patients and animal models (22, 23, 96, 100). This study reviewed the feasibility and efficacy of acupuncture for managing PCOS and summarized the potential mechanisms of acupuncture on treating PCOS. To the best of our knowledge, this is the first detailed review to address acupuncture-specific action on PCOS-related symptoms, including ovulatory dysfunction, hyperandrogenism, insulin resistance, obesity, and emotional disorders.

The disordered hypothalamic–pituitary–ovarian/adrenal axis is one of the most important pathological and physiological states of PCOS (125). As the main metabolic feature of PCOS, insulin resistance is considered to be a crucial pathophysiological basis for the pathogenesis of

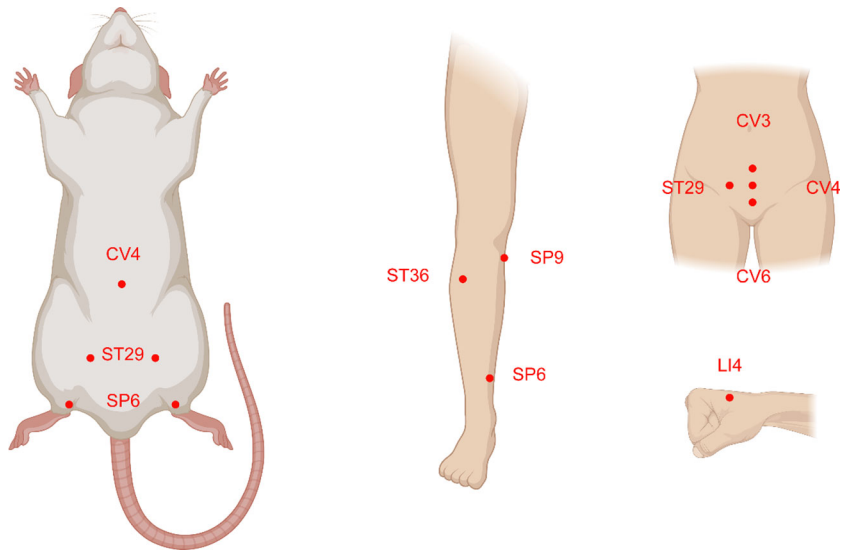

FIGURE 5
The acupoints common selected and their location distributions in animals and humans with polycystic ovary syndrome (PCOS). The majority of the acupoints are located on the abdomen, upper and lower extremities.

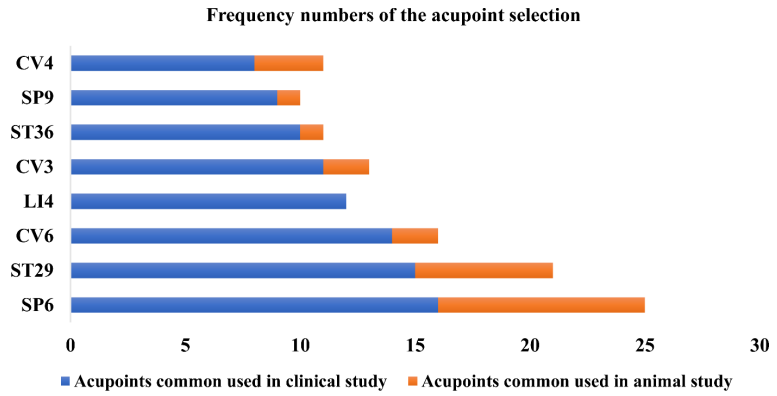

FIGURE 6
The use frequency of acupoints. SP6 (Sanyinjiao) and ST29 (Guilai) are the most frequently stimulated two acupoints both in animals and humans with polycystic ovary syndrome.

PCOS(107). Inhibiting phosphoinositide-3 kinase and phosphorylation of IRS-1 impairs insulin signaling by affecting GLUT-4 expression and glucose uptake (126, 127). Another vital pro-inflammatory agent relevant to the pathogenesis of PCOS is adipose tissue (128). It has been proven that adipose tissue-resident macrophages lead to the release of TNF-α and IL-6, which are implicated in the induction of insulin resistance (129). Hyperandrogenism causes the aberration of adipose tissue functions in PCOS. Insulin resistance, hyperandrogenism, chronic low-grade inflammation, and adipose tissue hypertrophy and dysfunction may affect a vicious cycle in the pathophysiology of PCOS (130, 131). Evidence has shown that acupuncture elevates the level of β-endorphin not only in the central endocrine system but also in the peripheral circulation (94), which is associated with both direct and indirect tonic inhibitions of GnRH and subsequent LH release (132). Aberrant sympathetic neurogenic regulation of the ovary is involved in the pathogenesis of PCOS (23), and acupuncture can also inhibit the overexpression of NGF to decrease sympathetic activity, resulting in a restoration to the normal level of the ovarian steroid response to gonadotropins (25). Moreover, acupuncture regulates the phosphorylation of insulin substrates and receptors and inhibits the abnormal expression of signaling pathways, thereby improving metabolic dysfunction such as insulin resistance (32, 56, 133). Acupuncture may also ameliorate cholesterol metabolism, affecting lipid metabolism enzyme activity, inhibiting the synthesis of fatty acids, and then promoting fat decomposition and energy metabolism (100, 134).

Many review articles concerning PCOS and acupuncture have been published during the last 10 years (23, 132, 135–137). However, our present paper is different from those published papers. Firstly, this review summarized current available information from both clinical studies and animal studies. Secondly, the mechanisms of acupuncture on PCOS-related main symptoms (ovulatory dysfunction, hyperandrogenism, insulin resistance, obesity, and negative emotion) were all overviewed. Thirdly, the acupoints that commonly used in PCOS patients and animals were also overviewed in this study. To our knowledge, this is the most comprehensive review that summarized current progress on acupuncture treatment for PCOS.

Some points should be noted. At present, most studies on the effect of acupuncture on PCOS are statistical comparisons, with insufficient depth and breadth of its mechanism of action. System biology and omics techniques have become a new trend, and transcriptomics technology will better analyze the specific expression factors and biological mechanisms of acupuncture treatment. Additionally, there is considerable heterogeneity in terms of animal models (dihydrotestosterone, dehydroepiandrosterone, and testosterone propionate), research intervention (acupoint selection, frequency, electrical current range, pulse width and length of stimulation) and major endpoints (live birth, multiple pregnancy rate, ovulation rate, clinical pregnancy rate, and miscarriage rate), lessening the generalizability of the results from those studies. Moreover, the majority of these studies were conducted in various phenotypes of patients and animal models with PCOS. Although the pathophysiology of the symptoms is similar between several phenotypes and models, there could be differences, making data from one not entirely applicable to the other. As a recent review reported, clinical practice and health policy underuse beneficial acupuncture therapies.

Comments and future perspectives

At present, a large number of clinical studies have confirmed that acupuncture could improve many symptoms in patients

with PCOS. These symptoms include chronic and continuous anovulation, hyperandrogenemia, insulin resistance, negative emotion, glucose and lipid metabolism dysfunction, etc. The effect of acupuncture may be induced by stimulating muscle conduction and chemical signals to induce the central release of key factors through sympathetic nerve conduction and then regulating the female reproductive axis. The selection of acupoints and EA frequency may also impact the therapeutic effect, which needs to be verified by more studies.

With recent advances in technology, the effect of acupuncture could be observed from a more microscopic point of view. Whether the clinical effect of acupuncture is associated with the traditional Chinese meridian theory is still unclear. This needs to be verified and discussed in the next few decades. Clarifying the mechanism of acupuncture in the treatment of PCOS will help to make acupuncture therapy accepted by more people.

Author contributions

YY and C-CZ contributed to writing the original draft, literature search, and data collection; H-QH contributed to figure presentation and manuscript editing; IF contributed to writing, corrections, and editing; H-LZ contributed to conceiving, designing, editing, and supervising. All authors contributed to this article and approved this 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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Therapeutic effect and safety of curcumin in women with PCOS: A systematic review and meta-analysis

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Background: Polycystic ovary syndrome (PCOS) is a multi-factorial heterogeneous syndrome that has both adverse reproductive and metabolic implications for affected women and its management is a challenging clinical problem. Curcumin, as a phenolic compound with potent anti-inflammatory and antioxidant properties exerting positive effects on the lipid profile and insulin resistance, appears to be a valuable treatment regimen for patients with PCOS.

Objective: This study aimed to evaluate the efficacy and safety of curcumin in the treatment of PCOS.

Methods: Chinese databases (Chinese National Knowledge Infrastructure, China Biology Medicine Databases, VIP database, Wanfang Database, and Chinese Clinical Trial Registry) and English databases (PubMed, Web of Science, Embase, Cochrane Library, Scopus and Clinical trials) were thoroughly investigated through screening randomized controlled trials on curcumin in PCOS published from the date of inception to May 2022. Standardized data search and abstraction were conducted following the preferred reporting items for systematic reviews and meta-analysis (PRISMA) statement. Quantitative and qualitative analyses were performed. Heterogeneity was assessed using I^2 statistics.

Results: A total of 447 patients from seven randomized controlled trials were included in the meta-analysis. Results showed that the ingestion of curcumin decreased body mass index (WMD -0.267, 95% CI -0.450 to -0.084, P = 0.004, I^2 = 0.0%), fasting plasma glucose (WMD -3.618, 95% CI -5.165 to -2.071, P < 0.001, I^2 = 20.4%), insulin (WMD -1.834, 95% CI -2.701 to -0.968, P < 0.001, I^2 = 8.4%), homeostatic model assessment for insulin resistance (WMD -0.565, 95% CI -0.779 to -0.351, P < 0.001, I^2 = 0.0%), total cholesterol (WMD -15.591, 95% CI -27.908 to -3.273, P = 0.013, I^2 = 68.9%), C-reactive protein (WMD -0.785,

95% CI -1.553 to -0.017, P = 0.045, I^2 = 23.9%), and increased the quantitative insulin sensitivity check index (WMD 0.011, 95% CI 0.005 to 0.017, P = 0.001, I^2 = 39.6%). As for safety, the treatment group did not cause significant adverse reactions than that in the control group.

Conclusion: In light of presented findings, curcumin has beneficial effects on serum markers of inflammation, weight loss and glucose and lipid metabolism in patients with PCOS. The incidence of adverse reactions does not increase with the application of curcumin. However, a larger, more definitive study is needed to further investigate these results.

Systematic review registration: https://www.crd.york.ac.uk/prospero/, identifier CRD42022332394.

KEYWORDS

curcumin, polycystic ovary syndrome, meta-analysis, systematic review, complementary therapy

Background

Polycystic ovary syndrome (PCOS), the most common endocrine disorder, is characterized by ovulatory dysfunction, hyperandrogenism, and polycystic ovaries. At present, the incidence of PCOS is from 6% to 25% in women of reproductive age worldwide (1, 2), of which the prevalence in China is 7.8%, and it has increased by 65% in the past 10 years (3). In addition, PCOS has been linked to a number of higher risks of metabolic disorders, including insulin resistance (IR), glucose intolerance, type 2 diabetes, obesity, dyslipidemia, and cardiovascular diseases (4). Among them, cardio metabolic diseases such as myocardial infarction and stroke, are major causes of death in women (5).

As a global epidemic disease, obesity is a 21st-century major public health challenge (6). Despite adiposity is not a defining criterion for PCOS, rates of obesity are estimated to be 2.8 times higher in PCOS than in the general population, with a prevalence of 50-80% (7). Excess adiposity, particularly around the abdomen, causes insulin resistance, a critical etiological component to PCOS (8, 9). Insulin resistance and consequent hyperinsulinemia lead to hyperandrogenism by acting on the adrenal gland, ovaries and liver to increase androgen production and decrease sex hormone binding globulin (SHBG) (10). In addition, androgen excess has been shown to induce visceral fat accumulation and possibly adipose tissue dysfunction (11). Meanwhile, some studies have found hyperandrogenism aggravates the symptoms of insulin resistance, leading to a vicious cycle that promotes PCOS development.

Over the recent years, numerous preclinical and clinical studies have demonstrated that PCOS is associated with a chronic inflammatory state, inflammatory cytokines in PCOS patients can induce adipocyte proliferation by modulation of signal transducer and activator of transcription 3 (STAT3) signaling (12, 13). Excessive inflammatory factors also produce redundant reactive oxygen species (ROS) and disrupt internal ROS homeostasis, thereafter inhibit insulin signaling and insulin-mediated glucose transport, aggravating insulin resistance (14). Decreasing plasma insulin level and ameliorating insulin resistance not only leads to an improvement in reproductive abnormalities, but also probably reduces the future risk of developing diabetes and cardiovascular disease in PCOS women (15). Besides lifestyle intervention, metformin, a biguanide, is a commonly prescribed agent for the management of PCOS (16). It works by inhibiting hepatic glucose production, reducing intestinal glucose absorption and improving glucose metabolism (17). However, it has been observed that 20-30% of people receiving metformin therapy develop gastrointestinal side effects, with approximately 5% being unable to tolerate metformin at all (18). Fortunately, complementary and phytomedicines medicines have shown satisfactory results to cure PCOS.

Curcumin (diferuloylmethane) is a natural polyphenol extracted from the roots of Curcuma longa (Zingiberaceae). For many years, as an Indian spice, it has been widely used as food additives, food pigments and seasonings (19). In view of its anti-inflammatory, hypolipidemic and anti-anxiety activities, it is also used to treat a variety of chronic diseases, such as diabetes, depression and so on (20). With the

deepening of research, a lot of evidence shows that curcumin is a natural regulator and protector in the process of female reproduction (21). Continuous (up to 4 months) and high-dose (up to 12 grams in human body) use of curcumin is also quite safe (22, 23). Curcumin has obvious protective effect on ovarian tissue. In fact, this compound seems to be involved in inhibiting the expression of vascular endothelial growth factor (VEGF), a proangiogenic factor closely related to the formation of PCOS, thereby inhibiting ovarian angiogenesis, preventing ovarian fibrosis and promoting matrix degradation (24). Nanocurcumin can significantly improve oxidative markers, glucose index and tumor necrosis factor α (TNF-α) level, restore phosphoinositol 3 kinase (PI3k)/threonine kinase (Akt)/mammalian target of rapamycin (mTOR) level, and then reduce insulin resistance and maintain the integrity of islet function (25).

Many clinical trials have shown that curcumin supplementation has a beneficial effect on improving insulin levels (26, 27). The latest clinical trial on the potential effectiveness of curcumin on PCOS also unanimously showed that Curcuma longa (CL) can increase insulin sensitivity in patients with PCOS (28). Although these findings are not supported by other studies (29). And most of the existing systematic reviews have observed the efficacy of curcumin and paid more attention to the effect of curcumin on blood glucose control and blood lipid level of PCOS, but there is a lack of the latest systematic review to evaluate safety of curcumin as an intervention group. Given the growing interest in alternative and complementary therapies and the global burden of PCOS, we attempt to provide an updated summary of the efficacy and safety on PCOS.

Materials and methods

This systematic review was conducted following the Preferred Reporting Items for Systematic Review and Meta-analysis (PRISMA) statement (30) and the study protocol was registered on PROSPERO (CRD42022332394).

Search strategy

Eligible literature published up to May 2022 was identified through a search in PubMed, Embase, Cochrane Library, Web of Science, Scopus, Clinical Trials, Chinese Clinical Trial Registry, Chinese Biomedical Literature Database (CBM), Chinese National knowledge Infrastructure (CNKI), VIP database, and Wanfang Database, and an additional search of grey literature and missed references to help minimise publication bias. The search strategy consisted of medical subject headings (MeSH) as well as free words and was slightly adjusted for the syntax appropriate for the different databases without restriction to race, ethnicity, or language. Details of the search strategies are presented in the Supplementary Appendix 1.

Eligibility criteria

Studies were considered eligible if they met the following criteria: 1) parallel-assignment randomized controlled trials (RCTs) of evaluation of the effects of curcumin on PCOS; 2) all patients, at any age, had PCOS as classified by the revised European Society for Human Reproduction and Embryology/American Society for Reproductive Medicine (ESHRE/ASRM) diagnosis, which were based on the Rotterdam criteria; 3) the interventions included curcumin/Curcuma longa or curcumin/Curcuma longa combined with medication (unlimited dosage form, dose, or duration); 4) the control group should be placebo or medication; and 5) the trial gives enough information to conduct the effect estimates for meta-analysis. Exclusion criteria were as follows: 1) editorials, reviews, book chapter, letter, meta-analyses, observational study, animal experiments and so on; 2) women who had other pathologies such as congenital adrenal hyperplasia, Cushing's syndrome, thyroid hormone abnormalities, hyperprolactinemia, ovarian/adrenal tumors or any severe medical problem or any neurological or psychiatric history.

Two investigators independently performed the eligibility assessment on the basis of inclusion, and any disagreement was resolved by discussion. After deletion of duplicates, they screened all titles and abstracts for primary screening. Subsequently, the full texts of remaining articles were scrutinized to determine eligible studies.

Data extraction

Two researchers independently scrutinized each eligible article, extracted data and cross-checked the results to ensure the data accuracy. Any discrepancy was resolved through discussion to reach consensus. The following parameters were collected from each study: basic information of the articles (first author, publication year, country), participants (race, mean age, and sample size), curcumin characteristics (dose, frequency, treatment duration and route of administration), comparison methods, every outcome parameter and adverse effects. For studies with missing or ambiguous data, if possible, we will attempt to contact the first or corresponding author *via* telephone or email for clarification or addition to ensure the integrity of the data.

Risk-of-bias assessment

Two authors used the Cochrane risk of bias tool to assess methodological quality of RCTs, which included the following seven specified domains: random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective reporting, and other bias. Each reviewer appraised bias according to the specific content within each item, designating a low, high, or unclear risk of bias by answering yes, no or unclear. Disagreements between the two reviewers were resolved through discussion or by consulting a third author until there was 100% agreement.

Statistical analyses

All statistical analyses were conducted with Stata software, version 14.0 (StataCorp) in accordance to the guidelines described in the Cochrane Handbook for systematic reviews of interventions. For dichotomous variables, the odds ratio (OR) with corresponding 95% confidence intervals (CIs) was calculated to summarize the difference between the groups. For continuous data, the results were presented as weighted mean difference (WMD) together with 95% CI of changes before and after the therapy in the curcumin group with those in the control group. Since some studies used different measures for the same outcome (e.g., AST and ALT), we calculated the standardized mean difference (SMD) (31). Heterogeneity among the included studies was estimated using Q statistic and the I2 statistic, results were deemed as low heterogeneity (I2 < 25%), medium heterogeneity (I2 = 25%-50%), or high heterogeneity (I2 > 50%) (32). Owing to the clinical heterogeneity inherent in our data such as ethnic differences, different use of curcumin preparations as well as duration of treatment, and so forth, random-effects models were performed for calculating pooled effect measures. We also conducted a sensitivity analysis to test the robustness of the findings.

Results

Study selection

Three hundred and eleven potentially relevant papers were imported into NoteExpress after searches across databases. After removal of duplicates across databases and reviewing of titles and abstracts, 158 papers were deemed to be of potential interest for

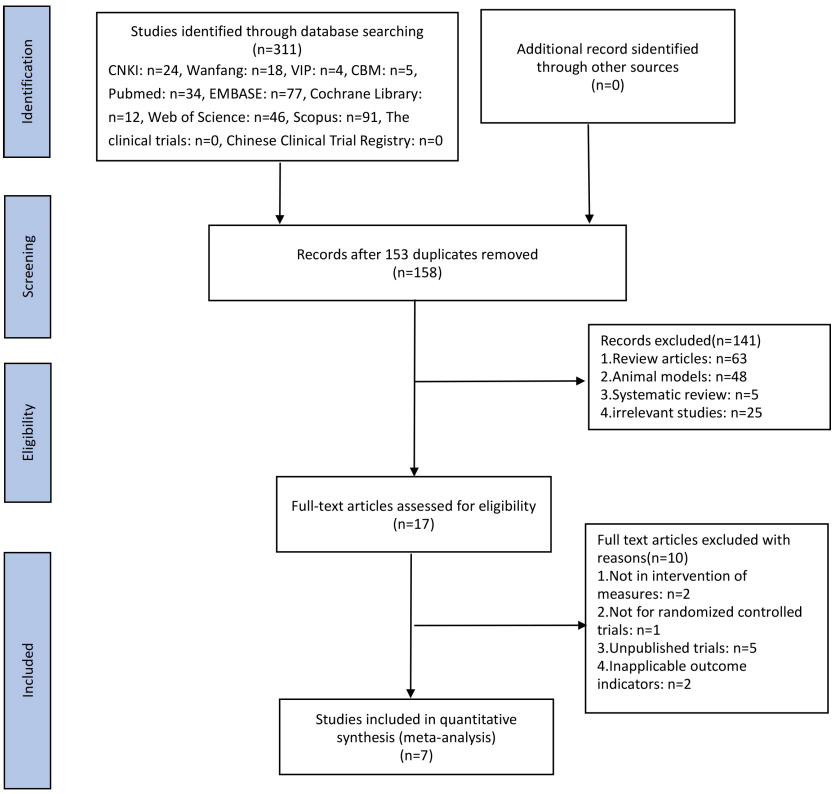

FIGURE 1
Flow diagram of the study selection process.

further consideration and full texts were retrieved. Review of the full text rapidly eliminated the majority of studies and in all 7 RCTs (26–29, 33–35) met the eligibility requirements and were included in the meta-analysis. The literature selection process is depicted in Figure 1.

Study characteristics

The main characteristics of the included studies in the present meta-analysis are described in Table 1. Overall, a total of 447 participants in the 7 RCTs that were conducted in Iran, Turkey and China was analyzed. Among the 7 included studies, 5 studies compared curcumin/CL water decoction with placebo (26, 28, 29, 33, 34), 2 studies compared curcumin/CL water decoction plus metformin with metformin alone (27, 35). The dosage of curcumin ranged from 80 to 1500 mg/day and CL water decoction's dosage was 90ml/day, the duration of the intervention varied from 6 weeks to 6 months. Primary outcome measures included fasting blood glucose (FBG), insulin (INS), homeostatic model assessment for insulin resistance (HOMA-IR) and C-reactive protein (CRP).

TABLE 1 The characteristics of the included studies.

Reference	Nation	Sample size (T/C)	Mean age (T/C)	Intervention	Comparison	Dosage (T/C)	Treatment duration	Outcomes	Adverse reaction
Jamilian M 2020 (26)	Iran	24/26	28.6 ± 4.7/27.2 ± 3.4	Curcumin	Placebo	Curcumin 500mg, qd/ Placebo Nr	12 weeks	Weight, BMI, FBG, INS, QUICKI, HOMA-IR, T, DHEAS, LH, FSH, LH/FSH, TG, TC, LDL-C, HDL-C	Nr
Sohrevardi SM2021 (27)	Iran	48/50	29 ± 2/ 28.8 ± 2.46	Curcumin +Metformin	Metformin	Curcumin 80mg, qd Metformin 500mg, tid/ Metformin 500mg, tid	12 weeks	Weight, BMI, FBG, INS, QUICKI, HOMA-IR, T, DHEAS, LH, FSH, LH/FSH, TG, TC, LDL-C, HDL-C	Nr
Sohaei S 2019 (29)	Iran	27/24	29.40 ± 5.33/ 29.58 ± 5	Curcumin	Placebo	Curcumin 500mg, bid/ Placebo Nr	6 weeks	Weight, BMI, FBG, INS, QUICKI, HOMA-IR, TG, TC, LDL-C, HDL-C, CRP	Nr
Asan SA 2020 (33)	Turkey	15/15	27.6 ± 3.6/28.3 ± 5.9	Curcumin	Placebo	Curcumin 93.34mg/ Placebo Nr	8 weeks	Weight, WC, BMI, FBG, INS, HOMA-IR, T, DHEAS, LH, FSH, TG, TC, LDL-C, HDL-C, CRP	Nr
Heshmati J 2021 (34)	Iran	34/33	30.97 ± 5.20/ 30.75 ± 7.97	Curcumin	Placebo	Curcumin 500mg, tid/ Placebo 500mg, tid	12 weeks	WC, BMI, FBG, INS, QUICKI, HOMA-IR, DHEAS, LH, FSH	Nr
Wu JL 2022 (28)	China	47/44	27.06 ± 4.99/ 27.16 ± 4.87	CL water decoction	Placebo	CL water decoction 45ml, bid/ Placebo 45ml, bid	6 months	BMI, WHR, FBG, INS, Glu120, Ins120, HbA1c, HOMA-IR, LH/FSH, FAI, RBC, WBC, Cr, ALT, AST	T: pruritus (n = 1), edema (n = 1), nausea (n = 1), dizzy (n = 1) C: none
Wu JL 2020 (35)	China	30/30	26.1 ± 4.9/25. 6 ± 5.0	CL water decoction+ Metformin	Metformin	CL water decoction 45ml, bid Metformin 0.85g, bid/ Metformin 0.85g, bid	3 months	BMI, WHR, FBG, INS, Glu120, Ins120, HbA1c, HOMA-IR, LH/FSH, FAI, TG, TC, LDL-C, HDL-C, RBC, WBC, Cr, ALT, AST	T:nausea (n = 2), Bloating (n = 2), diarrhea (n = 2), constipation (n=3), dizzy (n = 2), pruritus (n = 5), edema (n= 1) C: nausea (n = 2), Bloating (n = 2), diarrhea (n = 3), constipation (n=1), dizzy (n = 1), pruritus (n = 3)

CL, Curcuma Longa; WC, waist circumference; BMI, body mass index; WHR, waist-to-hip ratio; FBG, fasting blood glucose; INS, insulin; QUICKI, quantitative insulin sensitivity check index; Glu120, Blood glucose at 2 h after OGTT; Ins120, Insulin at 2 h after OGTT; HbA1c, Glycosylated hemoglobin A1c; HOMA-IR, Homeostatic model assessment for insulin resistance; T, testosterone; DHEAS, dehydroepiandrosterone-sulfate; LH, luteinizing hormone; FSH, follicle-stimulating hormone; LH/FSH, luteinizing hormone/follicle-stimulating hormone; FAI, free androgen index; TG, triglycerides; TC, Total Cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; CRP, C-reactive protein; RBC, red blood cell; WBC, white blood cell; Cr, Creatinine; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Nr, not report; ±, operator symbol, values are expressed as mean ± SD.

Risk-of-bias assessment

Figure 2 summarizes the risk of bias of the included studies according to the pre-defined criteria in Cochrane handbook. Adequate randomized sequence generation was reported in all included trials except one (33). Most randomized trials did not report whether allocation was concealed (26, 27, 33, 35), and participants were not blinded to randomization 3 trials (27, 33, 35). 2 studies (29, 34) specified that the evaluators of outcome assessors were blinded and were given a low risk of bias. There was a low risk of bias of incomplete outcome data, selective reporting, and other sources in all studies. We did not assess funnel plots for publication bias because fewer than 10 studies were included in the meta-analysis.

Outcome measures

Effect of curcumin on anthropometric parameters

4 studies (26, 27, 29, 33) with 229 patients were involved in this analysis. The result reported apparent trend for curcumin to decrease weight in PCOS patients and there was a medium degree of heterogeneity (WMD -0.924, 95% CI -2.009 to 0.162, P = 0.095, I^2 = 45.2%, Figure 3A). Only two RCTs (33, 34) reported waist circumference (WC), there was no significant difference in WC of the intervention groups when compared with the placebo groups, and with high heterogeneity (WMD -1.475, 95% CI -4.519 to 1.570, P = 0.342, I^2 = 81.6%, Figure 3B).

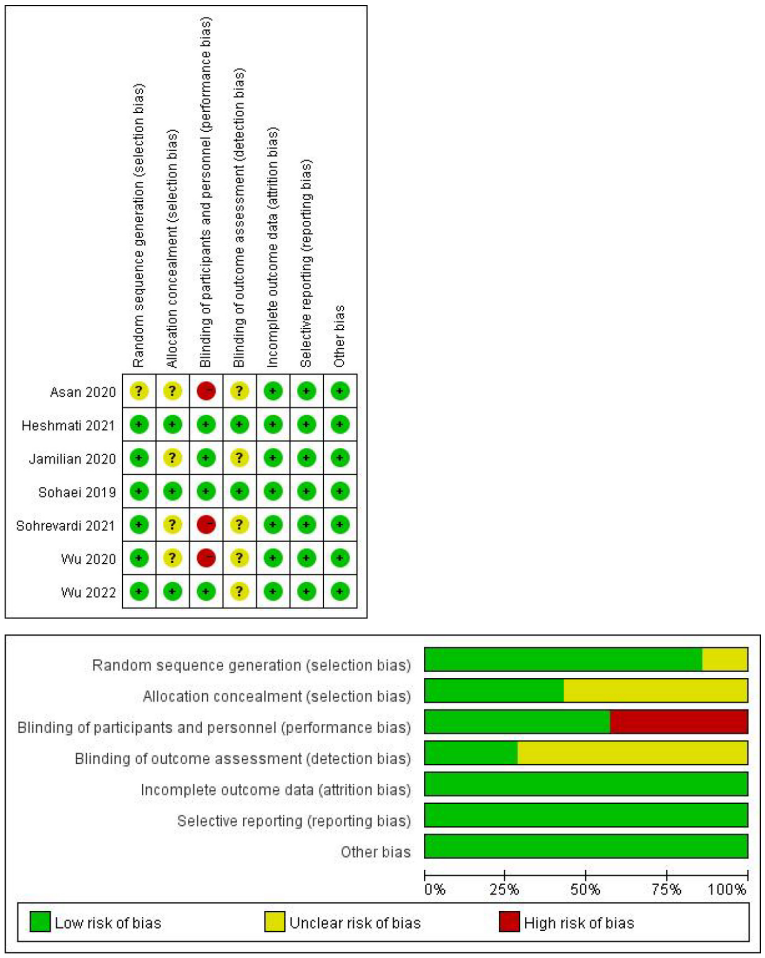

FIGURE 2
Risk of bias.

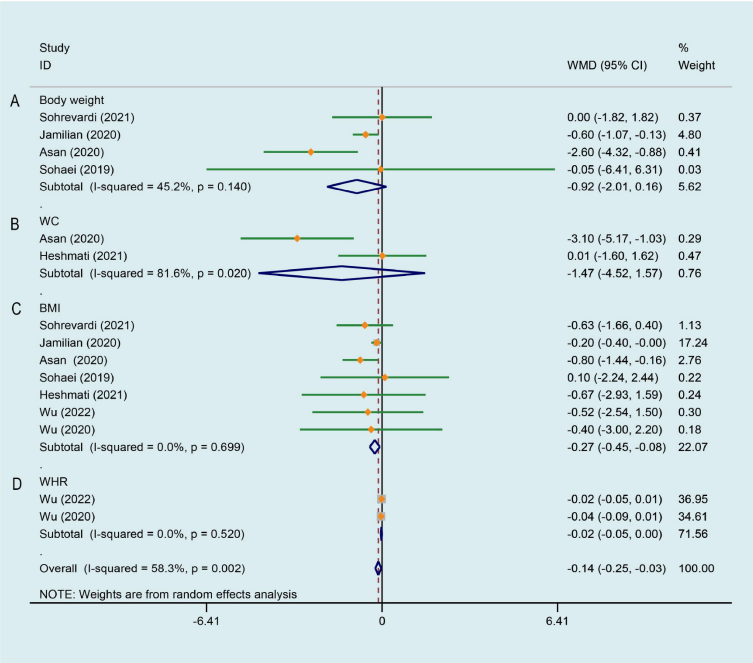

FIGURE 3
Meta-analyses of the effect of curcumin on anthropometric parameters. **(A)** Body weight, **(B)** WC, **(C)** BMI, **(D)** WHR.

7 trials (26–29, 33–35) evaluated the effects of curcumin on body mass index (BMI) in this review, there were 225 patients in the intervention group and 222 in the control group. Meta-analysis revealed a significant BMI-lowering effect favoring the experimental group compared to the control group (WMD -0.267, 95% CI -0.450 to -0.084, P = 0.004, I^2 = 0.0%, Figure 3C). 2 studies (28, 35) analyzed the effects of CL water decoction on waist-to-hip ratio (WHR) in PCOS patients. Compared with the control group, there was no significant difference in WHR in the intervention group (WMD -0.024, 95% CI -0.048 to 0.000, P = 0.052, I^2 = 0.0%, Figure 3D).

Effect of curcumin on CRP

The level of CRP was evaluated in the 2 trials comparing curcumin with placebo (29, 33). The meta-analysis revealed a significant reduction by the treatment of curcumin (WMD -0.785, 95% CI -1.553 to -0.017, P = 0.045, I^2 = 23.9%, Figure 4).

Effect of curcumin on glucose metabolism

As illustrated in Figure 5A, a significant decrease of PCOS patients' FBG was observed after curcumin treatment comparing to that of the control group, there was a low degree of heterogeneity across the study data (WMD -3.618, 95% CI -5.165 to -2.071, P < 0.001, I^2 = 20.4%). Figure 5B displays the effects of curcumin on INS across 7 RCTs (26–29, 33–35), the study data has low heterogeneity. Compared with the control group, PCOS patients treated with curcumin/CL water decoction had significantly lower INS (WMD -1.834, 95% CI -2.701 to -0.968, P < 0.001, I^2 = 8.4%). The effect of curcumin on quantitative insulin sensitivity check index (QUICKI) was evaluated in 4 studies (26, 27, 29, 34). Compared with the control condition, a significant improvement on QUICKI was observed by the experimental group (WMD 0.011, 95% CI 0.005 to 0.017, P < 0.001, I^2 = 39.6%, Figure 5C). For HOMA-IR, 7 studies (26–29, 33–35) involving 447 subjects suggested a significant improvement effect by the treatment group compared with the control group (WMD -0.565, 95% CI -0.779 to -0.351, P < 0.001, I^2 = 0.0%, Figure 5D). Blood glucose at 2 h after OGTT (Glu120) was evaluated in two studies (28, 35) that compared CL water decoction/CL water decoction plus metformin with placebo/metformin alone. There was not strong evidence that the treatment group had an effect on improving Glu120 because of no statistical difference (WMD -0.063, 95% CI -2.307 to 2.181, P = 0.956, I^2 = 87.4%, Figure 5E). Meta-analysis of 2 studies (28, 35) involving 151 patients showed that no significant difference between the treatment group and the control group was identified on the level of insulin at 2 h after OGTT (Ins120) (WMD -12.445, 95% CI -44.384 to 19.494, P = 0.445, I^2 = 0.0%, Figure 5F). As shown in Figure 5G, when the

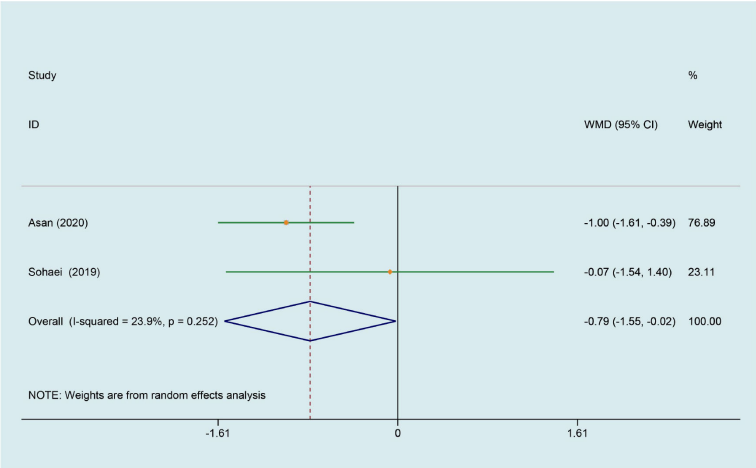

FIGURE 4
Meta-analyses of the effect of curcumin on CRP.

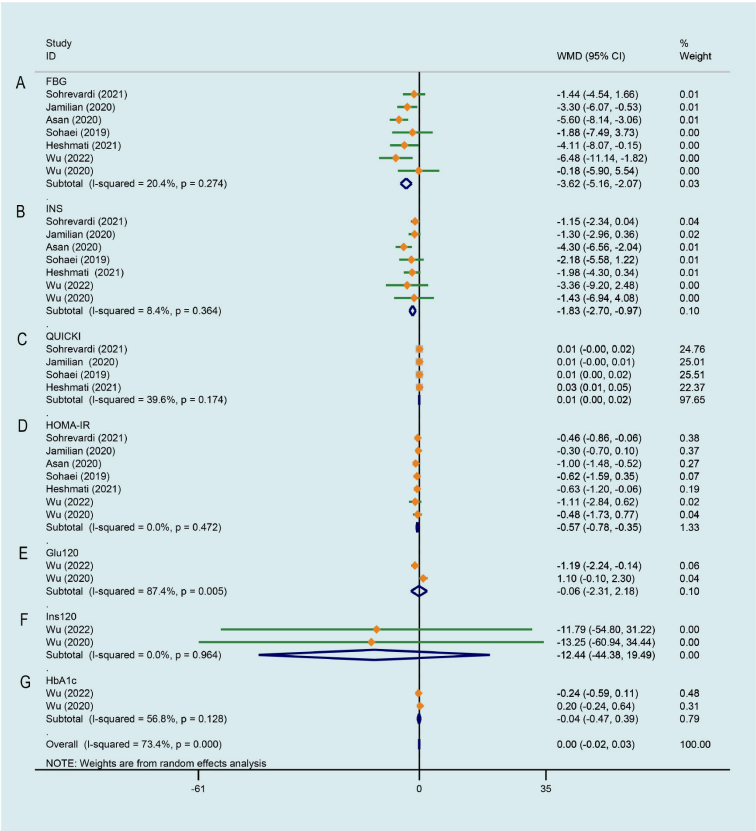

FIGURE 5
Meta-analyses of the effect of curcumin on glucose metabolism. **(A)** FBG, **(B)** INS, **(C)** QUICKI, **(D)** HOMA-IR, **(E)** Glu120, **(F)** Ins120, **(G)** HbA1c.

treatment group was compared with the control group, there was no significant difference in the level of glycosylated hemoglobin A1c (HbA1c) between the two groups (WMD -0.042, 95% CI -0.471 to 0.387, P = 0.849, I^2 = 56.8%) (28, 35).

Effect of curcumin on lipid metabolism

5 trials (26, 27, 29, 33, 35) evaluated the effects of curcumin on the level of total cholesterol (TC). Meta-analysis showed that curcumin/CL water decoction significantly decreased the level of TC in patients with PCOS (WMD -15.591, 95% CI -27.908 to -3.273, P = 0.013, I^2 = 68.9%, Figure 6A). The whole five data (26, 27, 29, 33, 35) were pooled and significant improving effects of curcumin on triglycerides (TG) (WMD -8.889, 95% CI -27.246 to 9.468, P = 0.343, I^2 = 91.5%, Figure 6B), low-density lipoprotein cholesterol (LDL-C) (WMD -6.427, 95% CI -17.343 to 4.489, P = 0.249, I^2 = 78.8%, Figure 6C) and high-density lipoprotein cholesterol (HDL-C) (WMD 3.713, 95% CI -0.786 to 8.211, P = 0.106, I^2 = 81.3%, Figure 6D) were not identified compared to the control group. The heterogeneities in the study data of TG, LDL-C and HDL-C were all high.

Effect of curcumin on hormone parameters

There was significant heterogeneity across the study data, and our result revealed curcumin had no significant effect on improving testosterone (T) level of PCOS patients in comparison with the control group (WMD -0.128, 95% CI -0.383 to 0.127, P = 0.326, I^2 = 98.6%, Figure 7A). Random effects meta-analysis found no significant effect for curcumin reducing level of dehydroepiandrosterone-sulfate (DHEA) in comparison with the control group (WMD -8.239, 95% CI -30.260 to 13.781, P = 0.463, I^2 = 62.3%, Figure 7B). As shown in Figures 7C, D, pooling 3 RCTs (27, 33, 34) together did not show any significant change in luteinizing hormone (LH) (WMD -0.003, 95% CI -0.007 to 0.000, P = 0.087, I^2 = 0.0%) and follicle-stimulating hormone (FSH) (WMD 0.002, 95% CI -0.024 to 0.029, P = 0.854, I^2 = 0.0%) of PCOS patients after curcumin treatment comparing to that of control group. Compared to the control group, an evident improvement on LH/FSH was not observed by curcumin in 3 studies (WMD -0.114, 95% CI -0.311 to 0.084, P = 0.259, I^2 = 0.0%, Figure 7E) (27, 28, 35). In terms of ameliorating free androgen index (FAI), there was not a significant difference between the intervention

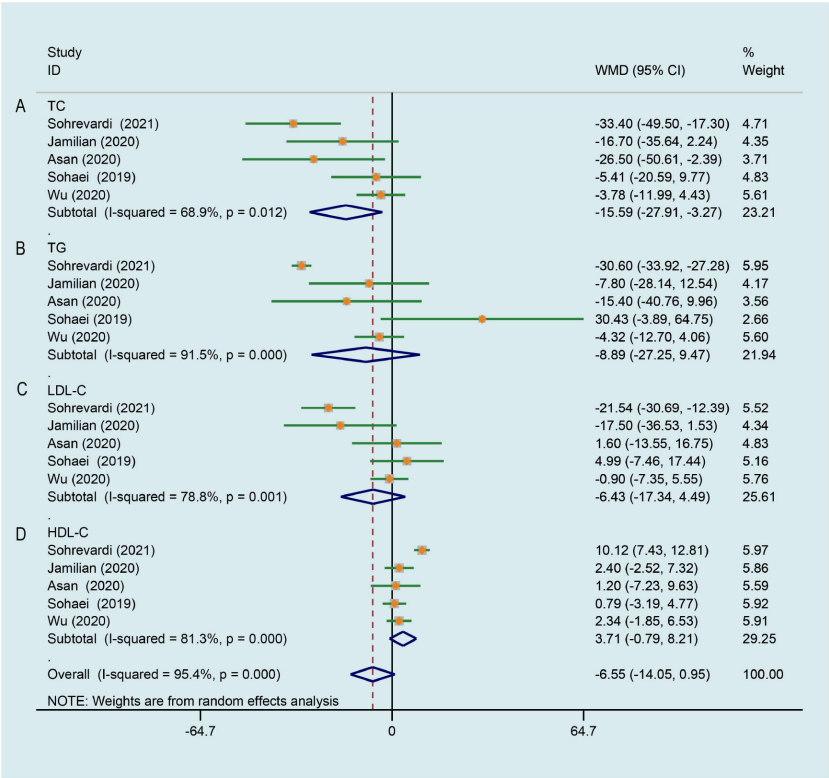

FIGURE 6
Meta-analyses of the effect of curcumin on lipid metabolism. **(A)** TC, **(B)** TG, **(C)** LDL-C, **(D)** HDL-C.

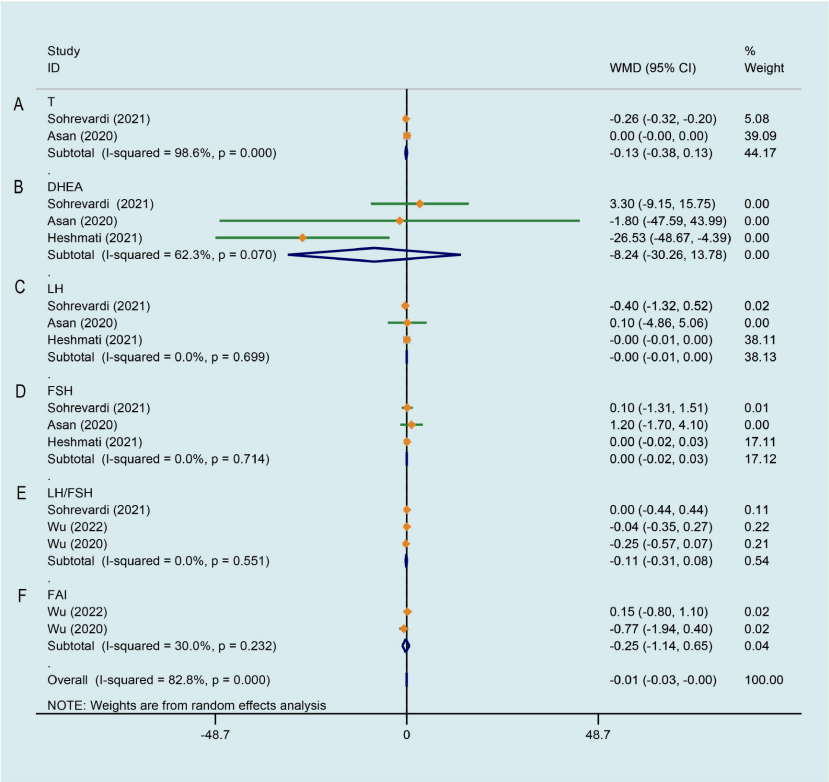

FIGURE 7
Meta-analyses of the effect of curcumin on hormone parameters. **(A)** T, **(B)** DHEA, **(C)** LH, **(D)** FSH, **(E)** LH/FSH, **(F)** FAI.

group and the control group (WMD -0.245, 95% CI -1.138 to 0.647, P = 0.590, I^2 = 30.0%, Figure 7F).

Adverse events

2 studies (28, 35) were included that evaluated the influence of curcumin on red blood cell (RBC), white blood cell (WBC) and creatinine (Cr). Meta-analyses found no obvious improvement on the level of RBC (WMD 0.077, 95% CI -0.124 to 0.279, P = 0.452, I^2 = 0.0%, Figure 8A), WBC (WMD 0.180, 95% CI -0.303 to 0.663, P = 0.465, I^2 = 0.0%, Figure 8B) and Cr (WMD 0.592, 95% CI -2.980 to 4.163, P = 0.745, I^2 = 0.0%, Figure 8C) in PCOS women after treatment with curcumin versus the comparison group. Meta-analysis of three studies (27, 28, 35) assessed the effect of curcumin on alanine aminotransferase (ALT) and aspartate aminotransferase (AST), there was no significant difference in ALT (SMD -0.325, 95% CI -1.124 to 0.473, P = 0.424, I^2 = 89.6%, Figure 8D) and AST (SMD -0.350, 95% CI -0.766 to 0.066, P = 0.099, I^2 = 62.6%, Figure 8E) between the groups. Two of the enrolled studies included adverse events, the meta-analysis showed that it was not more possible for curcumin to cause adverse events, it may be a safe therapeutic method (OR 2.215, 95% CI 0.516 to 9.512, P = 0.285, I^2 = 24.3%, Figure 8F).

Sensitivity analysis

Based on the results of our meta-analysis, we performed a sensitivity analysis for outcomes with high heterogeneity: WC, Glu 120, HbA1c, T, DHEA, TG, TC, LDL-C, HDL-C, ALT and AST. The results of sensitivity analyses showed that all the points fell in the confidence interval, indicating that none of the individual studies affected the final conclusion obviously (Supplementary Appendix 2).

Discussion

To the best of our knowledge, this is currently the most comprehensive systematic review and meta-analysis of the effect

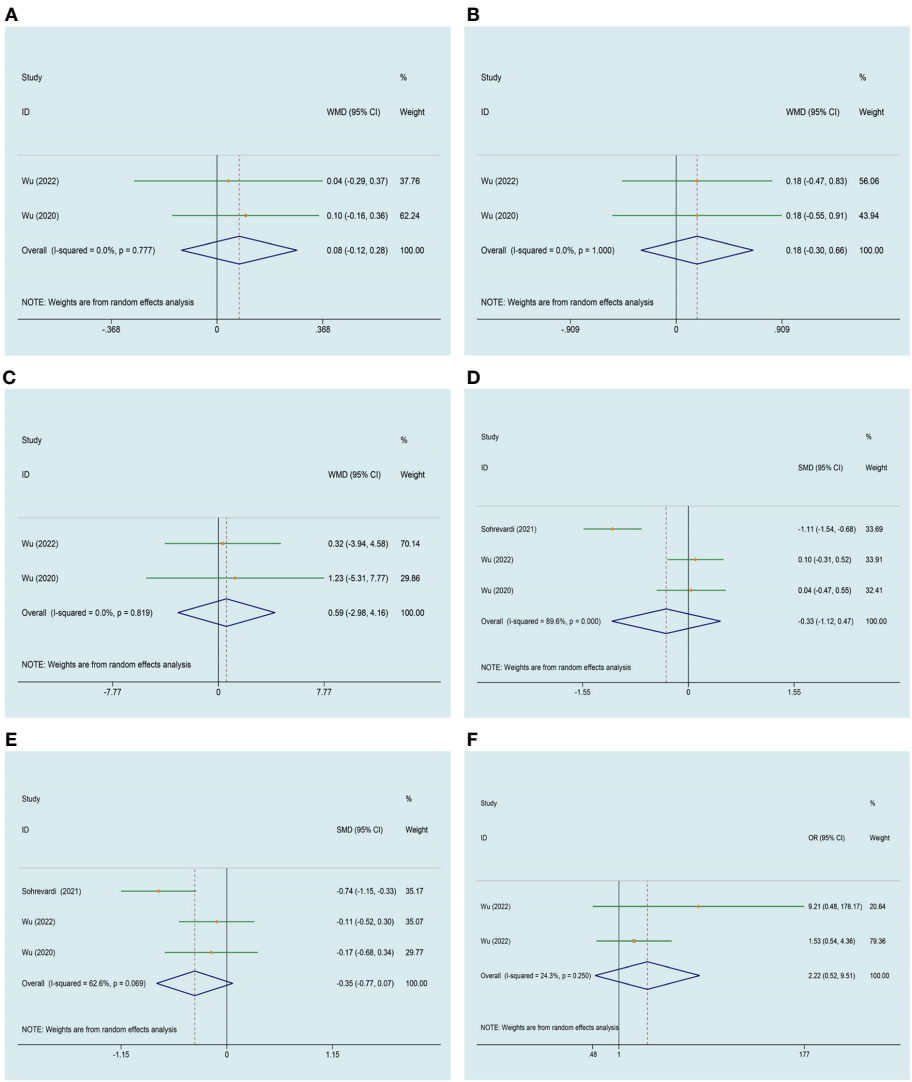

FIGURE 8
Meta-analyses of the effect of curcumin on adverse events. **(A)** RBC, **(B)** WBC, **(C)** Cr, **(D)** ALT, **(E)** AST, **(F)** adverse events.

of curcumin specifically for patients undergoing treatment for PCOS, in which we systematically searched and analyzed results from 7 eligible RCTs that involved 447 participants with PCOS. This analysis found that curcumin can significantly ameliorate HOMA-IR, FBG, INS, QUICKI and TC when compared with control group. In contrast to previous meta-analyses, curcumin also has a significant large positive effect size in CRP and BMI. Furthermore, we have also found that significantly decreasing trends of weight, LH and WHR after the curcumin intervention. As for the safety, curcumin appears to be well-tolerated with few adverse events reported by the included studies. However, this meta-analysis included a limited number of high-quality studies, therefore more longer-term and large sample trials evaluating the efficacy and safety of curcumin for PCOS are warranted.

Curcumin and anthropometric parameters

Women with PCOS report significant concern regarding weight gain, the rates of weight gain can be higher, which is more likely to be obese (36). Obesity is related to the infertility of PCOS and is a major risk factor for type 2 diabetes and cardiovascular disease in women (37, 38). In view of evidence-based guidelines on PCOS treatment, lifestyle management, including diet, exercise and behavioral strategies, is the first-line management in the intervention hierarchy in PCOS (39). In recent years, functional foods and nutraceuticals which have been shown as potential secondary therapies for the prevention of cardiovascular risk factors have been proposed for the

prevention against chronic diseases, glycemic and lipid metabolic disorders, and multiple metabolic syndrome components (40, 41). The results of our meta-analysis might confirm curcumin's effects on body composition indices. Our results highlighted an overall reduction in the level of BMI as a result of curcumin. This finding was in agreement with studies from a previous meta-analysis of 11 studies in which curcumin intervention significantly decreased the level of BMI in patients with overweight or obese (42). Several previous studies have reported the mechanisms that curcumin might affect body composition indices: curcumin can affect certain signal transduction and regulate the expression of specific cytokines (such as interleukin-1β, interleukin-6 (IL-6), TNF-α, monocyte chemoattractant protein-1, leptin and adiponectin), thereby maintaining energy homeostasis (43, 44). On the other hand, curcumin also induces the conversion of white adipocytes to a brown fat phenotype (45), which facilitates energy metabolism.

Curcumin and CRP

Previous studies have demonstrated that PCOS-related metabolic diseases, such as insulin resistance, obesity, type 2 diabetes and atherosclerosis are linked to chronic low-grade inflammation (46). In addition, pro-inflammatory factor can also promote the proliferation of ovarian granulosa cell and ovarian follicular membrane cells to produce more androgen leading to hyperandrogenemia (47). A number of studies have confirmed the anti-inflammatory properties of curcumin on PCOS in clinical research and animal models. Mohammadi et al. (48) found that the number of necrotic cells, IR index and IL-6 levels in adult female Wistar rats with PCOS were significantly reduced after curcumin treatment. Sohaei et al. (29) also observed, after curcumin therapy, a significant improvement in CRP after treatment of 27 patients with PCOS, which was consistent with the results of our studies. CRP is one of the members of the pentraxin family in hepatocytes, whose expression is mainly activated by IL-6 and regulated by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) signal path (49). Curcumin exhibits potent anti-inflammatory activity *via* suppression of IkB kinase activity and NF-kB signaling pathway (50).

Curcumin and glycolipid metabolism

The prevalence of metabolic syndrome (MS) among PCOS patients has been reported to be about 2 times higher compared to that in the general population (51). Both insulin resistance and dyslipidemia are associated with metabolic disorder in PCOS patients, which have been evidenced as risk factors for T2DM and cardiovascular diseases (52, 53). Curcumin has been widely investigated owing to its obvious effects on improving glucose metabolism and lowering blood lipids. Compared with the control group, it was observed a substantial decrease in FBG, INS and HOMA-IR and a marked increase in QUICKI of PCOS patients in this meta-analysis. Hypoglycemic properties of curcumin have been known since 1972 (54), the action is probably mediated by the stimulation of the PI3K/Akt pathway, which in turn promotes the translocation of glucose transporter 4 (GLUT4) to the plasma membrane, leading to an increase in glucose uptake and glycolysis (55). In the study by Wu et al (35), a remarkable rise of disposition index and glucose disposal rate has been observed after taking curcumin for 3 months, suggesting that curcumin ameliorates glucose homeostasis through protection of islet B cells. Recently, the importance of postprandial hyperglycemia has been highlighted by the fact that uncontrolled postprandial hyperglycemia gradually causes pancreatic β-cell exhaustion (56, 57). Furthermore, it has been shown that fluctuating glucose also produces oxidative stress, thereby inducing endothelial dysfunction and inflammation (56). A study in experimental animals has demonstrated that curcumin treatment for 8 weeks decreases both postprandial glycemia and HbA1c (58), which contradicted our findings. Nevertheless, we cannot deny the positive effect of curcumin on the postprandial glucose control of PCOS individuals, due to the limited number of studies. Possible mechanisms for the hypolipidemic effect of curcumin could involve increasing polyunsaturated sphingomyelin expression, improving the apoptotic status of liver tissue and inhibiting oxidative stress *via* downregulating malondialdehyde (MDA) levels and upregulating superoxide dismutase (SOD) levels (59, 60). But in our result, we did not find significant effects of curcumin on blood lipids (HDL-C, TG and LDL-C) other than TC. These inconsistent results may be ascribed, at least in part, to differences in study population, doses of curcumin and analytical approaches. The results, therefore, need to be interpreted with caution and larger studies are required to validate the results.

Curcumin and sex hormone

Hyperandrogenism is implicated as a key mediator of the pathogenesis of PCOS, which persists throughout reproductive life (61). The pathogenesis may include abnormal gonadotropin secretion and hyperinsulinism caused by IR. Abnormally increased LH pulse frequency and amplitude further enhance androgen synthesis in ovarian theca cell and promote hyperandrogenemia in patients with PCOS (62). Hyperinsulinemia may cause an augmented androgen production in the adrenal cortex and follicles *via* stimulation of LH secretion and a decreased SHBG production, resulting elevated androgen levels that may lead to the characteristic clinical manifestations like acne and hirsutism (63). The present meta-analysis has not demonstrated that curcumin has good efficacy on female reproductive hormones, however,

several studies provided strong justification for further exploration. A study by Heshmati et al (34), investigating the effect of curcumin on patients with PCOS, showed a significant reduction in DHEA after the curcumin than placebo. In another study, the experimental group of women that were diagnosed with PCOS, following the treatment with curcumin, manifested a clear descending trend of the levels of FAI (35). From these, curcumin has potential effects on lowering androgen levels in patients with PCOS. Most of the analysis results of our research are negative, but we cannot exclude that curcumin might be playing an active role in various reproductive hormones of PCOS patients.

Adverse effects

No serious side effects occurred as a result in our study, and only a small number of patients complained of minor side effects such as mild gastrointestinal discomfort and pruritus. Simultaneously, we observed that biochemical parameters such as RBC, WBC, Cr, AST and ALT did not show any gross abnormalities in expression, indicating that there was no obvious damage to the blood routine and liver and kidney function, which is one of the advantages of this meta-analysis. Moreover, a randomized, double-blind, placebo-controlled clinical trial found that when the clinical dose was 2400mg/d, curcumin supplementation could reduce systolic blood pressure and had no effect on cardiac metabolic risk parameters (64). In the United States, curcumin is approved as safe by the Food and Drug Administration (FDA) (65). From the current evidence, curcumin seems to be generally well tolerated and safe, although more clinical studies are needed to confirm the safety of curcumin in long-term treatment.

Strengths and limitations

To the best of our understanding, compared with the previously published results, this study is the first meta-analysis of RCTs to simultaneously evaluate the effects of anthropometric indicators, glucose and lipid metabolism, inflammatory factors, sex hormone levels and adverse reactions in PCOS, and provides evidence for curcumin as a non-toxic and safe drug to treat PCOS. In addition, all tests included in our analysis are clearly based on the Rotterdam standard, which is highly homogeneous. However, our review has several important limitations that need to be recognized. First, the limited sample size of the meta-analysis (a total of 447 randomized patients) resulted in weak evidence-based conclusion of the effectiveness of curcumin. Second, the descriptions of the allocation concealment or blinding were sparse in most of the included trials, which may lead to performance bias in outcome measurement. As such, these findings should be treated with caution until replicated. In addition, the duration of the involved studies was generally short-to-medium term (mostly 6 weeks to 3 months), and there was a lack of follow-up observation on the long-term efficacy of curcumin. Finally, most randomized controlled trials came from the Middle East (mainly Iran and Turkey) and the Asia Pacific region (especially East Asia, such as China), and there were no eligible studies from Western Europe and North America. Therefore, the representativeness of research results has some limitations. Collectively, there is an absence of more racially and ethnically high-quality data in our study. At present, we cannot provide robust support for the efficacy and safety of curcumin in treating PCOS, but it will lay the foundation for future large-scale trials.

Implications for future

More strictly designed studies are needed to confirm the impact of curcumin on PCOS, and large sample, longer-term multi center, high-quality and well-designed clinical trials should be registered to better understand the potential mechanism of curcumin's efficacy on patients with PCOS and provide decision-making for clinical evidence-based treatment. In addition, research that includes patient data from other countries or regions in the world will help to expand the applicability of the results.

Conclusion

Altogether, the results of this meta-analysis are inspiring and provide evidence supporting the potential effectiveness and safety of curcumin in orchestrating the inflammatory microenvironment and reducing the risk of abnormalities of glucose and lipid metabolism and obesity in patients with PCOS. However, the strength of this conclusion is tempered by the dearth of large-scale, high-quality reference datasets and the significant number of studies on this topic. Indeed, the effect sizes reported in this analysis merit further evaluation in a larger, well-designed, high-quality prospective randomized clinical trial. Studies that explore the different doses and types of the supplement are also required for access to high solubility and bioavailability curcumin.

Data availability statement

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

Author contributions

WJS and YZ conceptualized the research question. YFQ and HJ participated in drafting and writing the review. YFQ, HWW, and HJ participated in the formulation of retrieval strategies, data acquisition, data analysis and quality assessment. YJP and YHZ participated in the drawing of tables and figures. XKW and YHH participated in critical revision of the manuscript. All authors contributed to the research and approved the final 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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1051111/full#supplementary-material

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Effect of different timing of letrozole initiation on pregnancy outcome in polycystic ovary syndrome

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Objective: To investigate the efficacy of oral letrozole (LE) starting on day 3 or 5 of the menstrual cycle in patients with polycystic ovary syndrome (PCOS).

Design: Retrospective cohort study.

Setting: Reproductive Endocrinology Department of Hangzhou Women's Hospital.

Methods: In this retrospective analysis, we analyzed patients who received oral LE for ovulation induction (OI) at the Hangzhou Women's Hospital from January 2016 to January 2021. In total, 539 PCOS patients with fertility requirements were classified into the D3 group and D5 group according to the different starting times of oral LE, that is, from the 3rd or 5th day of the menstrual cycle or LE is taken orally for 5 days starting on day 3 or 5 of progesterone withdrawal bleeding. Treatment started with one tablet (LE 2.5 mg), continue the regimen from the previous cycle in non-responders and continued until pregnancy or for up to three ovulatory cycles, with visits to determine ovulation and pregnancy, followed by tracking of pregnancies. The primary outcome was to compare ovulation rates, conception rates, live birth rates, pregnancy complications, and pregnancy outcomes at different initiation times.

Results: Women who started LE on the 5th day of their menstrual cycle had more cumulative conception rates than those who started LE on the 3rd day (173 of 228[75.9%]*vs*. 201 of 311[64.6%], P= 0.005; rate ratio for conception, 1.174; 95% confidence interval,1.052 to 1.311) without significant differences in overall live birth rate, though there were 142 of 228[62.3%] in the D5 group versus 172 of 311[55.3%] in the D3 group (P= 0.105). The median (IQR) endometrial thickness was significantly (P = 0.013) greater during the D5 group treatment compared to the D3 group, which may be related to higher conception and clinical pregnancy rates. The median (IQR) maximum follicle diameter was not statistically (P = 0.073) different between the two groups. The

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cumulative ovulation per cycle rate was higher with D5 than with D3 (287 of 405 treatment cycles [70.9%] *vs*. 388 of 640 treatment cycles [60.6%], P=0.001). There were no significant between-group differences in pregnancy loss (31 of 173 conceptions in the D5 group [17.9%] and 29 of 201 conceptions in the D3 group [14.4%]) or multiples pregnancy (8.2% and 10.5%, respectively). Rates of other adverse events during pregnancy were similar in the two treatment groups.

Conclusion: As compared with D3 group, D5 group was associated with higher ovulation and conception rates, shorter time-to-pregnancy among infertile women with the PCOS.

KEYWORDS

polycystic ovarian syndrome, letrozole, ovulation induction, infertility, conception, pregnancy

Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women of reproductive age, affecting about 10% of women (1). PCOS is characterized by sparse ovulation, hyperandrogenism, and polycystic ovary morphology based on ultrasound evaluation. It is usually associated with metabolic syndromes such as insulin resistance, obesity, hyperlipidemia, and hypertension. It accounts for 90% of infertility in anovulatory women and is one of the important causes of infertility in women of childbearing age (1). Current research shows that although the natural conception rate for women with PCOS is low, the treatments and strategies currently being used in the clinic are highly effective in improving conception rates (2). Therefore, choosing simple but effective infertility treatment options for patients with PCOS is essential. Even though PCOS is a complex disorder of reproductive metabolism, the hypothalamic-pituitary axis remains the target of first-line ovulation treatment. Many treatment options aimed at achieving ovulation, pregnancy, and live birth have been used with varying success (eg. Aromatase inhibitors, clomiphene, metformin for patients with abnormal glucose metabolism, etc.) (3, 4).

Letrozole (LE), the third generation of an aromatase inhibitor, is a new type that stimulates ovulation drugs to inhibit androstenedione and the conversion of testosterone to estrogen in the ovary, decreasing estrogen levels. It acts on the hypothalamic-pituitary gland through positive feedback, promotes the secretion and release of FSH, and induces follicle development and mature discharge (5). Numerous randomized trials have found increased ovulation, pregnancy, and live birth rates in women with PCOS after LE ovulation promotion compared to clomiphene citrate ovulation (6–8). Currently, LE is started on the third to the fifth day of the menstrual cycle. The potential advantages of using LE during this period are its relatively short half-life (*45 h), accumulation of intraovarian androgens, and activation of estrogen receptors, which will enhance follicular sensitivity, resulting in rapid endometrial growth. Nevertheless, there is no consensus on the optimal start time (9). We designed a retrospective cohort study to compare effectiveness and safety when LE was started on the third or fifth day of menstruation, respectively, to explore the optimal timing of ovulation initiation.

Materials and methods

Study oversight

This retrospective cohort study was approved by the Ethics Review Committee of the Hangzhou Women's Hospital. Written informed consent was waived due to the retrospective nature of the study.

Participants

A total of 624 patients with PCOS who received LE ovulation-promoting treatment and visited the Reproductive Endocrinology Department of Hangzhou Women's Hospital from January 2016 to January 2021 were collected.

The inclusion criteria for participants eligible for the use of ovulation-promoting drugs were: 1) Women of childbearing age between 20 and 40 years old who have not been pregnant without contraception for ≥1 year; 2) The PCOS was defined according to

Rotterdam Consensus 2003 (Meet two of the three and exclude other causes of hyperandrogenism: Low ovulation/anovulation, clinical manifestations of high androgen (acne/hirsute) and/or biochemical manifestations (testosterone≥0.8ng/ml or free androgen index [FAI]≥5)[3], gynecological ultrasonography during the menstrual cycle or 3rd to 5th days after bleeding after progesterone withdrawal suggests polycystic changes in the ovary (small follicles with a diameter of 2 -9 mm, ≥12 small follicles, and/or ovarian volume >10 ml) (10); 3) The women and their partners agreed to have regular intercourse with the intention of conception during the study.

The exclusion criteria include: 1)Women with BMI > 30kg/m2; 2)Patients with tubal factor infertility; 3)Patients with uterine and reproductive tract malformation confirmed by gynecological ultrasound, HSG, laparoscopy, or hysteroscopy; 4) Patients with infertility due to abnormalities in the male partner's semen (normal sperm concentration of 15 million per milliliter and a normal activity rate of >40%, WHO 2010.); 5) Women who were pregnant before this ovulation induction drug started; 6)Women who have received ovulation induction (OI) treatment within 6 months and gonadotropin-releasing hormone agonists (GnRHa) within three months; 7) Patients with diabetes mellitus, hypertension, endometrial hyperplasia/cancer, thyroid disease, and hyperprolactinemia that cannot effectively control by medication; 8)Patients with major systemic illnesses; 9)Patients with a history of LE allergy and contraindications.

Study overview

The final 539 patients were included and divided into groups D3 (n=311) and D5 (n=228) according to the start of oral LE (Femara, Novartis Pharmaceuticals), that is, oral LE 2.5 mg/d for 5 days starting on days 3 and 5 of the menstrual cycle. All patients were tested for follicular growth by vaginal ultrasound from day 10 of the menstrual cycle.

HCG (human chorionic gonadotrophin) injection and corpus luteum support standard: When the largest diameter of the dominant follicle was ≥18mm or the urine LH was positive, an intramuscular injection of HCG5000-10000 IU was used to induce ovulation, and the patient was asked to have sex on the injection day or the next day; or intrauterine insemination was performed 24h or 36h after HCG injection. After that, vaginal ultrasound monitoring is done daily until the day of ovulation, or every 2-3 days until after the next menstrual period if ovulation has not occurred after 96 hours of HCG.

Urine HCG testing 10 days after ovulation to determine conception, the follow-up to 5-6 weeks after the last menstrual period, diagnosis of clinical pregnancy when a gestational sac is detected by ultrasound, and the obstetric records of those conceiving were reviewed for pregnancy outcomes. If no conception occurs, continue the regimen from the previous cycle, with no conception for 3 consecutive cycles considered a failure.

Criteria for interrupting the treatment cycle: At the risk of ovarian hyperstimulation: ≥3 dominant follicles, ovarian diameter ≥60mm, ascites, serum estradiol level ≥5500pmol-L-1.

Outcomes

The primary outcome of the study was the conception (serum or urine HCG was positive)and live birth rates. Secondary outcomes included the rate of ovulation(serum progesterone level over 5ng/ml within one cycle), endometrial thickness(on the day of intramural injection of HCG), maximum follicular diameter(on the day of intramural injection of HCG), treatment cycles received until pregnancy, pregnancy loss (including biochemical, miscarriage, ectopic), pregnancy outcome, multiples pregnancy, pregnancy complications, mode of delivery and other adverse events.

Blood examination

Days 2-5 of the menstrual cycle, after a period of 10 or more hours without food, blood samples were collected from all participants before breakfast. Anti-Mullerian hormone (AMH), Follicle-stimulating hormone (FSH), luteinizing hormone (LH), Progesterone(P), Testosterone (T), Prolactin (PRL) and thyroid stimulating hormone (TSH) were measured using the chemiluminescence method (Beckman Coulter UniCel Dxl-800). The Beckman Coulter AU5821 chemistry analyzer was used to measure fasting plasma glucose (FPG), fasting insulin (FINS), total bilirubin, total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), Apoa1, homocysteine (HCY), uric acid (UA), serum calcium, serum phosphorus, neutrophil to lymphocyte ratio (NLR), platelet (PLT), and C-reactive protein (CRP).

Statistical analysis

Statistical analysis was performed using SPSS Statistics 26. Participants' characteristics in the two allocated treatment sequences were compared using independent non-parametric tests. Generalized estimating equations were used for the analysis of the ovulation rate to account for the correlation of multiple ovulation cycles for each subject. The endometrial thickness and maximum follicle diameter were compared using independent non-parametric tests. Categorical data were

compared using the chi-squared test or Fisher's exact test. In addition, the rate ratio (RR) and the absolute difference (AD) (95% Confidence Interval) were estimated for conception and live birth rates. Kaplan–Meier curves were used for time-to-event analyses. Logistic regression models created odds ratios (ORs) with associated 95 percent confidence intervals (CIs), which were used to assess the relationship between characteristics associated with conception and live birth after controlling for potential confounders such as maternal age and BMI (body mass index), LH/FSH, AMH (anti-Müllerian hormone), T (testosterone), and TSH (thyroid stimulating hormone).

Results

Characteristics of the patients

Figure 1 illustrates the flow of participants throughout the trial. In total,539 patients were included in this study, with 311 patients receiving LE on the 3rd day of their menstrual cycle and 228 patients receiving LE on the 5th day. Based on the baseline characteristics of both groups in Table 1, no significant differences were found at baseline characteristics.

Primary outcomes (conception and live birth)

The conception and live birth rates were depicted in Figure 2 and Figure 3 for the overall and each stratum. Throughout the study, as compared with the D3 group, the D5 group exhibited a substantial increase in conception rates (173 of 228 women [75.9%] vs. 201 of 311 [64.6%], P=0.005; RR for conception on the 5th day, 1.174; 95% CI,1.052 to 1.311) (Figure 2).

We performed an analysis according to the maternal BMI, when BMI was 18.5-25(normal range), the D5 group had significantly greater conception rates than the D3 group (76.0% vs. 63.3%, p=0.009). In PCOS individuals with BMI >25, the D5 group had a significantly higher live birth rate than the D3 group (70.8% vs. 52.2%, p=0.045). There was no large discrepancy in conception or live birth rates between the two groups of PCOS patients with the BMI < 18.5.

Sub-analysis of conception rates based on different ages, LH/FSH, T, and TSH revealed between the two groups. When the age was <30, LH/FSH <2, AMH≥4.15, T<0.8, and TSH was 1.43-2.61, the D5 group had a greater conception rate than the D3 group, and all were statistically different.

There was no statistical difference in live birth rates between the two groups, although the 5th day arm had a trend toward

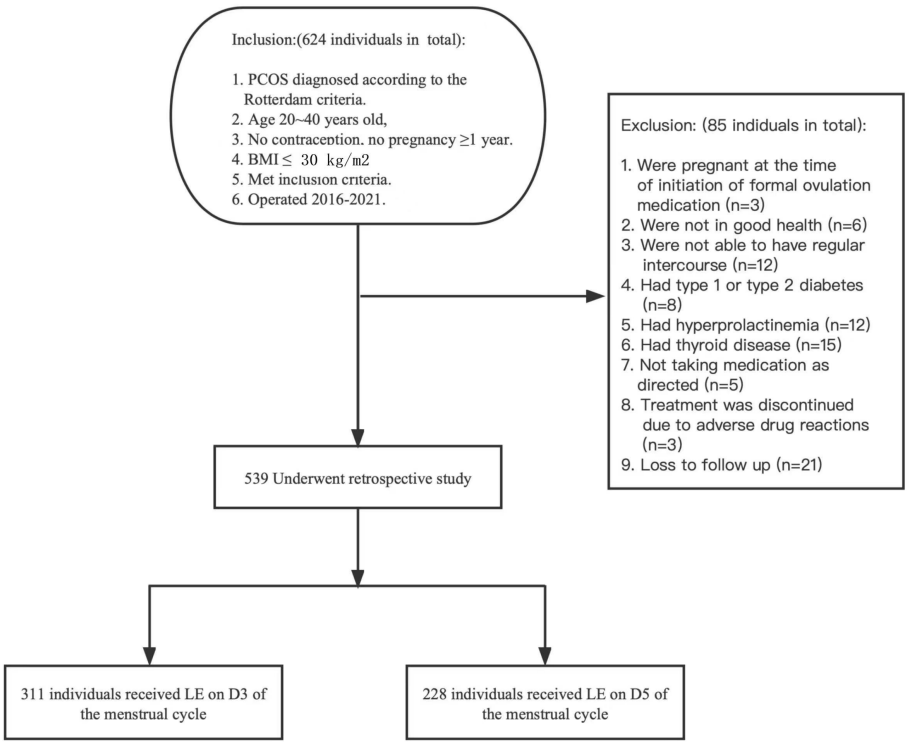

FIGURE 1
Flow Chart Depicting the Patient Selection.

10.3389/fendo.2022.1059609

TABLE 1 Baseline Characteristics of Participants.

	D3 (n = 311)	D5 (n = 228)	P
Age (years)	28.0 (26.0 to 31.0)	29.0 (26.0 to 31.0)	0.434
BMI (kg/m^2)	22.0 (19.8 to 24.3)	22.1 (19.8 to 24.1)	0.801
Infertility duration (years)	2.0 (2.0 to 2.0)	2.0 (2.0 to 3.0)	0.059
AMH (ng/ml)	6.5 (4.2 to 10.0)	7.0 (4.2to 10.0)	0.833
LH (IU/L)	7.4 (4.6 to 13.1)	7.8 (5.1 to 13.7)	0.39
FSH (IU/L)	6.7 (5.6 to 7.7)	6.4 (5.2 to 7.7)	0.208
P (ng/ml)	0.62 (0.37 to 1.02)	0.62 (0.43 to 1.08)	0.218
Testosterone (ng/ml)	0.57 (0.44 to 0.72)	0.61 (0.42 to 0.77)	0.064
FAI (%)	6.56 (3.8 to 11.0)	5.8 (2.9 to 11.2)	0.419
Prolactin (ng/ml)	11.9 (8.9 to 16.8)	12.2 (9.1 to 18.3)	0.414
Fasting insulin (mIU/L)	6.7 (4.6 to 10.6)	8.2 (5.7 to 10.7)	0.146
Fasting glucose (mmol/L)	5.0 (4.6 to 5.2)	4.9 (4.6 to 5.2)	0.605
TSH (mIU/L)	1.8 (1.4 to 2.6)	1.9 (1.5 to 2.6)	0.921
PLT (10^9/L)	245 (212 to 275)	237 (209 to 273)	0.313
NLR	1.8 (1.4 to 2.5)	2.0 (1.5 to 2.7)	0.126
UA (umol/L)	296 (253 to 347)	304 (255 to 354)	0.557
TC (mmol/L)	4.92 (4.32 to 5.54)	4.78 (4.27 to 5.52)	0.464
TG (mmol/L)	1.08 (0.74 to 1.72)	1.12 (0.82 to 1.66)	0.406
HDL (mmol/L)	1.52 (1.27 to 1.78)	1.50 (1.27 to 1.79)	0.79
LDL (mmol/L)	2.62 (2.21 to 3.04)	2.61 (2.16 to 3.04)	0.661
Apoa1 (g/L)	1.50 (1.29 to 1.71)	1.44 (1.28 to 1.74)	0.536

(Numerical data presented as median (25th to 75th percentile).
D3, using LE on the 3rd day of menstruation; D5, using LE on the 5th day of menstruation.
BMI, body mass index; AMH, anti-Müllerian hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; P, progesterone; FAI, free androgen index; TSH, thyroid stimulating hormone; NLR, neutrophil to lymphocyte ratio; UA, uric acid; TC, total cholesterol; TG, triglyceride; HDL, high density lipoprotein; LDL, low density lipoprotein.

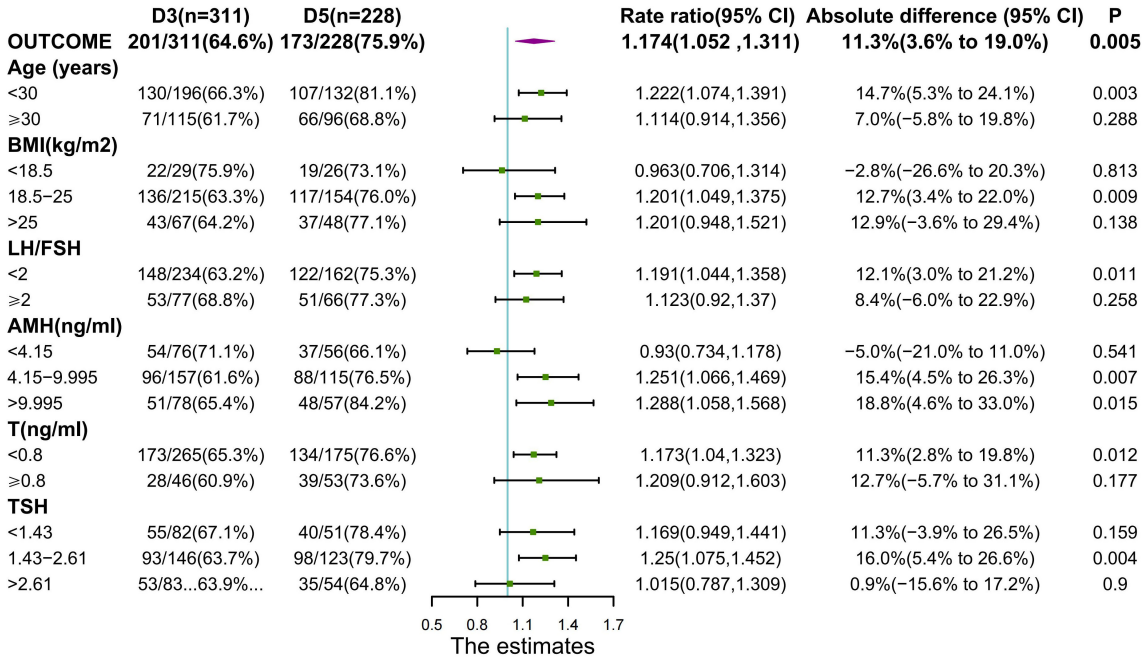

FIGURE 2
Outcomes with Regard to Conception Rate.

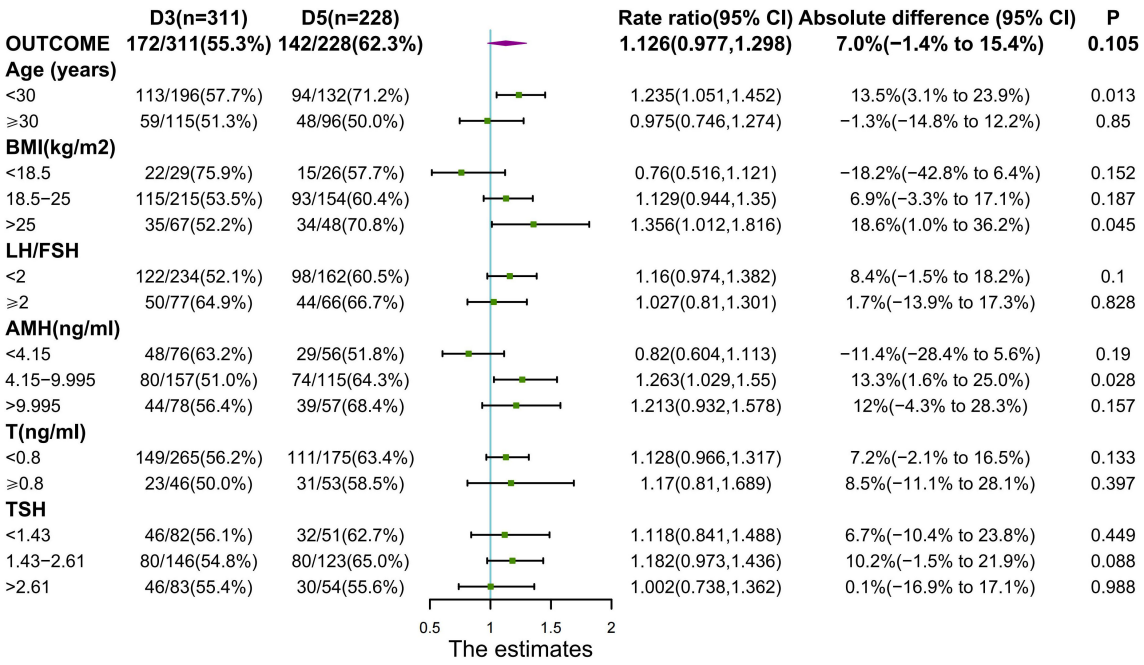

FIGURE 3
Outcomes with Regard to Live Birth Rate.

higher rates. To detect variations in live birth rates, larger sample size is necessary. The primary outcome of live birth was significantly influenced by age (p=0.027) (Table 2), when the age was less than 30, the live birth rate for subjects in the D5 group was considerably greater than for women in the D3 group (p=0.013) (Figure 3). At AMH between 4.15 and 9.995, live birth rates were higher in the D5 group than in the D3 group (p=0.028) (Figure 3).

Secondary outcomes (ovulation, pregnancy, and pregnancy loss)

On the day of HCG intramuscular injection, the median (IQR) endometrial thickness was substantially (P = 0.013) larger in the D5 group than in the D3 group. The maximum follicle diameter was not statistically (P = 0.073) different between the two groups (Table 3).

Per cycle analysis revealed considerably greater ovulation rates in the D5 group (P=0.001) (Table 3). As previously stated, however, the variations in ovulation rates did not result in a significant increase in the live birth rate among the D5 group. Rates of conception and Singleton pregnancy per cycle in which ovulation occurred were higher in the D5 group (P=0.029, P=0.069). There was statistically significant change in the frequencies of conception and singleton pregnancy per ovulated subject (P=0.002, P=0.024). But there was no statistically significant change in the frequencies of live births per ovulated subject.

The median number of treatment cycles received until pregnancy was significantly (log-rank P=0.0012) smaller with the D5 group (2[1–3] cycles) compared to the D3 group (1[1–3] cycles) (Figure 4).

The pregnancy rate in the D3 group was considerably lower than in the D5 group (P=0.042 for both comparisons). The rates of pregnancy loss after conception were equivalent in the two treatment groups. Four adverse events of pregnancy loss after observed heart motion occurred during infertility treatment in the D5 group (Table 3).

Between the D3 and D5 groups, there was no significant difference in the period from treatment initiation to live birth (log-rank P=0.66) (Figure 4).

Adverse events and mode of delivery

Table 4 summarizes adverse events and their mode of delivery. During pregnancy, gestational diabetes was the most prevalent consequence, followed by early membrane rupture, preterm labor, and HDP (hypertensive disorder in pregnancy), with no significant differences between treatment groups. There were two substantial congenital malformations (one with D3 and one with D5); the between-group difference was not significant. Both groups had similar delivery patterns.

TABLE 2 Logistic Regression of Live Birth Outcome.

Baseline Parameter	Category	aOR (95% CI)	P
Age (years)	per year increased	0.944 (0.896 to 0.993)	0.027
BMI (kg/m2)	per kg/m2 increased	0.992 (0.942 to 1.046)	0.779
LH/FSH	per increased	1.214 (1.011 to 1.458)	0.037
AMH (ng/ml)	per ng/ml increased	1.011 (0.964 to 1.059)	0.66
T (ng/ml)	per ng/ml increased	0.775 (0.358 to 1.678)	0.519
TSH (mIU/L)	per mIU/L increased	0.929 (0.785 to 1.101)	0.396
Type of treatment	D3 vs. D5	1.325 (0.929 to 1.888)	0.12

Analyses were adjusted for maternal age, maternal BMI, LH/FSH, AMH, T, TSH.
aOR, adjusted odd ratio.

TABLE 3 Rates of Ovulation, Pregnancy, and Pregnancy Loss.

Variable	D3 (n = 311)	D5 (n = 228)	Rate ratio (95% CI)	Absolute difference (95% CI)	P
No. of ovulations/total treatment cycles	388/640 (60.6%)	287/405 (70.9%)		10.2% (4.3% to 15.9%)	0.001
ET (mm) [median (IQR)]	8.0 (7.0 to 10.0)	9.0 (8.0 to 10.0)			0.013
Maximum follicle diameter	1.88 (1.77 to 1.97)	1.84 (1.75 to 1.95)			0.073
Conception	201/311 (64.6%)	173/228 (75.9%)	1.174 (1.052 to 1.311)	11.3% (3.6% to 19.0%)	0.005
Pregnancy	172/311 (55.3%)	146/228 (64.0%)	1.158 (1.007 to 1.331)	8.7% (0.4% to 17.0%)	0.042
Singleton	154/172 (89.5%)	134/146 (91.8%)	1.025 (0.955 to 1.1)	2.3% (-4.1% to 8.7%)	0.495
Twins	16/172 (9.3%)	12/146 (8.2%)	0.884 (0.432 to 1.807)	-1.1% (-7.3% to 5.1%)	0.734
Multiples	18/172 (10.5%)	12/146 (8.2%)	0.785 (0.391 to 1.576)	-2.3% (-8.7% to 4.1%)	0.495
Pregnancy loss					
Total losses among subjects who conceived	29/201 (14.4%)	31/173 (17.9%)	1.242 (0.781 to 1.975)	3.5% (-4.0% to 11.0%)	0.359
Biochemical factor or no fetal heart motion	21/201 (10.4%)	20/173 (11.6%)	1.107 (0.621 to 1.972)	1.1% (-5.3% to 7.5%)	0.731
Ectopic pregnancy	8/201 (4.0%)	7/173 (4.0%)	1.017 (0.376 to 2.746)	0.1% (-4.0% to 4.0%)	0.974
Loss after observed heart motion	0/201 (0.0%)	4/173 (2.3%)		2.3%0.1% to 4.5%)	0.045
Events among ovulated cycles					
Conception	201/388 (51.8%)	173/287 (60.3%)	1.164 (1.017 to 1.331)	8.5% (0.9% to 15.9%)	0.029
Singleton pregnancy	154/388 (39.7%)	134/287 (46.7%)	1.176 (0.988 to 1.4)	7.0% (-0.5% to 14.5%)	0.069
Singleton live birth	154/388 (39.7%)	130/287 (45.3%)	1.141 (0.956 to 1.362)	5.6% (-2.0% to 13.1%)	0.145
Events among subjects who ovulated					
Conception	201/287 (70.0%)	173/210 (82.4%)	1.176 (1.066 to 1.298)	12.4% (4.7% to 19.5%)	0.002
Singleton pregnancy	154/287 (53.7%)	134/210 (63.8%)	1.189 (1.025 to 1.379)	10.2% (1.4% to 18.6%)	0.024
Singleton live birth	154/287 (53.7%)	130/210 (61.9%)	1.154 (0.992 to 1.342)	8.3% (-0.6% to 16.8%)	0.067

Categorical data: % (n/N); Ovulation was defined as serum progesterone level over 5ng/ml within one cycle; IQR, interquartile; ET, endometrial thickness (on the day of intramural injection of HCG); Conception was defined as a serum level of human chorionic gonadotropin that was positive; Pregnancy was defined by the presence of fetal heart movements on ultrasound.

Logistic regression of conception and live birth outcome

Table 5 shows the outcomes of a logistic regression model for the factors that could have influenced the conception rate. The results showed that using LE on the fifth day of menstruation had a substantial positive (favorable) effect (OR = 1.71, 95% CI: 1.161–2.519).

However, there was no significant effect of LE use on 5th day or 3rd day and a favorable effect of LH/FSH on live birth (OR = 1.214, 95 percent CI: 1.011-1.458) in regression models of live birth rates. Maternal age showed a negative (adverse) effect on outcome (OR=0.944, 95% CI: 0.896-0.993) (Table 2).

Discussion

To the best of our knowledge, this is the first sufficiently powered clinical study exploring the optimal initiation time of LE. Our study found that starting LE on the fifth day of their menstrual cycle was more effective as a fertility treatment than starting LE on the third day in anovulatory women with PCOS.

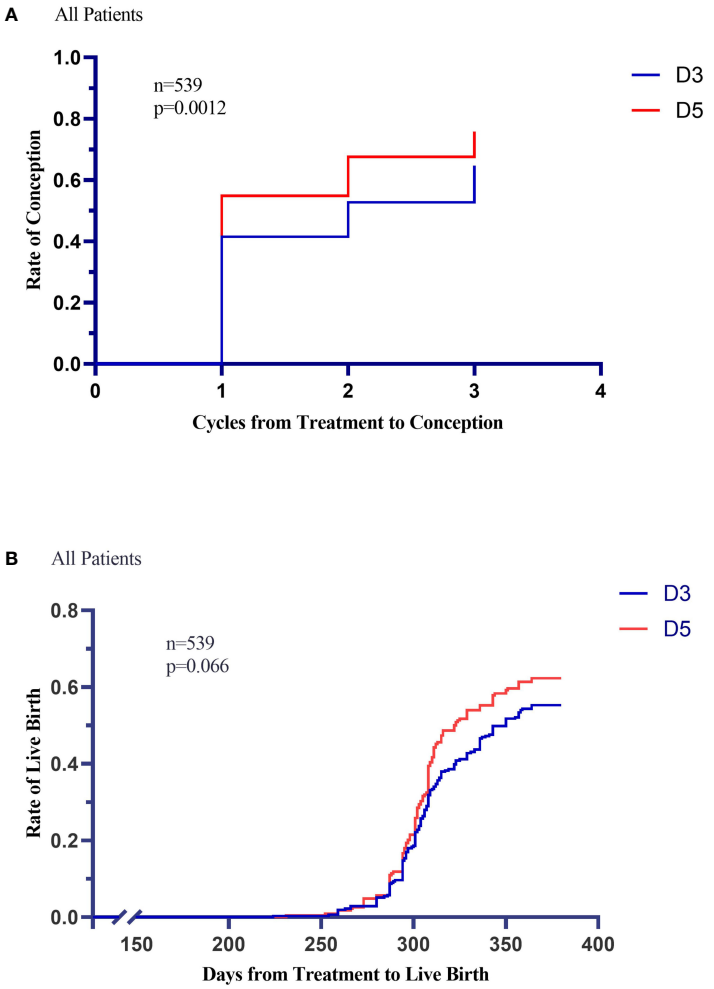

FIGURE 4
Kaplan-Meier Curves for Conception and Live Birth. Conception rates and Live-birth rates are shown according to treatment group in Panel **A** and Panel **B**.

Ovulation, endometrial thickness (on the day of intramural injection of HCG), conception, and pregnancy were significantly more likely after treatment with the D5 group. The rate of pregnancy loss, mode of delivery, multiples pregnancy, and rates of other adverse events during pregnancy (including anomalies) did not differ significantly between the two treatment groups. Our study also found that the D5 group required 1.39 cycles on average to conceive, while the D3 group required 1.54 cycles on average. When studying fertility treatment, we acknowledge that the ideal primary outcome would be live birth. The live birth rate was 10% higher in the D5 group than in the D3 group (RR = 1.126), but this did not reach statistical significance because the study was not strong enough to look at this outcome (power = 37.4%, alpha = 0.05), so we cannot rule out possible differences with small sample sizes. At the same time, we consider that the outcome of live birth is related to many other factors during pregnancy, such as environmental, physical, dietary, exercise and lifestyle factors. Our research was a retrospective analysis, and all pregnant women were not managed uniformly and followed up regularly during pregnancy, so we chose the conception and ovulation rates as the primary outcome, which is a limitation of our study. We will expand the sample size and provide appropriate management for prospective randomized clinical trials. Regular follow-up of all pregnant women included in the trial criteria to exclude other factors that interfere with pregnancy outcomes where possible and further explore the effect of different LE initiation times on pregnancy outcomes.

The triad that determines the success of human reproduction is the embryo, the endometrium, and their interactions. Past high-quality studies have shown that women with PCOS exhibit a clinically significant increased risk of obstetric and neonatal complications (11). Similar results have been published previously, confirming that endometrial dysfunction and altered oocyte capacity in women with PCOS are well-documented mechanisms contributing to increased pregnancy

TABLE 4 Adverse Events.

Event	D3 n (%)	D5 n (%)	Rate ratio (95% CI)	P
sPTB	21 (10.4%)	17 (9.8%)	0.941 (0.513 to 1.725)	0.843
Eutocia	95 (47.3%)	77 (44.5%)	0.942 (0.755 to 1.175)	0.594
Cesarean section	71 (35.3%)	63 (36.4%)	1.031 (0.786 to 1.353)	0.826
Forceps delivery	6 (3.0%)	2 (1.2%)	0.387 (0.079 to 1.894)	0.295
GDM	40 (19.9%)	29 (16.8%)	0.842 (0.546 to 1.298)	0.435
HDP	16 (8.0%)	15 (8.7%)	1.089 (0.555 to 2.138)	0.804
PROM	34 (16.9%)	32 (18.5%)	1.094 (0.706 to 1.694)	0.689
Fetal anomalies	1 (0.5%)	1 (0.6%)	1.162 (0.073 to 18.437)	1.0
LBW	7 (3.5%)	7 (4.0%)	1.162 (0.416 to 3.247)	0.775
VLBW	2 (1.0%)	2 (1.2%)	1.162 (0.165 to 8.161)	1.0
Macrosomia	5 (2.5%)	4 (2.3%)	0.929 (0.254 to 3.407)	1.0

sPTB, spontaneous preterm birth; GDM, gestational diabetes; HDP, hypertensive disorder in pregnancy; PROM, premature rupture of membranes; LBW, Infant low birth weight; VLBW, Infant very low birth.

TABLE 5 Logistic Regression of Conception Outcome.

Baseline Parameter	Category	aOR (95% CI)	P
Age (years)	per year increased	0.96 (0.909 to 1.014)	0.146
BMI (kg/m2)	per kg/m2 increased	0.982 (0.929 to 1.039)	0.538
LH/FSH	per increased	1.184 (0.968 to 1.45)	0.1
AMH (ng/ml)	per ng/ml increased	1.03 (0.979 to 1.085)	0.256
T (ng/ml)	per ng/ml increased	0.743 (0.323 to 1.707)	0.484
TSH (mIU/L)	per mIU/L increased	0.856 (0.716 to 1.023)	0.087
Type of treatment	D3 *vs.* D5	1.71 (1.161 to 2.519)	0.007

Analyses were adjusted for maternal age, maternal BMI, LH/FSH, AMH, T, TSH.
aOR, adjusted odd ratio.

complications and outcomes (12, 13). Women with PCOS can exert a potentially adverse effect on the endometrium with pre-conception, conception, and post-conception endometrial function. LE is a third-generation, highly effective, and specific aromatase inhibitor that does not bind to estrogen receptors and does not affect the quality of cervical mucus or the thickness, morphology, and tolerability of the endometrium. Past studies have demonstrated the ability of LE to improve endometrial development, and it is more conducive to embryo implantation (14). During controlled ovarian stimulation (COS), LE administration during the early follicular phase significantly increases testosterone and androstenedione levels in the follicular fluid, thereby improving the follicular sensitivity to FSH stimulation and optimizing pregnancy outcomes. Our data indicate that multiples pregnancy and rates of other adverse events during pregnancy (including anomalies) are similar in the two treatments we evaluated. The rate of multiple pregnancies is consistent with a randomized, double-blind study (6.1%) (6). Our Pregnancy loss with LE on D3 and D5 was similar (p=0.359), and this data was in general agreement with the results of a study that included 42 RCTs in which Pregnancy loss was 19% (7). Our data show that using LE on the fifth day of menstruation does not increase the risk of teratogenicity or miscarriage compared to using LE on the third day.

There have been conflicting reports about the timing of LE's medication. Kaitlin et al. found that a single oral dose of LE 25 mg for 1 day versus 5 mg/d for 5 days resulted in comparable cycle pregnancy rates in both groups (14.2% vs. 11.6%) (15); Mitwally et al. showed that a single oral dose of LE 20 mg on day 3 of the menstrual cycle compared with 2. 5 mg/d on days 3-7 of the menstrual cycle resulted in comparable cycle pregnancy rates in both groups (15% vs. 18%) (16); Badawy et al. discovered that oral administration of LE 2.5mg/d on days 1-10 of the menstrual cycle resulted in a higher pregnancy rate than patients who received 5mg/d on days 1-5 of the menstrual cycle (17. 4% vs.12. 4%) (17); Other scholars believe that if there is no dominant follicle in the ovary and endometrial lesions are ruled out, any time during the follicular phase can be used as the time to initiate ovulation-promoting drugs. Nonetheless, the most widespread clinical use is for 5 days starting on day 3 or 5 of the menstrual cycle or progesterone withdrawal bleeding. Because LE is taken orally from day 3 or 5 of the menstrual cycle, it can pass a half-

life (45 hours), which is right in the selection period of dominant follicles (5-7 days of the menstrual cycle) (18). Therefore, this method of medication has long been considered the clinic's most reasonable ovulation induction plan.

However, no studies have compared the effect of oral LE starting on the third day and fifth day of the menstrual cycle. In our study, LE initiation on the 5th day of the menstrual cycle had higher ovulation rates, endometrial thickness (on the day of HCG intramuscular injection), pregnancy rates, and clinical pregnancy rates in women with PCOS than LE initiation on the 3rd day of the menstrual cycle. In the present study, though the median (IQR) maximum follicle diameter in the D3 and D5 groups were comparable on the day of intramuscular injection of HCG(P=0.0730), the median (IQR) endometrial thickness was significantly better in D5 group [9.0(8.0 to 10.0)mm] compared with D3 group [8.0(7.0 to 10.0)mm]. Roy et al. reported similar results in a randomized control trial of LE versus CC in women undergoing superovulation, which showed that vascular penetration of the endometrium was associated with thicker endometrium (19). At the same time, Chien et al. confirmed a significant increase in pregnancy rate with deeper endometrial vascular penetration (20). Therefore, we believe that the endometrium on the day of ovulation when LE started on the fifth day of menstruation is thicker than when LE started on the third day of menstruation. For the D5 group, the thickness of the endometrium is more compatible with the dominant follicles, and the permeability of the endometrial vascular is more profound. HCG was routinely administered to allow follicle maturation and precisely the time the intercourse for these couples to increase the conception rate of patients with PCOS favorably.

LE has a high cumulative ovulation rate in the trial by Legro et al.(61.7%), comparable to the cumulative ovulation rate in our D3 group (60.6%). However, the study was biased towards obese subjects, which may account for the lower live birth rate (8). The D5 group resulted in an increased pregnancy rate compared to the D3 group (RR 1.158, 95% CI 1.007 to 1.331). Our pregnancy rate was higher than that of the most recent Cochrane review (35%) (21), and we had obese patients lose weight before our OI treatment may have contributed to this. Moreover, our small sample size could be a contributing factor. However, a high-quality study found that PCOS patients using LE had a pregnancy rate of 61.2%, which is comparable to our findings (6).

A possible explanation for the higher success of using LE on D5 is the greater ovulation rate per cycle in the D5 group. Aromatase activity occurs in granulosa cells of follicles larger than 6-8 mm on Day 5-8 of the menstrual cycle. The dominant follicle produces more estradiol-17 than the cohort's other follicles, inhibiting FSH. LE administered on day 5 of menstruation effectively inhibits aromatase activity, enlarges the FSH window to stimulate follicle growth, and enables the simultaneous selection of multiple follicles. However, past researches indicates that around one-fourth of apparently healthy women may experience more than one follicle selection during a single menstrual cycle. It is conceivable that the variability of follicular dynamics in women of reproductive age is more significant than previously believed. We speculate that more than one follicular selection was probably generated in the D5 group (22).

Many studies have pointed to a decreasing trend in pregnancy rates as the age of the mother increases (23). In our research, we found that the conception rate was significantly higher in the D5 group than in the D3 group when the age was <30 years (81.1% vs. 66.3%, P=0.003), and the live birth rate was better in the D5 group than in the D3 group (71.2% vs. 57.7%, P=0.013). Therefore, for PCOS patients of optimal reproductive age, initiating LE on the 5th day of the menstrual cycle is the best time to promote ovulation.

BMI is one of the most important factors affecting fertility and pregnancy outcomes in women of childbearing age, obesity is responsible for an increased risk of subfecundity and infertility, and obesity has a significant impact on different PCOS phenotypes (24). For PCOS patients in different BMI groups, our study found that among PCOS patients whose BMI was 18.5-25 (normal weight), the pregnancy rate in D5 group was higher than that in D3 group (76.0% *vs*.63.3%, P=0.009). Our PCOS participants met the generally accepted Rotterdam diagnostic criteria for PCOS and had a median BMI of about 22 kg/m2. We believe that this cohort is well representative of PCOS women who receive fertility treatment at most fertility centers. Therefore, our results can be generalized to clinical practice on a global scale.

Past studies have shown that in the pathogenesis of PCOS, the secretion pattern of gonadotropin-releasing hormone (GnRH) is disrupted, leading to a relative increase in the release of LH and FSH. Although the LH/FSH ratio is not part of the androgen excess society's diagnostic criteria for PCOS, in healthy women, the ratio of LH/FSH is usually between 1 and 2. In women with PCOS, this ratio flips. In some cases, it can reach 2 or 3. Ovulation does not occur in patients with PCOS due to the high LH/FSH ratio (25). Because the LH/FSH cut-off point is thought to indicate the responsiveness of ovaries to ovulation-stimulating medications, studies on the link between the LH/FSH cut-off point and pregnancy outcomes have been done since 1995 (26). Su et al. found that baseline LH/FSH level was a significant independent risk factor for live birth ($p<0.05$) (27). Our study found that when LH/FSH<2, the D5 group had a higher conception rate than the D3 group, with significant statistical differences. After adjusting for confounders, each unit increase in LH/FSH resulted in a 21.4% increase in a live birth. The small sample size could be the reason for this difference.

AMH is a product of the granulosa cells of the antral follicles, and serum AMH levels are 2-5 times higher in women with PCOS than in normal subjects due to the increased number of small follicles and excessive production of AMH per follicle (27, 28). We performed an analysis according to the quartile of maternal AMH. When AMH is in the 25th to 100th percentile range, the pregnancy rate of the D5 group is significantly higher than that of the D3 group. When AMH is in the 25th to 75th percentile range, The live birth rate of the D5 group was better than the D3 group, and the difference was statistically significant.

Therefore, it can be inferred that the initiation of LE ovulation induction therapy on the fifth day of the menstrual cycle is more suitable for most anovulation PCOS patients.

Hyperandrogenemia is a prominent feature of PCOS and plays a significant role in its pathogenesis (29). Studies have shown that hyperandrogenemia in PCOS may alter the growth and function of early-onset follicles, leading to abnormal follicular development, which may affect conception and pregnancy (30). Our study found that among patients with T <0.8ng/mL, the pregnancy rate in the D5 group was significantly higher than that in the D3 group (76.6% vs. 65.3%, P=0.012). When T≥0.8ng/mL, the pregnancy rate in the D5 group was higher than that in the D3 group, but the difference was not statistically significant (P=0.177). Considering the sample size, we cannot rule out potential differences, which need to be confirmed by further studies.

Furthermore, we discovered that conception rates were significantly higher in the D5 group when TSH was in the 25th-75th percentile range. Logistic regression studies revealed that TSH levels were not substantially related to conception outcomes. Some studies have reported that TSH levels of 2.5 mIU/L did not link with fecundity, pregnancy loss, or live birth (31). That is similar to our findings, where higher D5 group conception rates contributed to identifying novel treatment options in the long term. The increase in E2 (estradiol) levels reduces free thyroid-hormone levels and increases the release of thyrotropin-releasing hormone (TRH), causing TSH levels to rise as a result of ovarian stimulation (32). That might explain the difference in conception rates between the two groups with high TSH levels.

Our retrospective study had several following limitations. First of all, the sample size was still relatively small, which may affect the results of this study. Then, this study did not manage patients uniformly during the pregnancy because it only analyzed the outcome data based on the completed cases, these intrinsic limitations, may impact its results. Third, this study did not utilize randomization and blinding, which may increase the risk of case selection. Finally, the 2018 guidelines proposed multiple phenotypes of PCOS (33), and our study did not further explore the optimal timing of ovulation induction for LE initiation in PCOS patients with different phenotypes.

In summary, our data showed that women who started LE on the fifth day of their menstrual cycle were superior to those who started LE on the third day as a treatment for anovulatory infertility in women with PCOS. The D5 group was associated with higher ovulation and conception rates and shorter time-to-pregnancy. These novel results suggest that this simple strategy may be an alternate low-risk, low-cost infertility treatment that offers superior reproductive results. Therefore, we recommend initiating LE on the fifth day of the menstrual cycle may be the best time for OI therapy with PCOS women. A further larger size of prospective randomized controlled studies is needed to clarify the optimal start time of LE OI therapy.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by The Ethics Review Committee of the Hangzhou Women's Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

ZZ and LS contributed to the conception of study. LS and SY were responsible for study designing, statistical analyses, and manuscript writing. ZZ and XJ contributed to revising the manuscript. LS, MG, and YC contributed to collecting data. All authors contributed to the article and approved the submitted version. LS and SY have contributed equally to this work.

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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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Cardiovascular disease risk in offspring of polycystic ovary syndrome

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Polycystic ovary syndrome (PCOS) is the most common endocrine disorder affecting women at reproductive age. PCOS diagnosis (Rotterdam criteria) is based on the presence of two out of three criteria; clinical and/or biochemical hyperandrogenism, oligo- or an-ovulation and polycystic ovaries. PCOS women suffer from a constellation of reproductive and metabolic abnormalities including obesity and insulin resistance. PCOS women also have increased blood pressure and increased risk of cardiovascular diseases (CVD). *In-utero*, offspring of PCOS women are exposed to altered maternal hormonal environment and maternal obesity (for most of PCOS women). Offspring of PCOS women could also be subject to genetic susceptibility, the transgenerational transmission of some of the PCOS traits or epigenetic changes. Offspring of PCOS women are commonly reported to have an abnormal birth weight, which is also a risk factor for developing CVD and hypertension later in life. Although studies have focused on the growth pattern, reproductive and metabolic health of children of PCOS women, very limited number of studies have addressed the risk of hypertension and CVD in those offspring particularly as they age. The current narrative review is designed to summarize the available literature (both human studies and experimental animal studies) and highlight the gaps in addressing hypertension and CVD risks in offspring of PCOS women or hyperandrogenemic female animal models.

KEYWORDS

cardiovascular disease, hypertension, polycystic ovary syndrome, offspring, sex differences

Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder affecting women at reproductive age (1). Although the guidelines for diagnosis of PCOS have evolved over time, the currently-used Rotterdam criteria developed by the European Society of Human Reproduction and Embryology/American Society for Reproductive

Medicine Rotterdam consensus (ESHRE/ASRM) requires the presence of two out of three features to establish a PCOS diagnosis: oligo- or an-ovulation (OA), clinical and/or biochemical hyperandrogenism (HA), and polycystic ovarian morphology (PCOM) on ultrasound (2).

In addition to being a reproductive and endocrine disorder, PCOS women suffer from metabolic anomalies and obesity, which is their highest priority reason for seeking medical care (3). PCOS women also have increased blood pressure (BP) and increased risk of cardiovascular diseases [CVD] (4), with various involved mechanisms including obesity, sympathetic nervous system activation, renin-angiotensin system (RAS) activation and increased 20-hydroxyeicosatetraenoic acid (20-HETE) in the renal microvasculature [reviewed in (5)]. Given the myriad of signs/symptoms associating PCOS, a lot of questions arise about the health of the offspring. In part they are subjected to the altered *in-utero* environment including maternal hyperandrogenemia. They are also subjected to maternal obesity which has been shown to negatively impact the cardiovascular and metabolic health of the offspring (6–9). The altered maternal environment persists across the lactation period if their mothers are nursing them. There is also a genetic component and the possible transgenerational transmission of PCOS traits (10, 11). Studies have addressed the growth patterns and metabolic and reproductive health of the offspring (12–14). However, very limited studies addressed the risk of developing hypertension in the offspring, which represents a major risk factor for CVD (15), and whether those risks are sex-specific.

Pregnancy in PCOS women, and associated complications

PCOS women have difficulty getting pregnant and they often require assisted reproduction [e.g. invitro fertilization] (16). PCOS women diagnosed according to the Rotterdam criteria could have one of 4 different phenotypes; full-blown phenotype A (having PCOM, OA and HA), phenotype B (having HA and OA), phenotype C (ovulatory PCOS having HA and PCOM) and phenotype D (non-hyperandrogenic PCOS having OA and PCOM] (17, 18). Decreased fertility in women with PCOS could not be fully explained on the basis of ovulatory dysfunction (19, 20). A comprehensive review utilizing literature from 1970 – 2020 showed that PCOS women exhibit endometrial dysfunction which partly underlies their poor reproductive outcomes. This dysfunction could be due to hyperandrogenemia, insulin resistance, obesity or even chronic inflammation (21). Decreased fertility in PCOS women could also be a result of decreased oocyte competence, which could be influenced by PCOS comorbidities (obesity and insulin resistance) in addition to the differences between the PCOS phenotypes (20).

Studies addressing PCOS pregnancy-associated complications have some discrepancy in their findings. For example, Haakova and colleagues have shown that PCOS is not associated with increased risk of gestational diabetes mellitus (GDM) or pregnancy-induced hypertension (PIH) along with no significance in the gestational weight gain between PCOS and control women (22). On the contrary, the majority of studies found that PCOS women have increased risk of miscarriage, PIH, GDM, pre-eclamptic toxaemia and preterm delivery (23, 24). Homburg and colleagues showed that results of different studies are related to their cohort size, where studies with fairly-small cohorts (n = 22-47) show increased incidence of hypertensive disorders of pregnancy in PCOS, while large-scale studies found no correlation between PCOS and hypertensive disorders of pregnancy [reviewed in (25)]. Another important consideration is the variability of the criteria used for diagnosis of PCOS women enrolled in those studies starting by the NIH 1990 criteria and including the currently-used Rotterdam criteria, with some studies even not mentioning the criteria used. It is also important to think of this discrepancy from the view of the control group, it is possible that the variation in the control group used (whether they are just age-matched healthy women or age-matched and body mass index (BMI)-matched women) is the reason behind the variation seen between the findings of those studies. An extensive review summarizing clinical and pathophysiological features of pregnancy in PCOS concluded that PCOS women have a clinically-significant increased risk (3-4 fold) of pregnancy complications (including PIH, PE, GDM and premature delivery) compared with controls without adjusting for other confounders (including BMI, infertility treatments and others). Women with PCOS still had increased risks of the same pregnancy complications after adjusting for confounders (1.5-2 fold) compared to women without PCOS (26). Unfortunately, the exact pathophysiological mechanism of pregnancy complications in PCOS remains unclear.

The question remains as to whether pregnancy complications are linked to a certain PCOS phenotype. PCOS women diagnosed according to the Rotterdam criteria could have one of 4 different phenotypes; full-blown phenotype A (having PCOM, OA and HA), phenotype B (having HA and OA), phenotype C (ovulatory PCOS having HA and PCOM) and phenotype D (non-hyperandrogenic PCOS having OA and PCOM] (17, 18). Palomba et al., (27), reported increased cumulative rates of adverse obstetric and neonatal outcomes in PCOS women compared to BMI-matched controls. The authors compared the ovulatory PCOS women to the oligo- or an-ovulatory PCOS women and found that the latter group had higher risk of miscarriages, PIH, GDM and operative delivery. Although the risk of pre-eclampsia (PE) was higher in PCOS women than controls, it was similar between ovulatory and oligo- or an-ovulatory women with PCOS (27). The same study categorized women into the 4 Rotterdam phenotypes and showed that the full-blown PCOS (phenotype A) and phenotype B had the highest incidence (93% and 86%, respectively) of adverse neonatal and obstetric outcomes,

followed by phenotypes D and C (60% and 22%, respectively) (27). On the other hand, a recent retrospective study that used data extracted from computerized database in France showed that oocyte morphology (essential for fertilization and subsequent fetal development) and percentage of normal oocytes was similar between women with PCOS phenotypes A, C and D (28). In an opinion paper, the author suggested that PCOS women have reduced fertility that is caused by altered oocytes, embryo and endometrial competence, regardless of their ovulatory status (19).

Offspring birth weight in PCOS; human studies versus experimental animal studies

Studies indicated that PCOS pregnancy could be associated with increased risk of abnormal birth weight in the offspring. The majority of studies showed offspring are born small-for-gestational age (SGA), an indication of intrauterine growth restriction [IUGR] [(24, 29, 30) and reviewed in (23, 25)], and some studies showed offspring are born large-for-gestational age [LGA] (16, 31). The variation could be attributed to the different phenotypes of PCOS, different diagnostic criteria used, different ethnicities of PCOS women or environmental factors surrounding the PCOS women (32) or differences in the pre-pregnancy BMI (33) or maternal diet consumed during pregnancy and lactation. Palomba et al. showed an increased incidence of SGA and LGA and decreased incidence of appropriate-for-gestational age (AGA) babies in women with PCOS compared to controls. The authors showed that only babies from PCOS mothers of phenotypes A and B had significantly higher risk of being born SGA than babies from mothers with phenotypes D and are less likely to be born AGA than babies from mothers with phenotypes D or C, suggesting that the combination of hyperandrogenemia and ovulation disturbance as a key factor for abnormal birth weight in the offspring (27). Interestingly, women with phenotype C and D in this study had a similar percentage of AGA babies as seen in control women of the same study (~ 80 – 86%) (27). Fux-Otta et al. compared pregnancy outcomes from two Latin American populations of women with PCOS and found that offspring from Argentinian PCOS women had higher incidence of SGA babies compared to Chilean PCOS women after adjusting to different maternal factors (32).

Theories behind having SGA babies in PCOS included insulin resistance and insulin-dependent growth dysfunction (25). Another hypothesis is that fetal exposure to excess androgens can induce changes in differentiating tissues, which would also cause the PCOS phenotype to develop in adult life (34). PCOS women with clinical signs of hyperandrogenism have been shown to have decreased endometrial and sub-endometrial blood flow indices (35), which could affect offspring birth weight. Chekir et al. also reported impaired uterine artery perfusion in women with PCOS that correlates with their hyperandrogenemia (36). According to the Barker hypothesis, SGA offspring are at increased risk of metabolic disease, obesity and hypertension as they age (37, 38). LGA offspring are also at increased risk of adverse cardiovascular outcomes with aging (39). Unfortunately, the impact of birth weight on cardiovascular health later in life or the correlation between the maternal PCOS phenotype and the later cardiovascular health in PCOS offspring has not been clearly studied. Gunning and colleagues showed that there was no correlation between maternal androgen levels and offspring BMI or blood pressure at infancy or early childhood (2.5-8 years). Unfortunately, although mothers in this study were diagnosed according to the Rotterdam criteria, the study did not aim at differentiating between the different PCOS phenotypes (33).

On the other hand animal studies have consistently described IUGR in offspring exposed to maternal hyperandrogenemia. It is important to highlight that androgen injection or implantation is the tool utilized by those studies to induce maternal conditions that mimic human PCOS. In sheep, testosterone injections in the dams during early-mid pregnancy (gestational day (GD)30 - GD90; term is 147 days) caused females offspring only (40, 41) or offspring of both sexes (42) to be born with IUGR. In rats, testosterone injections in dams during late pregnancy (GD15 - GD19 of pregnancy; term is 21 days) results in IUGR in female offspring (43) or male offspring (44) or offspring of both sexes (45). Similarly, our studies have shown that maternal exposure to 5 alpha-dihydrotestosterone (DHT) starting prepubertally and continuing throughout pregnancy and lactation results in obesity and insulin resistance in the dams. Importantly, under those conditions of impaired maternal metabolic health and maternal hyperandrogenemia, but no maternal hyperglycemia, offspring of both sexes were still born with IUGR (46–48). Prenatal androgen-induced fetal growth restriction has been attributed to defective transfer of amino acids to the fetus (45), impaired placental function (34) or decreased insulin growth factor availability (42). This could support the suggestion that maternal factors other than hyperandrogenemia (e.g. maternal diet or GDM or other comorbidities) are responsible for having LGA offspring in some PCOS women.

Do daughters of PCOS women develop hypertension? data from human studies

The pathogenesis behind the development of PCOS is not clear. The origin of PCOS is thought to be multi-factorial and

involves an interaction of environmental and genetic factors over the life span. Environmental factors associated with PCOS can be classified into prenatal (intra-uterine environment and fetal developmental programming) or postnatal [diet, obesity, sedentary life style, etc.] (49). Therefore daughters of PCOS women were often studied for their potential to develop PCOS themselves. Some studies show that female offspring of PCOS mothers develop symptoms of PCOS (hyperandrogenemia, cystic ovaries, abnormal menses) with adolescence and young adulthood, and some studies do not, hence whether female PCOS offspring develop PCOS themselves is controversial. For example, some studies found PCOS daughters to have increased insulin (50–52), BMI (53), ovarian volume (52), increased anti-Mullerian hormone (53, 54), menstrual disturbances (54), and increased levels of testosterone pre- and post-pubertal (51, 52, 54). Meanwhile other studies found PCOS daughters to have similar BMI (52, 55) and ovarian volume as controls (56), with no change in insulin sensitivity and glucose tolerance post-puberty (57), and no increase in testosterone in childhood (55) when compared to BMI-matched controls (53, 54). De Leo, et al., suggested a cross-generational relationship between the degree of maternal hyperandrogenism and the development of PCOS in their daughters (58).

Despite those extensive studies on the reproductive and metabolic health of daughters of PCOS women, little is known about their risk for hypertension and CVD. Daughters (4-17 years of age) of PCOS mothers (diagnosed according to the older NIH criteria) had similar BMI, body composition and importantly BP (cuff method) compared to controls at all assessed ages (56). Another study showed that normal weight eumenorrheic daughters of PCOS mothers that have PCOM are still at increased risk of CVD as demonstrated by increased ambulatory BP and decreased plasma nitric oxide (NO) metabolites, along with hyperinsulinemia and hyperglycemia at 24-26 years of age, despite the absence of hyperandrogenemia in those daughters (59).

Lessons from animal studies in female offspring

PCOS is diagnosed as early as menarche. PCOS women have a 2-3 fold increase in their androgen levels. Studies have shown that women with PCOS maintained a high androgen level throughout pregnancy (60–63) and lactation (64). In addition to this, the majority of PCOS women suffer from a myriad of signs/symptoms that include obesity, insulin resistance and glucose intolerance, all of which could induce reproductive dysfunction and adverse effects on the offspring.

Our group used a rat model of hyperandrogenemia, originally developed by Manneras and colleagues (65), that is induced by subcutaneous implantation of DHT pellets in female Sprague Dawley (SD) rats starting at 4 weeks of age (pre-pubertal) to induce hyperandrogenemia as seen in PCOS women (66). Manneras, et al., showed that the DHT-treated rats exhibited irregular cycles along with PCOM (65). The pellet releases the androgen over a period of 90 days and is replaced every 85 days so hyperandrogenemia is maintained throughout the rat life, as in PCOS, making it suitable for studying different age changes in PCOS (47, 67–69). DHT, unlike testosterone, is a non-aromatizable androgen, therefore does not cause an increase in estradiol levels, neither does it suppress endogenous synthesis of estradiol in rats (66). Upon induction of hyperandrogenemia, rats developed obesity (increased fat mass), insulin resistance, glucose intolerance and an increase in their BP, same as what happens in PCOS women (66). Upon breeding DHT-treated SD females with vendor-supplied SD males, pregnancy occurred in 60% of DHT-treated females, compared to 99% in controls, and offspring are born smaller than control offspring despite the similar liter size, suggesting IUGR as seen in SGA babies from PCOS women (46–48). Thus offspring born to this rat model provide a very realistic tool to assess the CVD risks and their mechanisms in offspring of hyperandrogenemic dams as an experimental model of PCOS.

Female offspring born to DHT-treated females had similar estradiol and testosterone levels compared to control offspring as adults. They did not develop obesity (increased fat mass), had a normal serum lipid profile and normal renal function (indicated by normal proteinuria levels) as adults (46). Surprisingly, female offspring of DHT-treated dams had lower urinary nitrate/nitrite excretion, but remained normotensive. Nitrates/nitrites are metabolites of the vasodilator NO and are used as a measure of endogenous NO levels. We tested their response to exogenous angiotensin (Ang) II after blocking their endogenous RAS by enalapril, an Ang converting enzyme inhibitor, and found that they had a suppressed response to Ang II which could indicate being protected against CVD risks (46). Our results from the female offspring of DHT-treated dams are summarized in Table 1. Similar to our studies, using a mouse model injected with DHT during pregnancy (GD16.5 - GD18.5, late pregnancy in mice), female offspring developed cardiac hypertrophy with no change in their BP. The findings in the mouse model were independent of maternal diet and the metabolic profile of the female offspring (70).

Chinnathambi et al. used a rat model to show that female offspring of dams injected with testosterone (GD15 – GD19, late pregnancy in rats) developed hyperandrogenemia and hypertension as adults. The authors concluded that hypertension in those female offspring was mediated by gonadal testosterone because ovariectomy normalized BP (71). They also showed that female offspring develop a decrease in the levels of NO synthase in their mesenteric arteries, with a decrease in NO-mediated vascular relaxation (72). More and colleagues used a similar model and showed that female offspring develop hypertension as adults, along with an increased contractile response to Ang II in mesenteric arteries due to downregulation of Ang II type-2 receptors (73). King et al.

used a sheep model to show that female offspring of dams injected with testosterone (GD30 – GD90, early-mid pregnancy in sheep) also develop mild hypertension as adults. However hypertension is independent of gonadal hormones. It was also independent of plasma aldosterone and catecholamine levels (74). Importantly, testosterone is an aromatizable androgen that is converted to estradiol causing high levels of estradiol during gestation, which raises the question whether the hypertensive effect in the female offspring in those studies is purely androgenic or partly estrogenic.

Do sons of PCOS develop hypertension, and are there sex differences in CVD risks in offspring of PCOS? data from human studies

Despite the accumulating evidence of the existence of sex differences in the etiology of hypertension and CVD risks (75, 76), studies have not clearly addressed sex differences in the risk of CVD in children born to PCOS mothers. For instance, children of PCOS women, aged 2.5 - 8 years (not separated by sex), had higher aortic pulse pressure, left ventricular internal diameter, and carotid intima-to-media thickness compared to control children (77). The same study pointed out that children of PCOS mothers also had higher triglycerides (TG) and low-density lipoprotein cholesterol (LDL-c) compared to controls (77). Another study that used data from the Western Australia Data linkage system pointed out that PCOS offspring (not separated by sex) are at increased risk of postnatal hospitalizations and were at higher risk of being born with congenital cardiovascular anomalies [1.5% compared with 1.0%, odds ratio 1.37, 95% CI 1.01–1.87] (78), which suggests their increased risk of CVD later in life.

A systematic review and meta-analysis of nine observational studies including offspring of PCOS women from Chile, Netherlands and the US re-analyzed the data with/without stratifying for the sex of the offspring and summarized cardiometabolic health as metabolic sum scores 1 (BMI + systolic BP (SBP) + insulin levels + TG + high-density lipoprotein cholesterol (HDL-c)) and 2 (waist-to-height ratio + SBP + glucose + TG + HDL-c). The authors showed that both metabolic sum scores 1 and 2 were not different between PCOS offspring and control offspring when the data are not stratified for sex; however, with the stratification there was a significant interaction between both sexes, suggesting that sex of the offspring was a significant mediator of cardiometabolic outcomes, when comparing PCOS offspring versus control offspring (13).

A Chilean study recruited sons of women with PCOS (NIH criteria) at 2-3 months of age (infants), 4-7 years of age (children), and 18-30 years of age (adults) to study their metabolic health. The authors showed that sons of PCOS women had increased body weights at all tested ages compared to controls, and had insulin resistance with hyperinsulinemia as adults (79). Later on, the same group showed that sons of women with PCOS (NIH criteria) at 7-18 years of age had hypercholesterolemia and increased LDL-c compared to controls (80). Unfortunately, neither study addressed BP in PCOS sons. Thus it remains unclear whether sons of women with PCOS are at increased risk of developing hypertension or CVD as adults or not.

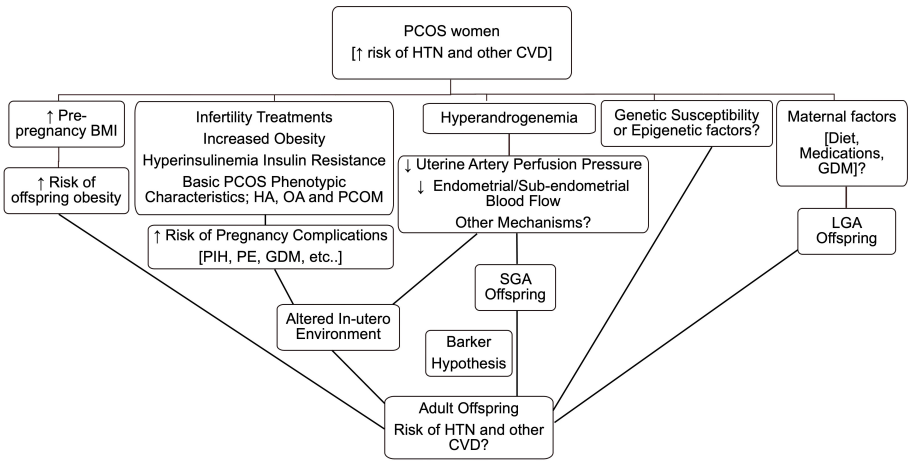

FIGURE 1
Rationale and possible mechanisms behind increased risk of hypertension (HTN) and other cardiovascular diseases (CVD) in offspring of polycystic ovary syndrome (PCOS) women. BMI, body mass index; HA, hyperandrogenism; OA, oligo- or an-ovulation; PCOM, polycystic ovarian morphology; GDM, gestational diabetes mellitus; PIH, pregnancy-induced hypertension; PE, pre-eclampsia; LGA, large-for-gestational age; SGA, small-for-gestational age.

Lessons from animal studies in male offspring

Using our DHT-treated rat model explained earlier, adult male offspring had a decrease in their body weights that is mediated by a decrease in their lean mass. They also developed hypercholesterolemia and increased urinary protein excretion (marker of renal injury). Importantly, although those males remained normotensive at baseline, they had an exaggerated pressor response to chronic Ang II after blocking their endogenous RAS with enalapril (Table 1) (48). This could suggest an increased risk of hypertension and CVD with aging in male offspring of hyperandrogenemic dams.

Chinnathambi, et al., showed that although both male and female offspring of testosterone-treated dams (rat model) develop hypertension as adults, the increase in BP in prenatal testosterone-exposed adult males was more pronounced than in females (71). Unlike female offspring, male offspring of testosterone-treated dams developed a decrease in endothelium-derived hyperpolarizing factor-mediated relaxation of mesenteric rings (72). Injecting testosterone directly into the flanks of male fetuses (GD62 - GD82, post-sexual differentiation in sheep fetus) resulted in dyslipidemia and altered metabolic health (hyperinsulinemia with normal testosterone levels) (81), but the authors did not address BP.

What happens to BP in offspring of PCOS with aging?

Currently-used criteria for diagnosis of PCOS (Rotterdam criteria) have been in place since 2003/4, which makes offspring of the population of women diagnosed according to this diagnosis paradigm still in their late teens (2). Older criteria (NIH) were in place in 1990, so offspring of this population are now in their third decade of life. Therefore, human studies so far have not addressed the question of whether maternal hyperandrogenemia as seen in PCOS would impact cardiovascular health of the offspring as they age. In fact, studies addressing the impact of aging on the cardiovascular health of PCOS women themselves are still lacking. It is well-accepted that males are at increased risk of hypertension and CVD when they are young, compared to age-matched women [reviewed in (82)]. According to the National Health and Nutrition Examination Survey conducted on 9,623 participants, the prevalence of stage 2 hypertension (SBP/DBP >/= 140/90 mmHg) among women older than 75 years was 78% compared to 71% in men. Also the prevalence of stage 1 hypertension (SBP/DBP >/= 130/80 mmHg) among women older than 75 years was 85% compared to 79% in men (83). The question still remains as to whether male and female offspring of PCOS mothers will carry exaggerated risks of CVD with aging or not.

Using the DHT-treated female rats, we have shown that female offspring at 16-18 months of age (post-estrous cycling) remained normotensive, despite decreased renal function (higher proteinuria). They had lower heart rates compared to the adult female offspring of DHT-treated females, suggesting further damage to the heart function with further aging. Post-estrous cycling female offspring of DHT-treated dams also had similar fat mass and body weight, but higher serum total cholesterol compared to age-matched controls. We challenged them with exogenous angiotensin II after blocking their endogenous RAS with enalapril to determine their pressor response. Post-estrous cycling female offspring of DHT-treated dams had a suppressed pressor response to Ang II and Ang II plus 4% salt diet, which could be partly due to increased intrarenal Ang 1-7 (vasodilator arm of RAS). Our future studies will aim at addressing CVD risk in the female offspring with further aging, and in male offspring (46).

TABLE 1 Characteristics of female and male offspring of DHT-treated dams compared to their age- and sex-matched controls.

	Adult male offspring (4-6 months of age)	Adult female offspring (4-6 months of age)	Post-estrous cycling female offspring (16-20 months of age)
Born smaller than controls (intra-uterine growth restriction)	Yes (47, 48)	Yes (46, 47)	
Body weight	Lower (48)	Similar (46)	Similar (46)
Lean mass	Lower (48)	Similar (46)	Similar (46)
Fat mass	Similar (48)	Similar (46)	Similar (46)
Serum total cholesterol	Higher (48)	Similar (46)	Higher (46)
Proteinuria (marker of renal injury)	Higher (48)	Similar (46)	Higher (46)
Urinary nitrates/nitrites (measure of the endogenous vasodilator NO)	Similar (48)	Lower (46)	Similar (46)
Baseline blood pressure	Similar (48)	Similar (46)	Similar (46)
Pressor response to Ang II after blocking endogenous RAS	Exaggerated (48)	Suppressed (46)	Suppressed (46)
Pressor response to Ang II plus salt diet after blocking endogenous RAS	Not tested	Not tested	Suppressed (46)

NO, nitric oxide; Ang, angiotensin; RAS, renin-angiotensin system.

Impact of life style, diet or different medications received during PCOS pregnancy on offspring cardiovascular health

Another great gap in our knowledge is the impact of different factors including the PCOS mother`s life style and medications on the offspring cardiovascular health and their risk of developing hypertension. For example, some studies pointed out an increased prevalence of PCOS in western diet (WD)-consuming females (84, 85). Szczuko, et al., even suggested that the improper diet is the main reason behind metabolic abnormalities in PCOS (86). However, studies addressing the impact of maternal diet in PCOS on offspring cardiovascular health or risk of hypertension are lacking. Bishop and colleagues have shown that in Rhesus Macaques, testosterone-treated females require increased time to achieve pregnancy; however, WD-fed Macaques had decreased numbers of pregnancies and overall fertility to 70%. Importantly, testosterone-treatment plus WD consumption simultaneously decreased the number of viable fetuses compared to either testosterone or WD alone (87). Testosterone plus WD also promoted an increase in fasting blood glucose level and impairment of glucose tolerance during pregnancy (87), which could suggest further metabolic derangements in the offspring as they age. However, using a mouse model, Risal, et al., showed that prenatal androgen exposure, but not maternal WD consumption, causes transgenerational reproductive and metabolic dysfunction in female offspring (11). Neither addressed the cardiovascular health or BP of offspring with aging.

Another example is the use of metformin by pregnant PCOS women and its effect on their offspring BP as they go into adulthood. Various studies have addressed the role of metformin in reducing the incidence of miscarriage and GDM in pregnant PCOS [(88) and reviewed in (89, 90)]. Others found that metformin treatment during the whole pregnancy in PCOS could reduce the risk of IUGR in offspring [from 17%-22% in groups untreated with metformin or treated for only certain period during their pregnancies to 2% in group treated with metformin across the whole pregnancy] (88). On the contrary, a follow-up study on two randomized controlled trials showed that metformin-exposed children had higher BMI and increased prevalence of overweight/obesity at 4 years of age (91). Using a rat model of PCOS, Xie, et al., showed that metformin treatment during pregnancy reduces the risks of insulin resistance and obesity in female offspring (92).

Torstein and colleagues performed a follow-up study on a randomized clinical trial to include children of PCOS women (~ half of them received metformin during pregnancy and the other half were placebo-treated) with an average age of 8 years. In both metformin and placebo groups, BP in the offspring was within the normal range for the gender and age studied. However, SBP was higher in the metformin group (106 mmHg vs. 101 mmHg) with a borderline significance of $p = 0.05$. Meanwhile, DBP was not different between the groups. This study has a very limited number of participants with the majority of the metformin group being boys and the majority of the placebo group being girls, and data were not stratified according to sex most likely because of the limited number of participants (93).

Conclusion

Based on their abnormal birth weight, exposure to altered maternal environment both *in-utero* and during lactation and possible trans-generational transmission of some PCOS traits, the risk of hypertension and CVD in offspring of PCOS may be strongly predicted as shown in Figure 1. However, studies have not yet clearly addressed those risks in either adult or aging offspring. Sex differences are strongly suggested and should be examined further. Animal studies paying attention to duration and timing of exposure of dams to hyperandrogenemia and the associated metabolic abnormalities could provide a useful tool for determining the impact of maternal hyperandrogenemia plus maternal obesity (as seen in obese PCOS) on offspring cardiovascular health as adults, and even more importantly with aging.

Author contributions

NS conceptualized and designed the study, performed the literature search, drafted and revised the review article.

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Conflict of interest

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

Publisher's note

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Effects of follicular output rate on cumulative clinical pregnancy rate and cumulative live birth rate in PCOS patients with different characteristics

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Objective: We aim to explore the effects of follicular output rate (FORT) on cumulative clinical pregnancy rate (CCPR) and cumulative live birth rate (CLBR) in polycystic ovary syndrome (PCOS) patients with different characteristics undergoing *in vitro* fertilization (IVF) treatment.

Methods: This retrospective study analyzed 454 patients with PCOS undergoing their first IVF cycle at our center from January 2016 to December 2020. FORT was calculated as pre-ovulatory follicle count (PFC) × 100/antral follicle count (AFC). Multivariate regression analyses were conducted to explore the relationships between FORT and CCPR and CLBR. Curve fitting and threshold effect analyses were established to find nonlinear relationships. Effect modification in different subgroups were examined by stratification analyses.

Results: Based on the FORT values, individuals were classified into the following three groups: low-FORT group, middle-FORT group and high-FORT group. Multivariate regression analyses revealed that FORT was an independent factor affecting the CCPR and CLBR significantly (OR = 1.015, 95% CI: 1.001, 1.030 and OR = 1.010, 95% CI:1.001, 1.020). Curve fitting and threshold effect analyses showed that the CCPR and CLBR had a positive correlation with FORT when the FORT was less than 70% (OR = 1.039, 95% CI: 1.013, 1.065 and OR = 1.024, 95% CI: 1.004, 1.044). Stratification analyses showed that the CLBR increased by 1.3% with each additional unit of FORT for patients with hyperandrogenic manifestations (OR = 1.013, 95% CI: 1.001, 1.025). Compared with the low-FORT group, in the high-FORT group, CCPR increased 1.251 times for patients with polycystic ovarian morphology, while CCPR and CLBR increased 1.891 times and 0.99 times for those with ovulation disorder, respectively (OR = 2.251, 95% CI: 1.008, 5.028 and OR = 2.891, 95% CI: 1.332, 6.323 and OR = 1.990, 95% CI: 1.133, 3.494).

Conclusion: In patients with PCOS, cumulative IVF outcomes have a positive correlation with FORT when the FORT is less than 70%. For PCOS patients with polycystic ovarian morphology, ovulation disorder or hyperandrogenic manifestations, a high FORT could be conductive to achieving better pregnancy outcomes.

KEYWORDS

follicular output rate, cumulative clinical pregnancy rate, cumulative live birth rate, polycystic ovarian syndrome, PCOS characteristics, *in vitro* fertilization-embryo transfer

Introduction

Polycystic ovary syndrome (PCOS) typically manifests with hyperandrogenism, oligo-anovulation and polycystic ovarian morphology. Studies have shown that PCOS is the main cause of anovulatory infertility, affecting about 8-13% of childbearing age women (1–3). For PCOS patients, *in vitro* fertilization (IVF) treatment is an assisted reproductive option, where controlled ovarian stimulation (COS) is the main step. However, COS in these patients frequently leads to large quantities of poor-quality oocytes and increased incidence of ovarian hyperstimulation syndrome (OHSS).

Anti-Mullerian hormone (AMH), antral follicle count (AFC), and basal follicle stimulating hormone (FSH) levels have been used to adjust ovarian stimulation (OS), minimize risks and optimize assisted reproductive technology (ART) outcomes, but all of them have certain limitations (4–7). They do not reflect the dynamic nature of follicular growth in response to exogenous gonadotrophins (Gn) and their ability to predict clinical outcomes after ART is limited (7, 8).

In 2011, Genro et al. (9) proposed the concept of the follicular output rate (FORT), the ratio of pre-ovulatory follicle count (PFC) on the trigger day to the AFC, to quantify the follicular development potential. Gallot et al. (10) further explored the correlation between pregnancy rate and FORT in patients who underwent IVF treatment with regular menstrual cycles. They found that better pregnancy outcomes were related to high FORT values. In addition, Hassan et al. (11) studied women with unexplained infertility and found that FORT had an independent effect on clinical pregnancy rate.

Several studies have suggested that FORT may reflect clinical outcomes after ART in PCOS patients, but consensus has not been reached (12–14). In an early study of 140 patients with PCOS, the fertilization rate and high quality embryo rate were highest in the middle-FORT group, although the FORT groups did not differ significantly in the clinical pregnancy rate (12). However, Tan et al. (13) found that the clinical pregnancy rate and high quality embryo rate increased with FORT in PCOS patients. A recent study of PCOS patients performed by Yang et al. (14) showed that the cumulative live birth rate (CLBR) was highest in the high-FORT group but lowest in the middle-FORT group.

Although Yang et al. (14) investigated the association of FORT with cumulative ART outcomes, the impact of clinical characteristics of PCOS was not taken into account in this study. A previous research showed that PCOS patients with different phenotypic characteristics reflected varied ovarian responses to COS (15). Furthermore, AFC and CLBR were significantly different in PCOS patients with different phenotypic features (15–17). To date, conclusive and definite data about the role of clinical characteristics of PCOS on the relationship between FORT and cumulative ART outcomes are still lacking.

In this study, we investigated the relationship between FORT and cumulative ART outcomes in PCOS patients with different characteristics after one IVF cycle including all fresh and subsequent frozen-thaw embryos, in order to guide COS medication and help PCOS patients get better reproductive outcomes.

Materials and methods

Subjects

This was a retrospective study of 454 PCOS patients undergoing the first IVF cycle from January 2016 to December 2020 in the Second Hospital of Hebei Medical University. The diagnosis of PCOS was assessed by the Rotterdam criteria (18), which requires at least two of the following: (1) oligoanovulatory ovarian dysfunction (OAD); (2) biochemical or clinical evidence of hyperandrogenism (HA); (3) polycystic ovarian morphology (PCOM). Exclusion criteria included endocrine abnormalities (such as abnormal thyroid function or Cushing's syndrome), uterine cavity abnormalities, endometrial diseases, histories of ovarian surgery, chromosomal abnormalities, oocyte freezing, female age >38 years. Also, women who did not get a live birth

and did not run out of all embryos were excluded. This study was approved by our hospital ethical committee.

Treatment protocol

Patients adopted the gonadotropin-releasing hormone antagonist (GnRH-ant) protocol, gonadotrophin-releasing hormone agonist (GnRH-a) long protocol or GnRH-a prolonged protocol. In the GnRH-ant protocol, ovarian stimulation was started from the 2nd or 3rd day of the menstrual cycle with Gn (Recombinant Human Follitropin Alfa, MerckSerono, Italy, 75 IU) until one follicle reached 14 mm or more than six follicles reached 11-13 mm in diameter or serum E2 reached 400pg/ml, then GnRH-ant (Cetrorelix, MerckSerono, Switzerland, 0.25 mg) was administered daily. In the GnRH-a long protocol, triptorelin (Decapeptyl, Ferring, Germany, 1 ml; 0.1 mg) was used for pituitary down regulation, 0.1 mg once daily, from the middle luteal phase of the previous menstrual cycle. When the down regulation was confirmed, Gn was administered until triggering for oocyte maturation. In the GnRH-a prolonged protocol, GnRH-a (Decapeptyl, Ferring, Germany, 3.75mg) was injected in early follicular phase, and Gn was initiated 28-30 days later along with confirmation of pituitary down regulation.

For all the COS protocols, blood tests and ultrasound were used to monitor hormone levels and follicle growth. When the diameter of the leading follicle reached 18 mm or more than two follicles reached 17 mm, human chorionic gonadotropin (hCG) or GnRH agonists was used to trigger the oocyte final maturation referred to patients' hyperstimulation risk. Oocytes were collected under ultrasound scan 36–37h after triggering.

Collected oocytes from each woman were inseminated through conventional IVF and fertilization assessment was carried out 17h after insemination. Based on the Istanbul Consensus (19) and Vienna Consensus (20), the embryos were classified into grades I-IV referred to their morphology, cell number and the percentage of fragmentation at 72h after fertilization. Grade I embryos on day 3 of culture were taken as high quality embryos. Grade I-II embryos on day 3 were considered available embryos which could be transferred or frozen. The remaining cleavage embryos were cultured to blastocysts and those with a Gardner score above 3CC on day 5 or 6 were considered suitable for vitrification.

For fresh embryo transfer, the embryo transfer was carried out on the 3rd or 5th day following oocyte retrieval. For frozen embryo transfer, the patients started taking 2-3 mg of oral estradiol twice daily from the third day of the menstrual cycle for endometrial preparation. Vaginal progesterone was administered for corpus luteum support when the thickness of the endometrium reached 8 mm under ultrasound scan. Frozen embryo transfer was scheduled on the 4th or 6th day of corpus luteum support.

After embryo transfer, progesterone was given for corpus luteum support. The β-hCG level in peripheral blood was measured 2 weeks after embryo transfer. The transvaginal ultrasound examination of the gestational sac and heart beat was carried out 28-30 days following embryo transfer. Clinical pregnancy was defined as one or more intrauterine gestational sacs with heartbeat visualized under ultrasound scan.

Observation indicators

The AFC was recorded with a diameter of 3–10 mm at baseline. On the trigger day, the PFC was recorded with a diameter of 14–22 mm. FORT was calculated as PFC×100/AFC. The main outcomes of our study were cumulative clinical pregnancy rate (CCPR) and CLBR. CCPR was calculated as the number of clinical pregnancy cycles/number of first oocyte retrieval cycles. CLBR was calculated as the number of live birth cycles/number of first oocyte retrieval cycles.

Statistical analysis

We performed all statistical analyses with SPSS26.0 software and EmpowerStats (X&Y solutions, Inc., Boston, MA). Continuous variables were presented as mean ± SD or median (Q1-Q3). Categorical variables were presented as percentages.

If the variables were in normal distribution, variance analysis method and two-independent sample test were conducted for group comparisons. If the variables followed non-normal distribution, non-parametric Mann-Whitney U tests were applied to compare continuous variables. Fisher's exact test or Chi-square test was performed when comparing categorical variables. Univariate analyses were conducted with logistic regression models to detect the possible variables which may affect cumulative ART outcomes. Multivariate logistic regression analyses were carried out to estimate the associations between FORT and CCPR and CLBR. Curve fitting and threshold effect analyses were established to find nonlinear relationships. The relationships between FORT and cumulative ART outcomes in different subgroups were examined by stratification analyses. A p-value <0.05 was considered significant statistically.

Results

Baseline status

There were 454 PCOS patients included in our study after exclusions (Figure 1). Based on the FORT values, individuals were classified into the following three groups: low-FORT group (n = 145) with FORT values below the 33rd percentile (FORT

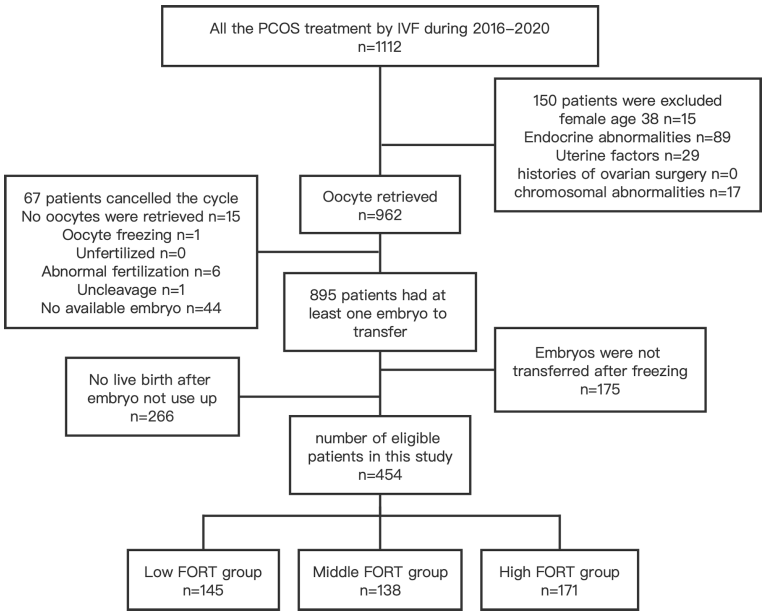

FIGURE 1
Flow chart for selection of patients from January 2016 to December 2020.

<0.54), middle-FORT group (n = 138) with FORT values between the 33rd and 67th percentiles (FORT 0.54~0.66), and high-FORT group (n = 171) with FORT values above the 67th percentile (FORT >0.66). Table 1 showed the patients' baseline characteristics. The age, body mass index (BMI), years of infertility, basal estradiol (E_2), basal FSH, starting dose of Gn, dose of Gn and duration of Gn were similar among the three groups. The AFC in the high-FORT group was lower than that in the middle-FORT and low-FORT group ($p < 0.05$). The AMH in the high-FORT group was higher than that in the low-FORT group ($p < 0.05$). The basal testosterone (bT), PFC were significantly higher in the high-FORT group than in the middle-FORT group and low-FORT group ($p < 0.05$). Patients with the high basal testosterone level accounted for a large proportion in the high-FORT group (62.573%). Table 2 showed the laboratory indicators and clinical outcomes of study participants. The number of retrieved oocytes, number of 2PN zygotes and number of cleavage embryos in the high-FORT group were significantly higher than that in the middle-FORT and low-FORT group ($p < 0.05$). The fertilization rate, available embryo rate, number of available embryos and number of high-quality embryos were significantly higher in the high-FORT group than in the low-FORT group ($p < 0.05$). The CCPR significantly increased with FORT ($p < 0.05$). The CLBR increased with increasing FORT although the p-value was not significant.

Follicular output rate is an independent factor for IVF outcome

The consequences of the univariate analyses were given in Supplementary Table 1. The multivariate logistic regression analysis adjusted for the following confounders: age, BMI, years of infertility, AMH, treatment plan, type of PCOS. In the adjusted model, we found that the FORT was an independent factor significantly affecting the CCPR and CLBR (OR = 1.015, 95% CI: 1.001, 1.030 and OR = 1.010, 95% CI:1.001, 1.020) (Tables 3, 4). The CCPR and CLBR increased by 1.5% and 1.0%, respectively, with each additional unit of FORT. The CCPR increased 2.017 times and the CLBR increased 1.188 times in the high-FORT group compared with the low-FORT group (OR = 3.017, 95% CI: 1.433, 6.355 and OR = 2.188, 95% CI: 1.256, 3.813).

The results of curve fitting revealed a curvilinear relationship between FORT and cumulative IVF outcomes, after adjustment for age, BMI, years of infertility, AMH, treatment plan and type of PCOS (Figure 2). Threshold effect analyses showed that the CCPR increased by 3.9% and the CLBR increased by 2.4% with each additional unit of FORT when the FORT was lower than 70% (OR = 1.039, 95% CI: 1.013, 1.065 and OR = 1.024, 95% CI: 1.004, 1.044) (Table 5). However, when the FORT was higher than 70%, the growth trend in the CCPR and CLBR with FORT was no longer significant (OR = 0.999, 95% CI: 0.983, 1.016 and OR = 1.003, 95% CI: 0.990, 1.015).

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TABLE 1 Patient's baseline characteristics.

FORT	Low (<0.54, n=145)	Middle (0.54~0.66, n=138)	High (>0.66, n=171)	F/χ^2	*p*-value
Age (years)	28.269 (2.935)	28.725 (3.311)	28.661 (3.310)	0.869	0.420
BMI (kg/m2)	25.650 (3.258)	25.416 (3.595)	24.777 (3.498)	2.740	0.066
Years of infertility (years)	3.000 (2.000-5.000)	3.000 (2.000-5.000)	3.000 (2.000-4.000)	2.941	0.054
bFSH (mIU/mL)	6.905 (1.869)	6.542 (1.708)	6.489 (1.773)	2.415	0.091
bE2 (pg/mL)	41.000(28.000-59.000)	42.000 (32.000-60.000)	41.000 (30.000-57.000)	0.260	0.771
bP (ng/mL)	0.650 (0.390-0.970)	0.665 (0.472-1.015)	0.650 (0.390-1.010)	0.495	0.61
bLH (mIU/mL	8.060 (4.740-12.560)	8.185 (4.855-11.985)	8.280 (4.145-13.270)	0.462	0.631
bT (ng/mL)	0.650 (0.490-0.820)*	0.660 (0.490-0.835)*	0.750 (0.595-0.880)	8.181	<0.001
AMH (ng/mL)	5.820 (4.170-8.810)*	6.405 (4.720-9.158)	7.050 (4.960-11.260)	5.276	0.005
No. of AFC	23.579 (4.445)*	23.203 (2.365)*	21.064 (4.865)	17.406	<0.001
Starting dose of Gn (IU	178.017 (55.282)	171.830 (45.811)	176.681 (47.704)	0.609	0.545
Dose of Gn (IU	2250.000 (1650.000-2925.000)	2193.750 (1650.000-2765.625)	2000.000 (1500.000-2475.000)	3.023	0.050
Duration of Gn (IU)	11.262 (2.789)	11.797 (4.215)	11.029 (2.753)	2.152	0.163
No. of PFC	10.159 (2.394)*#	13.529 (1.567)*	18.789 (7.028)	141.728	<0.001
FORT	45.830 (37.500-50.000)*#	58.330 (54.170-62.500)*	79.170(70.830-100.000)	274.479	<0.001
Type of infertility				0.824	0.662
Primary	93 (64.138%)	83 (60.145%)	111 (64.912%)		
Secondary	52 (35.862%)	55 (39.855%)	60 (35.088%)		
Type of PCOS				28.464	<0.001
A	44 (30.345%)	39 (28.261%)	59 (34.503%)		
B	10 (6.897%)	7 (5.072%)	33 (19.298%)		
C	8 (5.517%)	11 (7.971%)	15 (8.772%)		
D	83 (57.241%)	81 (58.696%)	64 (37.427%)		
PCOM				19.454	<0.001
no	10 (6.897%)	7 (5.072%)	33 (19.298%)		
yes	135 (93.103%)	131 (94.928%)	138 (80.702%)		
OAD				1.266	0.531
no	8 (5.517%)	11 (7.971%)	15 (8.772%)		
yes	137 (94.483%)	127 (92.029%)	156 (91.228%)		
HA				17.361	<0.001
no	83 (57.241%)	80 (57.971%)	64 (37.427%)		
yes	62 (42.759%)	58 (42.029%)	107 (62.573%)		
Treatment plan				5.244	0.263
GnRH-ant protocol	33 (22.759%)	19 (13.768%)	35 (20.468%)		
GnRH-a long protocol	35 (24.138%)	35 (25.362%)	34 (19.883%)		
GnRH-a prolonged protocol	77 (53.103%)	84 (60.870%)	102 (59.649%)		
Cycle outcome				34.161	<0.001

(Continued)

TABLE 1 Continued

FORT	Low (<0.54, n=145)	Middle (0.54~0.66, n=138)	High (>0.66, n=171)	F/χ^2	*p*-value
Fresh embryo transfer	97(66.897%)	67(48.551%)	58(33.918%)		
Frozen embryo transfer	48(33.103%)	71(51.449%)	113(66.082%)		
Number of transferred cycles				4.507	0.342
1	136 (93.793%)	128 (92.754%)	152 (88.889%)		
2	9 (6.207%)	10 (7.246%)	19 (11.111%)		

#P<0.05 compared with middle group.
*P<0.05 compared with high group.
Categorical variables are presented as number (%). Continuous variables are presented as mean (SD) or median (interquartile range). BMI, body mass index; bFSH, basal follicle-stimulating hormone; bE2, baseline estradiol; bP, baseline progesterone; bT, baseline testosterone; bLH, baseline luteinizing hormone; AMH, anti-Mullerian hormone; Gn, Gonadotropin; PCOM, polycystic ovarian morphology; OAD, oligoanovulatory ovarian dysfunction; HA, hyperandrogenism.

Stratification analysis

Stratification analysis was performed separately based on age, BMI and clinical characteristics of PCOS. When the patients' age was over 30, the CCPR increased by 3.3% and the CLBR increased by 1.6% with each additional unit of FORT (OR = 1.033, 95% CI: 1.005, 1.062 and OR = 1.016, 95% CI: 1.000, 1.033). When the patients were younger than 30, the CCPR and CLBR did not correlate significantly with FORT (Table 6).

When BMI was lower than 25, the CCPR increased by 2.4% and the CLBR increased by 1.3% with each additional unit of FORT (OR = 1.024, 95% CI: 1.002, 1.046 and OR = 1.013, 95% CI: 1.001, 1.026). When BMI was 25 or larger than that, the CCPR and CLBR did not correlate significantly with FORT (Table 6).

Among patients with hyperandrogenic manifestations, the CLBR increased by 1.3% with each additional unit of FORT (OR = 1.013, 95% CI: 1.001, 1.025). The CCPR increased 1.541 times and the CLBR increased 1.451 times in the high-FORT group compared with the low-FORT group (OR = 2.541, 95% CI: 1.041, 6.202 and OR = 2.451, 95% CI: 1.169, 5.139). Among patients with polycystic ovarian morphology, the CCPR increased 1.251 times in the high-FORT group compared with the low-FORT group (OR = 2.251, 95% CI: 1.008, 5.028). Among patients with ovulation disorder, the CCPR increased 1.891 times and the CLBR increased 0.99 times in the high-FORT group compared with the low-FORT group (OR = 2.891, 95%

TABLE 2 Patient's laboratory indicators and clinical outcomes.

FORT	Low (<0.54, n=145)	Middle (0.54~0.66, n=138)	High (>0.66, n=171)	F/χ^2	*p*-value
No. of oocyte	11.000 (8.000-17.000)*#	18.000 (13.000-22.750)*	19.000 (15.000-27.000)	35.612	<0.001
No. of 2PN	7.000 (4.000-10.000)*#	11.000 (7.000-15.000)*	12.000 (8.000-17.000)	32.156	<0.001
No. of cleavage embryo	9.000 (6.000-14.000)*#	15.000 (11.000-18.750)*	16.000 (12.000-23.000)	34.336	<0.001
No. of available embryo	3.000 (2.000-4.000)*#	4.500 (3.000-6.000)	5.000 (3.000-7.000)	14.353	<0.001
No. of high quality embryo	1.000 (0.000-2.000)*	1.000 (0.000-3.000)	1.000 (0.000-4.000)	4.845	0.008
Total number of transferred embryo	2.021 (0.520)	2.058 (0.509)	2.099 (0.620)	0.789	0.391
Fertilization rate	0.700 (0.213)*	0.733 (0.182)	0.762 (0.178)	4.027	0.018
2PN Fertilization rate	0.600 (0.440-0.730)	0.620 (0.500-0.750)	0.640 (0.500-0.775)	2.434	0.089
Cleavage rate	0.975 (0.124)	0.961 (0.152)	0.989 (0.032)	2.494	0.084
Available embryo rate	0.300 (0.220-0.400)*	0.270 (0.180-0.350)	0.250 (0.170-0.350)	5.462	0.005
High quality embryo rate	0.080 (0.000-0.200)	0.080 (0.000-0.190)	0.070 (0.000-0.180)	0.004	0.996
Cumulative clinical pregnancy	120 (82.759%)	124 (89.855%)	157 (91.813%)	6.688	0.035
Cumulative live birth	101 (69.655%)	101 (73.188%)	138 (80.702%)	5.397	0.067

#P<0.05 compared with middle group.
*P<0.05 compared with high group.
Categorical variables are presented as number (%). Continuous variables are presented as mean (SD) or median (interquartile range).

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TABLE 3 Follicular output rate and cumulative clinical pregnancy rate.

	Non-adjusted		Adjusted	
	OR (95%CI)	*p*-value	OR (95%CI)	*p*-value
FORT				
Low	1		1	
Middle	1.845(0.916, 3.719)	0.08663	2.008 (0.975, 4.138)	0.05871
High	2.336(1.165, 4.686)	0.01688	3.017 (1.433, 6.355)	0.00366
FORT	1.011(0.998, 1.024)	0.10493	1.015 (1.001, 1.030)	0.03496

Adjusted age, BMI, AMH, years of infertility, type of PCOS, treatment plan.

CI: 1.332, 6.323 and OR = 1.990, 95% CI: 1.133, 3.494) (Tables 7, 8).

Discussion

In this retrospective study of 454 PCOS patients, we found that FORT was an independent factor affecting the cumulative IVF outcomes. The CLBR and CCPR were positively correlated with FORT when the FORT was less than 70%.

FORT, as a simple and noninvasive tool in our clinical practice, could objectively reflect dynamic changes of follicular growth in response to exogenous Gn. Genro et al. found a negative association between FORT and AMH levels in peripheral blood, which might be explained by the hypothesis that AMH inhibited the sensitivity of antral follicles to Gn (9). Hassan et al. showed that no significant difference in the serum AMH levels was found among the FORT groups (11). We found that FORT was positively associated with AMH levels in peripheral blood. The difference between our results and other studies may be due to the disparities in the studied populations. We studied patients with PCOS, whereas Genro et al. and Hassan et al. studied patients with different infertility causes (9, 11). In the studies conducted on PCOS cases only, the serum AMH levels can be used as a marker of ovarian responsiveness and there was a positive association between AMH levels and assisted reproductive outcomes (21). In our study, basal testosterone levels in the high-FORT group were dramatically higher than that in the low-FORT and middle-FORT groups. This finding supports the previous studies which showed that basal testosterone level positively correlated with ovarian response and follicular count on trigger day (≥ 14 mm) (22, 23). The possible mechanism is that androgens could enhance FSH receptor expression in granulosa cells and are considered to promote follicular development by amplifying the effects of FSH (24). Additionally, androgens also augment the expression of insulin-like growth factor 1 (IGF-1) in the primate ovary, which is crucial for regulating follicular growth (22, 25).

TABLE 4 Follicular output rate and cumulative live birth rate.

	Non-adjusted		Adjusted	
	OR (95%CI)	*p*-value	OR (95%CI)	*p*-value
FORT				
Low	1		1	
Middle	1.189(0.709, 1.994)	0.51119	1.275(0.749, 2.170)	0.37060
High	1.822(1.084, 3.062)	0.02356	2.188(1.256, 3.813)	0.00572
FORT	1.007(0.998, 1.016)	0.10747	1.010(1.001, 1.020)	0.03268

Adjusted age, BMI, AMH, years of infertility, type of PCOS, treatment plan.

This study found that the CCPR, cleavage embryos, 2PN zygotes, number of retrieved oocytes and PFC increased progressively from the low to high FORT groups. And the numbers of available embryos and high quality embryos were significantly lower in the low FORT group. These results are in agreement with the earlier reported results (10–12). In the high-FORT group, patients have better ovarian responsiveness to exogenous gonadotrophins, resulting in increased mature and retrieved oocytes, and consequently better clinical outcomes. In our study, the fertilization rate was significantly higher in the high FORT group. Our findings are in agreement with those of Hassan et al. (11), but they differ from that obtained in other studies (10, 12, 14), which did not show any difference in fertilization rate among the FORT groups. The difference between other studies and our findings may be attributed to the disparities in the number of cases investigated and study populations. We also found that the available embryo rate was higher in the low FORT group. This may be due to the relatively low number of retrieved oocytes in the low-FORT group. Despite these contradictions, the findings revealed that FORT can be used as a qualitative reflector of the follicular responsiveness to FSH, oocyte competence, and embryo quality.

The central finding of our study is the positive correlation between FORT and CCPR and CLBR in PCOS patients. The CCPR and CLBR in the high-FORT group were significantly higher than that in the low-FORT group. With the widespread use of embryo cryo-resuscitation technology, the CCPR and CLBR, defined as the pregnancy and live birth after using up all fresh and frozen embryos derived from one single COS cycle, appear to be more accurate and comprehensive measures to reflect the effectiveness and safety of IVF treatment (26–29). After reviewing the published literatures regarding PCOS and FORT, we found that there were few researches used CCPR and

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CLBR as clinical outcome indicators. Yang et al. (14) investigated the relationship between FORT and CLBR and showed that the CLBR was highest in the high-FORT group and lowest in the middle-FORT group. Differences between this study and our results may be due to the disparities in the study populations and the COS protocols. Also, in their study, there were significant differences in Gn dosage and stimulation days among three groups. This may affect the outcome because PCOS patients are usually high ovarian responders.

With a threshold effect model, our results showed that when the FORT was lower than 70%, the CCPR increased by 3.9% and the CLBR increased by 2.4% with each additional unit of FORT. When the FORT was greater than 70%, the CCPR and CLBR did not increase significantly even if the FORT increased. The positive association between the FORT and the cumulative ART outcomes in the first segment of the curve suggested the importance of the high FORT values for the success of IVF. Patients with high FORT values may have more oocytes and produce more euploid embryos that can be used for embryo transfer, thereby increasing the chance of pregnancy and live birth. However, when the FORT reached a certain value, there was no longer a significant beneficial relationship. This may be due to the fact that pregnancy outcomes are influenced by many other factors, such as obesity, environmental exposure (including smoking and alcohol), stress and antiphospholipid syndrome (30, 31). Therefore, for PCOS patients, we should also pay attention to the general conditions and lifestyle rather than simply increasing the FORT.

According to our results, patients with hyperandrogenemia (HA) can increase the CCPR and CLBR by increasing the FORT. HA is the core etiology and primary endocrine characteristic of PCOS. HA leads to premature granulosa cell luteinization and abnormal oocyte maturation by altering follicular fluid microenvironment and the feedback of ovarian hormones to hypothalamic-pituitary-ovarian (HPO) axis (32). In addition, due to the expression of androgen receptors in pancreas and hepatocytes, high testosterone levels could lead to hyperinsulinemia, which seriously impairs ovarian function resulting in premature arrest of follicular development and oligo-anovulation (33). Furthermore, high testosterone levels in PCOS patients also influence glucose metabolism of endometrium, which leads to local insulin resistance and subsequently endometrial lesion (34). Evidence show that the CLBR of patients with hyperandrogenemia is significantly lower than that of individuals without hyperandrogenemia (16). Therefore, improving FORT is a good choice for these poor-prognosis patients with HA to improve the CCPR and CLBR.

TABLE 5 The threshold effect analysis of the follicular output rate and clinical outcomes.

Cut points	N	OR	95%CI	*p*-value
The cumulative pregnancy rate				
< 70	316	1.039	(1.013, 1.065)	0.0029
>70	138	0.999	(0.983, 1.016)	0.9188
The cumulative live birth rate				
< 70	316	1.024	(1.004, 1.044)	0.0170
>70	138	1.003	(0.990, 1.015)	0.6813

Adjusted age, BMI, AMH, years of infertility, type of PCOS, treatment plan.

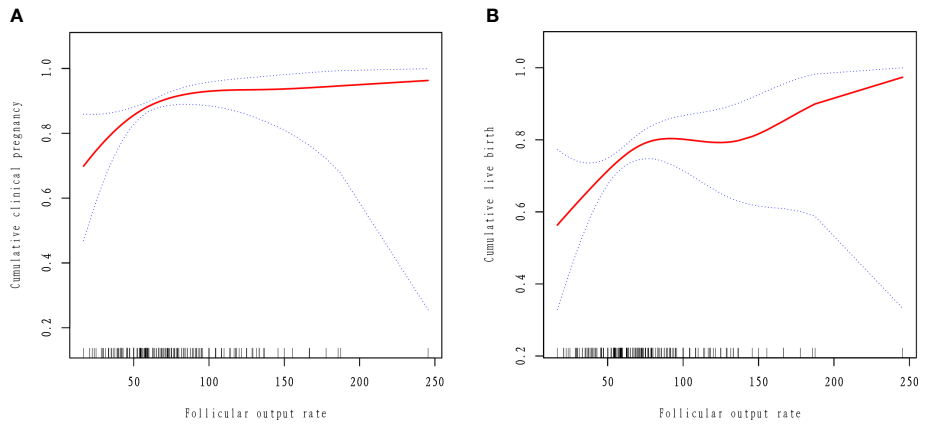

FIGURE 2
Curve fitting between follicular output rate and clinical outcomes. The adjusted smoothed plots between the follicular output rate with the cumulative clinical pregnancy rate and the cumulative live birth rate based on two-piece-wise regression model **(A, B)**. The nonlinear relationship between the follicular output rate and the cumulative clinical pregnancy rate and the cumulative live birth rate, respectively. Adjustment factors included age, BMI, AMH, years of infertility, type of PCOS, treatment plan. The solid line and dashed line represent the estimated values and their corresponding 95% confidence intervals.

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TABLE 6 Stratification analysis of follicular output rate and cumulative clinical outcomes.

	N	The cumulative pregnancy rate		The cumulative live birth rate	
		OR (95%CI)	*p*-value	OR (95%CI)	*p*-value
Age					
<30	287	1.007 (0.990, 1.025)	0.4064	1.007 (0.995, 1.020)	0.2489
>=30	167	1.033 (1.005, 1.062)	0.0198	1.016 (1.000, 1.033)	0.0496
BMI					
<25	210	1.024 (1.002, 1.046)	0.0311	1.013 (1.001, 1.026)	0.0398
>=25	244	1.016 (0.996, 1.036)	0.1189	1.012 (0.997, 1.027)	0.1272

Adjustment factors included age, BMI, AMH, years of infertility, type of PCOS, treatment plan, if not stratified by its.

Furthermore, we also found that patients with polycystic ovarian morphology or ovulation disorder had better cumulative IVF outcomes in the high-FORT group. These results together suggest that FORT, as a noninvasive and simple tool in our clinical practice, may contribute to improving the CCPR and CLBR for PCOS patients with typical clinical characteristics.

TABLE 7 Stratification analysis of follicular output rate and cumulative clinical outcomes.

	N	The cumulative pregnancy rate		The cumulative live birth rate	
		OR (95%CI)	*p*-value	OR (95%CI)	*p*-value
PCOM					
No	50	1.007 (0.979, 1.036)	0.6339	1.005 (0.980, 1.029)	0.7173
Yes	404	1.014 (0.997, 1.031)	0.0983	1.010 (0.999, 1.021)	0.0821
OAD					
No	34	1.022 (0.974, 1.074)	0.3731	1.038 (0.986, 1.094)	0.1576
Yes	420	1.013 (0.998, 1.028)	0.0833	1.007 (0.998, 1.017)	0.1330
HA					
No	227	1.013 (0.986, 1.041)	0.3437	1.006 (0.990, 1.022)	0.4734
Yes	227	1.013 (0.997, 1.030)	0.1206	1.013 (1.001, 1.025)	0.0386

Adjusted age, BMI, AMH, years of infertility, treatment plan.
PCOM, polycystic ovarian morphology; OAD, oligoanovulatory ovarian dysfunction; HA, hyperandrogenism.

Our results showed that the CCPR and CLBR increased with FORT in PCOS patients over 30 years old, whereas the relationship was not statistically significant in patients under the age of 30. This indicated that the older the age, the more positive correlation between the FORT and cumulative ART outcomes. We know that oocyte quality gradually declines with women aging and the competence of women's oocytes begins to deteriorate around their third decade (35). Multiple potential mechanisms may be responsible for this, such as meiotic spindle abnormalities, mitochondrial dysfunction and chronic exposure to oxidative stress, which usually lead to aneuploidy of the embryo and a higher incidence of adverse pregnancy outcomes (36–39). In general, younger women have better oocyte quality, which may somewhat attenuate the impact of FORT on pregnancy outcomes. Therefore, for the older age group of PCOS patients, due to the decline of oocyte quality, higher FORT values and more oocytes retrieved are needed to achieve better cumulative ART outcomes.

In this study, we found that the FORT was significantly related with the CCPR and CLBR in PCOS patients without overweight and obesity. By boosting FORT, we can increase the CCPR and CLBR in these individuals. Obesity and overweight are known risk factors for cumulative ART outcomes (40), and PCOS patients are more likely to be obese and overweight, which contributes to diminished response to ART, adverse pregnancy outcomes and higher incidence of other complications (41, 42). Obesity could impair endometrial function through inflammation, oxidative stress or other ways, which could cause decidual formation abnormalities and embryo implantation failure (43). Additionally, obesity affects oocyte function by inducing abnormal chromosome pairing and altering follicles' liquid microenvironment (32). Therefore, weight control and alleviating metabolism disorders are more beneficial to the prognosis of overweight and obese patients than increasing the FORT.

The main strengths of our study rest on the following aspects. First, it was the first study to investigate the role of clinical characteristics of PCOS on the relationship between FORT and cumulative IVF outcomes. Second, the present study first uncovered a curvilinear relationship between FORT and CCPR and CLBR, and this relationship might be useful in establishing an optimal treatment strategy for PCOS patients to obtain better reproductive outcomes. Additionally, our research used CCPR and CLBR as main outcome measures, which is an important advantage over other metrics.

Despite the strengths, this study has some limitations. One disadvantage of the index FORT is related to its operator-dependent characteristic. It is unlikely to rule out the variation in marking AFC and PFC by different sonographers. Secondly, the association between FORT and the incidence of OHSS was not investigated in our study. Other limitations of our study lie in the retrospective and monocentric character, as well as the small study population. Prospective and multicentric

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TABLE 8 Stratification analysis of follicular output rate and cumulative clinical outcomes.

	N	The cumulative pregnancy rate		The cumulative live birth rate	
		OR (95%CI)	*p*-value	OR (95%CI)	*p*-value
PCOM					
No					
Low FORT	10	1		1	
Middle FORT	7	10.270(0.270, 391.238)	0.2098	4.866 (0.265, 89.481)	0.2868
High FORT	33	11.392(0.692, 187.649)	0.0887	5.950 (0.735, 48.200)	0.0947
Yes					
Low FORT	135	1		1	
Middle FORT	131	1.816 (0.857, 3.852)	0.1196	1.166 (0.672, 2.021)	0.5857
High FORT	138	2.251 (1.008, 5.028)	0.0479	1.768 (0.978, 3.197)	0.0592
OAD					
No					
Low FORT	8	1		1	
Middle FORT	11	3.772 (0.198, 72.002)	0.3776	0.486 (0.041, 5.760)	0.5676
High FORT	15	1.848 (0.070, 48.735)	0.7130	7.929 (0.282,223.082)	0.2239
Yes					
Low FORT	137	1		1	
Middle FORT	127	1.862 (0.883, 3.925)	0.1025	1.345 (0.773, 2.342)	0.2946
High FORT	156	2.891 (1.322, 6.323)	0.0079	1.990 (1.133, 3.494)	0.0166
HA					
No					
Low FORT	83	1		1	
Middle FORT	80	1.588 (0.562, 4.484)	0.3824	1.136 (0.549, 2.353)	0.7309
High FORT	64	3.029 (0.730, 12.576)	0.1270	1.700 (0.715, 4.039)	0.2297
Yes					
Low FORT	62	1		1	
Middle FORT	58	2.161 (0.779, 5.996)	0.1388	1.271 (0.577, 2.801)	0.5523
High FORT	107	2.541 (1.041, 6.202)	0.0405	2.451 (1.169, 5.139)	0.0176

Adjusted age, BMI, AMH, years of infertility, treatment plan.
PCOM, polycystic ovarian morphology; OAD, oligoanovulatory ovarian dysfunction; HA, hyperandrogenism.

investigations with larger simple size and longer duration of observation would be necessary to further validate the findings.

In summary, the present findings indicate that cumulative IVF outcomes have a positive correlation with FORT in PCOS patients when the FORT was less than 70%. For PCOS patients with polycystic ovarian morphology, ovulation disorder or hyperandrogenic manifestations, a high FORT could be conductive to achieving better pregnancy outcomes.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by the research ethics committee of the Second Hospital of Hebei Medical University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

Study design: ZZ. Data acquisition: RJ, PC, and YL. Data analysis: RLJ, MC, and HH. Writing manuscript: Rulan Jiang. All authors read the article and agreed to the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1079502/full#supplementary-material

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Circulating apelin and chemerin levels in patients with polycystic ovary syndrome: A meta-analysis

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Background: Polycystic ovary syndrome (PCOS) is one of the most common gynecological endocrine disorders. Apelin and chemerin are newly identified adipokines, which are higher in obesity and diabetes. Studies have found that the serum apelin and chemerin levels in patients with PCOS are significantly increased. However, other studies showed the opposite results. Therefore, the relationship between those two adipokines and PCOS is still controversial.

Aim: This meta-analysis was conducted to statistically evaluate the apelin and chemerin levels of patients with PCOS.

Methods: We searched the Web of Science, Embase, PubMed, and Google Scholar databases for potential studies. "Polycystic ovary syndrome" or "PCOS" in combination with the terms "apelin" or "chemerin" were used as keywords search titles or abstracts. The publication period examined was between 1990 and 2021. Standardized mean differences (SMD) with corresponding 95% confidence intervals (CIs) were determined as the results of the meta-analysis.

Results: A total of 148 articles were initially retrieved, and 18 qualified articles were finally obtained through preliminary screening and quality evaluation. The publications together contain 1,265 cases and 894 controls. The results of the meta-analysis showed that the circulating chemerin levels in patients with PCOS were significantly higher than those in the controls (SMD: 0.79, 95% CI [0.36, 1.23]), and there was no significant difference in circulating apelin between patients with PCOS and controls (SMD: 0.57, 95% CI [-0.21, 1.35]).

Conclusions: This meta-analysis is the first to evaluate circulating apelin and chemerin levels in patients with PCOS. Our findings suggest that circulating chemerin levels of patients with PCOS are significantly higher than those of healthy controls.

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KEYWORDS

apelin, chemerin, polycystic ovary syndrome, PCOS, meta- analysis

Introduction

Polycystic ovary syndrome (PCOS) is one of the most common gynecological endocrine disorders with a complex pathogenesis (1). It is characterized by dysmenorrhea, amenorrhea, infertility, hairiness, and obesity, all of which seriously affect the physical and mental health of women of childbearing age. PCOS also increases the risk of endometrial cancer, gestational diabetes mellitus, and hyperlipidemia. Approximately 5%–10% of women of childbearing age are affected by PCOS. Patients with PCOS account for 15%–20% of infertility cases (2, 3). Studies have shown that abnormal follicular development in patients with PCOS is not only regulated by sex hormones but is also closely related to disorders in the follicular development microenvironment caused by ovarian autocrine/paracrine dysfunction. Recent studies suggested that insulin resistance may initiate PCOS development (4).

Apelin and chemerin are newly identified adipokines. Apelin is an APJ receptor ligand, is widely expressed in different organs, and plays an important role in glucose and lipid metabolism (5). Recent studies found that serum apelin levels are significantly correlated with type 2 diabetes mellitus and obesity (6, 7). Chemerin, also called TIG2 or RARRES2, is secreted as an 18 kDa precursor protein (chem163s), which can be transformed into a 16 kDa active molecule only after C-terminal cleavage (8). The precursor protein has multiple restriction sites, which can produce a variety of subtypes upon treatment with different proteases. Chemerin also contributes to adipogenesis, glucose homeostasis, food intake, and body weight and is associated with elevated levels of obesity, diabetes, and cancer (9–11).

Studies have found that serum apelin and chemerin levels in patients with PCOS are significantly increased (12–15), and are potential targets for the treatment of PCOS (16, 17). However, other studies showed that the serum apelin and chemerin levels of PCOS patients are lower than those of healthy individuals (18, 19). Therefore, the relationship between these adipokines and PCOS remains controversial. This meta-analysis aimed to statistically evaluate apelin and chemerin levels in patients with PCOS.

Methods

Search design

We searched the Web of Science, Embase, PubMed, and Google Scholar databases for potential studies. "Polycystic ovary syndrome" or "PCOS" in combination with the terms "apelin" or "chemerin" were used as keywords search titles or abstracts. The full electronic search strategy is provided in the Supplementary Data Sheet 1. The publication period examined was between 1990 and 2021. Concurrently, manual retrieval of relevant literature was performed and the references included in clinical trials were consulted to uncover relevant studies that might have been omitted. This review and meta-analysis were conducted according to the recommendations of the Cochrane Collaboration and following the PRISMA statement. The PRISMA list is provided in the Supplementary Data Sheet 2 and the PROSPERO registration number is CRD42020218316.

Inclusion criteria

The studies included in this meta-analysis met the following criteria: (1) case-controlled or prospective design; (2) detailed data on circulating apelin or chemerin levels in patients with PCOS and healthy controls; and (3) written in English. All the patients with PCOS included in the studies had no medical history or evidence of diabetes, hypertension, hyperprolactinemia, thyroid disease, Cushing's syndrome, and congenital adrenal hyperplasia. Patients taking drugs such as insulin-sensitizing drugs, oral contraceptives, corticosteroids, anti-androgens, and gonadotropin-releasing hormone agonists or antagonists within 3 months were also excluded from the study.

Data extraction and risk of bias

Two independent evaluators screened the studies according to the inclusion and exclusion criteria. First, the evaluators read the topic and abstract and eliminated duplicate studies and those who did not meet the inclusion criteria. Next, they read the full

text of the documents marked for inclusion and cross-checked the results. Finally, the two reviewers discussed and came to consensus on any publications with objections. If they still could not reach an agreement, a third researcher was invited for further evaluation. For documents with questions or missing data, we contacted the author or corresponding author to obtain as much confirmation or supplemental data as possible. The extracted content of the original publication data included the first author, publication year, study period, region, study design, and details of cases and controls.

The Newcastle Ottawa Scale (NOS) was used as the standard to evaluate the quality of the included literature. The NOS is applicable to the evaluation of cohort and case-controlled studies. It consists of three parts: selection of exposure and control populations, comparability, and evaluation of exposure or outcome. It has eight entries. NOS uses the semi-quantitative principle of a star scale to evaluate the quality of literature with a maximum of nine stars (20, 21).

Statistical analysis

Standardized mean differences (SMD) with corresponding 95% confidence intervals (CIs) were determined as the results of the meta-analysis. Cochran's Q test and I^2 statistics were used to test the heterogeneity among the studies. When $I^2 \leq 50\%$, there was no heterogeneity and a fixed effects model was used for combined analysis. When $I^2 > 50\%$, there was significant heterogeneity and the random effects model was used for combined analysis. The stability of the meta-analysis results was evaluated by a sensitivity analysis. Low-quality literature was excluded and the impact of a single study on the overall research results was excluded for each study. Begg's test was used to analyze publication bias. The significance level was set at $P < 0.05$. Stata 12.0 (College Station, TX, USA) was used for analysis.

Results

A total of 148 articles were initially retrieved, and 18 qualified articles were finally obtained through preliminary screening and quality evaluation (5–25). The publications together contain 1,265 cases and 894 controls (12–15, 18, 19, 22–33). The literature retrieval process is shown in Figure 1 and the baseline data and quality evaluation of the included case-controlled studies are shown in Table 1.

Results of the meta-analysis

The results of the meta-analysis showed that there was no significant difference in circulating apelin between patients with PCOS and controls (SMD: 0.57, 95% CI [-0.21, 1.35]; I^2 = 96.6%). Forest plots of circulating apelin levels in patients with PCOS compared with controls are shown in Figure 2. The circulating chemerin levels in patients with PCOS were significantly higher than those in the controls (SMD: 0.79, 95% CI [0.36, 1.23]; I^2 = 91.7%). Forest plots of circulating chemerin levels are shown in Figure 3. The funnel plots of circulating apelin and chemerin were presented in Supplementary Figures 1, 2.

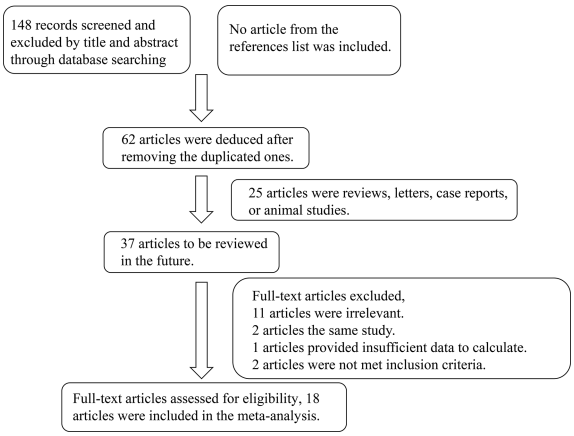

FIGURE 1
Flowchart of the detailed procedure for the inclusion or exclusion of selected studies.

Sensitivity analysis and publication bias

Using the sensitivity analysis by excluding individual studies one by one, the results showed little difference, suggesting that the results of this study were relatively credible (Figures 4, 5). A comprehensive search of articles obtained from the database was performed. Begg's test was also performed to determine whether there was a potential publication bias in the reviewed literature. The results ($P > 0.05$) suggest that there was no publication bias.

Discussion

This systematic review is the first to evaluate circulating apelin and chemerin levels in patients with PCOS. Although most studies have shown that circulating apelin and chemerin levels in patients with PCOS are higher than those in healthy controls, some found that they are lower. In this meta-analysis, 18 independent studies were included and analyzed. We concluded that the circulating chemerin levels in patients with PCOS were significantly higher than those in the healthy controls (SMD: 0.79, 95% CI [0.36, 1.23]), whereas there was

TABLE 1 Study characteristics of the published studies included in the meta-analysis.

Author	Publication Year	Study Period	Region	Study design	Case (n)	Control (n)	Case factor	Control factor	Indicator
Goren	2011	–	Turkey	Case-control study	32	31	PCOS patients, 15 – 35 years, BMI 22.51 ± 3.20 kg/m^2	healthy volunteers, 15 – 35 years, BMI 21.94 ± 1.63 kg/m^2	Apelin
Chang	2011	–	China	Case-control study	50	34	PCOS patients recruited from the outpatient Department of Obstetrics and Gynecology, 24.8 ± 5.0 years, BMI 22.2 ± 4.1 kg/m^2	healthy controls, 28.9 ± 5.0 years, BMI 21.0 ± 2.7 kg/m^2	Apelin
Cekmez	2011	2007 – 2008	Turkey	Case-control study	48	37	Obese PCOS patients recruited among the adolescents who attended the outpatient clinic of the Department of Pediatric Endocrinology, 16.9 ± 0.3 years, BMI 35.1 ± 4.3 kg/m^2	Obese, healthy children enrolled from patients who attended the hospital for minor illnesses such as the common cold, conjunctivitis etc, 17.2 ± 0.2 years, BMI 30.7 ± 2.2 kg/m^2	Apelin
Choi	2012	–	Korea	Prospective observational study	82	33	PCOS recruited from outpatients of the Department of Obstetrics and Gynecology, 24.51 ± 5.02 years, BMI 20.27 ± 2.34 kg/m^2	women visited our hospital for annual comprehensive medical examinations without specific health problems, 24.58 ± 2.72 years, BMI 19.88 ± 1.56 kg/m^2	Apelin
Wang	2014	July 2012 – April 2013	China	Case-control study	67	20	PCOS patients recruited consecutively from infertility and endocrine clinics, 24.46 ± 4.97 years, BMI 25.77 ± 3.23 kg/m^2	healthy volunteers, 23.55 ± 4.99 years, BMI 22.49 ± 2.29 kg/m^2	Chemerin
Ademoglu	2014	–	Turkey	Case-control study	70	38	newly diagnosed or untreated PCOS patients, 25.1 ± 5.7 years, BMI 27.4 ± 7.0 kg/m^2	aged–match healthy women, 26.2 ± 4.9 years, BMI 21.3 ± 2.7 kg/m^2	Chemerin
Guzel	2014	2011 – 2012	Turkey	Case-control study	80	57	PCOS patients recruited from the outpatient endocrinology and gynecology clinics, 25.73 ± 6.02 years, BMI 26.23 ± 6.58 kg/m^2	healthy volunteers, 24.89 ± 4.27 years, BMI 24.54 ± 4.29 kg/m^2	Chemerin
Benk	2014	–	Turkey	Case-control study	30	30	PCOS patients recruited from the Outpatient Clinic of Obstetrics and	BMI- and age-matched healthy volunteers, 23.66 ±	Apelin

(Continued)

TABLE 1 Continued

Author	Publication Year	Study Period	Region	Study design	Case (n)	Control (n)	Case factor	Control factor	Indicator
							Gynaecology Department, 22.46 ± 4.11 years, BMI 20.76 ± 2.08 kg/m^2	7.08 years, BMI 20.04 ± 2.22 kg/m^2	
Altinkaya	2014	–	Turkey	Case-control study	45	45	PCOS patients, 23.5 ± 5.3, BMI 25.3 ± 3.9 kg/m^2	age-matched women who had regular menses and no clinical or biochemical hyperandrogenism or PCO were eligible, 25.1 ± 5.7 years, BMI 22.8 ± 2.3 kg/m^2	Apelin
Yang	2015	January 2013 – June 2014	China	Case-control study	118	114	PCOS patients recruited from the outpatient endocrinology and gynecology clinics, 25.07 ± 4.27 years, BMI 24.63 ± 4.37 kg/m^2	healthy volunteers with normal ovulatory menstruation, 24.62 ± 3.69 years, BMI 23.08 ± 3.34 kg/m^2	Chemerin
Huang	2015	March 2012 – June 2014	China	Case-control study	148	88	newly diagnosed PCOS patients, 28.69 ± 5.69 years, BMI 25.80 ± 5.18 kg/m^2	healthy volunteers, 25.79 ± 5.11 years, BMI 23.24 ± 3.05 kg/m^2	Chemerin
Olszanecka–Glinianowicz	2015	2010 – 2011	Poland	Prospective observational study	83	67	newly diagnosed PCOS patients, 25.4 ± 5.5 years, BMI 29.4 ± 8.8 kg/m^2	regularly menstruating women without clinical symptoms of hyperandrogenism, 25.7 ± 4.9 years, BMI 28.3 ± 7.0 kg/m^2	Apelin
Guvenc	2016	–	Turkey	Case-control study	40	30	PCOS patients recruited from the endocrinology and gynecology, 25.40 ± 5.62 years, BMI 24.87 ± 5.02 kg/m^2	women who had visited the clinic for non–hormonal or non–menstrual irregularities. 31.50 ± 7.5 years, BMI 23.7 ± 4.46 kg/m^2	Chemerin
Kiyak Caglayan	2016	–	Turkey	Prospective observational study	55	55	PCOS patients recruited from the obstetrics and gynecology polyclinic, 26.42 ± 4.77 years, BMI 26.81 ± 4.76 kg/m^2	age- and BMI-matched healthy volunteer, 28.44 ± 6.28 years, BMI 25.78 ± 4.93 kg/m^2	Apelin
Martinez–Garcia	2019	–	Spain	Case-control study	17	17	newly diagnosed PCOS patients, 26.82 ± 6.87 years, BMI 30.12 ± 7.59 kg/m^2	age- and BMI-matched healthy volunteers recruited from the hospital's staff and by noticeboard advertising, 26.47 ±	Chemerin

(Continued)

TABLE 1 Continued

Author	Publication Year	Study Period	Region	Study design	Case (n)	Control (n)	Case factor	Control factor	Indicator
								5.34 years, BMI 29.12 ± 7.33 kg/m^2	
Foda	2019	January 2016 – July 2018	Egypt	Prospective observational study	100	70	untreated PCOS patients recruited from Department of Obstetrics and Gynecology, 21 –26 years, BMI 27.90 ± 3.37 kg/m^2	women with regular periods and normal findings on pelvic ultrasound scan, 26.47 ± 5.34 years, BMI 27.67 ± 3.90 kg/m^2	Chemerin
Ozegowska	2019	2014 – 2016	Poland	Case-control study	94	68	PCOS patients recruited at the Department of Infertility and Reproductive Medicine, 27.0 (24.0 – 29.0) years, BMI 21.0 (20.0 – 22.6) kg/m^2	age- and BMI-matched healthy volunteers with regular menstrual cycles, 28.0 (26.0 – 30.0) years, BMI 20.5 (19.5 – 22.3) kg/m^2	Apelin
Abruzzese	2020	April 2009 – September 2017	Argentina	Case-control study	106	60	PCOS patients recruited from Department of Endocrinology, 26.42 ± 5.36 years, BMI 31.8 (18.5 – 49) kg/m^2	unrelated women recruited from voluntary donors at the Department of Hemotherapy and Endocrinology, 27.70 ± 4.85 years, BMI 23 (18 – 45.65) kg/m^2	Chemerin

no significant association between circulating apelin and PCOS (SMD: 0.57, 95% CI [-0.21, 1.35]).

A 5-dihydrotestosterone (DHT)-induced rat model was used to simulate the reproductive and metabolic phenotypes of PCOS. These animal experiments showed that recombinant chemerin inhibits basal estradiol secretion in DHT-induced rat granulosa cells. *In vitro*, chemerin suppressed follicle-stimulating hormone-induced progesterone and estradiol secretion in cultured preantral follicles and granulosa cells (34). Chemokine-like receptor-1 (CMKLR1), an orphan G-protein-coupled receptor, is specifically expressed by monocyte-derived dendritic cells, macrophages, and circulating plasmacytoid dendritic cells. Chemerin is a chemoattractant ligand for CMKLR1. *CMKLR1* gene deletion

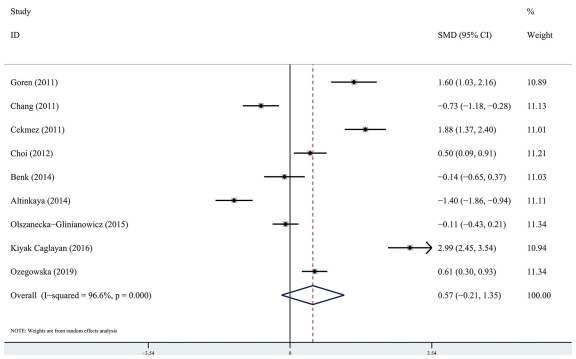

FIGURE 2
Forest plots of circulating apelin levels in patients with polycystic ovary syndrome compared with controls. Diamond represents the pooled odds ratio at 95% confidence interval.

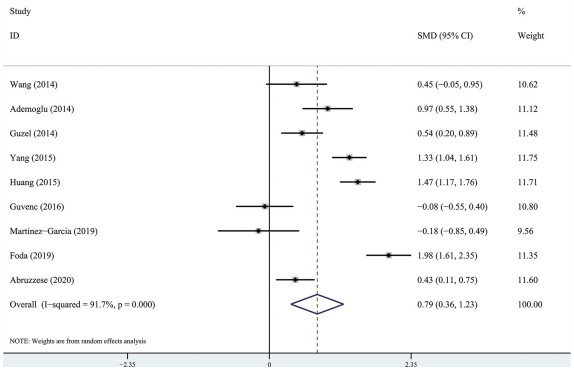

FIGURE 3
Forest plots of circulating chemerin levels in patients with polycystic ovary syndrome compared with controls. Diamond represents the pooled odds ratio at 95% confidence interval.

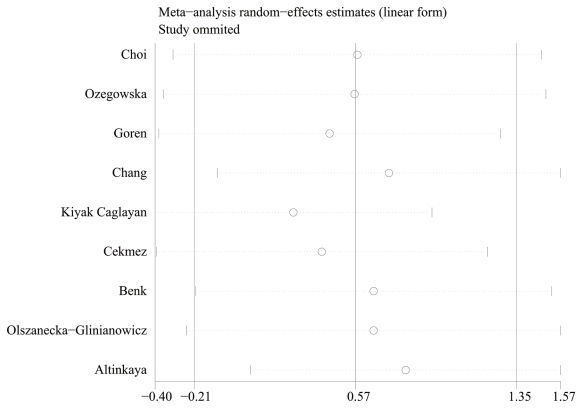

FIGURE 4
The sensitivity analysis results of circulating apelin levels in patients with polycystic ovary syndrome compared with controls.

attenuates the effects of chronic DHT treatment on ovarian function in mouse models of DHT-induced PCOS, likely *via* BMP4 signaling (35). Chemerin also reduces IGF-1-induced steroidogenesis and cell proliferation by decreasing the activation of the IGF-1R signaling pathway in primary human granulosa cells (36). Additionally, metformin treatment has been shown to significantly reduce serum chemerin levels in PCOS patients (32, 37). Chemerin treatment *in vitro* stimulates the process of angiogenesis (38). Therefore, Anusha et al. hypothesized that the increased expression of ovarian chemerin protein in PCOS subjects may cause derangements in ovarian steroidogenesis or angiogenesis that may trigger the development and progression of metabolism related reproductive disorder (39). In addition, murine model of polycystic ovaries when treated with pioglitazone and metformin showed improved insulin

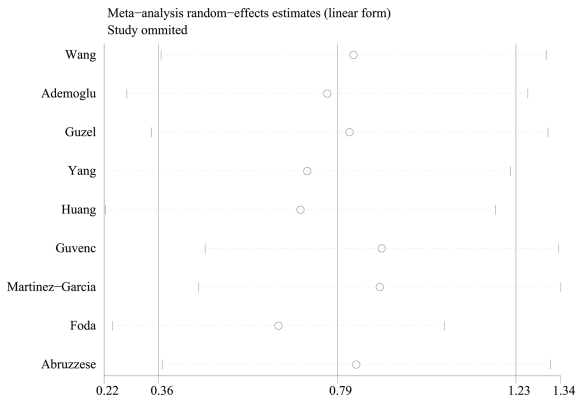

FIGURE 5
The sensitivity analysis results of circulating chemerin levels in patients with polycystic ovary syndrome compared with controls.

resistance and abnormal steroid production by attenuating the ovarian chemerin gene expression (40). These findings suggest that chemerin is a novel negative regulator that may contribute to PCOS pathogenesis. However, further investigation is necessary to understand the effects of chemerin on PCOS.

Although circulating apelin levels are significantly associated with diabetes, in our meta-analysis, there was no significant association between circulating apelin levels and PCOS. Apelin is expressed in granulosa cells, follicles, and follicular fluid and participates in the normal development of follicles, selection of dominant follicles, and proliferation and apoptosis of granulosa cells (41, 42). However, the influence of apelin on PCOS pathogenesis seems to be more complicated, as indicated by controversial data regarding the association between apelin levels, HOMA-IR, and body mass index (BMI) (12, 18, 22, 27). More high-quality studies are needed to better support the association between apelin and PCOS.

This meta-analysis aimed to statistically evaluate circulating apelin and chemerin levels in PCOS patients. However, this study had some limitations. Due to the lack of large sample case-controlled studies, most of the studies included in this meta-analysis were small. Additionally, some studies did not use BMI-matched healthy controls. Different detection methods for apelin and chemerin were used in these studies. All these factors may have affected the results; therefore, the results of this meta-analysis should be interpreted cautiously, as further research is needed.

Conclusion

This meta-analysis is the first to evaluate circulating apelin and chemerin levels in PCOS patients. Our findings suggest that circulating chemerin levels in PCOS patients are significantly higher than those in healthy controls. More high-quality studies are needed to better support the association between serum apelin levels and PCOS.

Data availability statement

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

Author contributions

XS designed the study. YG and XS searched databases and collected the data. HF and HW assessed the quality of the study. XS performed the analysis. HF and XS wrote the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1076951/full#supplementary-material

SUPPLEMENTARY DATA SHEET 1
The full electronic search strategy.

SUPPLEMENTARY DATA SHEET 2
Preferred reporting items for systematic review and meta-analyses (PRISMA) checklist

SUPPLEMENTARY FIGURE 1
The funnel plots of circulating apelin levels in patients with polycystic ovary syndrome compared with controls.

SUPPLEMENTARY FIGURE 2
The funnel plots of circulating chemerin levels in patients with polycystic ovary syndrome compared with controls.

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Add-on effect of the Guizhi Fuling formula for management of reduced fertility potential in women with polycystic ovary syndrome: A systematic review and meta-analysis of randomized controlled trials

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Background: Guizhi Fuling (GZFL) pill, a traditional Chinese herbal formula including *Semen Persicae*, *Ramulus Cinnamomi*, *Poria*, *Radix Paeoniae Alba*, and *Cortex Moutan*, has been widely applied in the management of gynecological diseases.

Objective: To evaluate the add-on effect of the GZFL formula for treating reduced fertility potential in women with polycystic ovary syndrome (PCOS) by conducting a systematic review and meta-analysis.

Methods: Two reviewers independently searched the PubMed, Embase, Cochrane Library, Wanfang, SinoMed, and CKNI databases until 09/11/2022. Eligible studies were randomized controlled trials (RCTs) of the GZFL formula plus Western medicine versus the Western medicine for treating PCOS. The primary endpoint was the ovulation, pregnancy, and miscarriage rate. The secondary endpoints included the serum follicle-stimulating hormone (FSH), total testosterone, luteinizing hormone (LH), estradiol, and homeostasis model assessment insulin resistance (HOMA-IR).

Results: There were 16 RCTs with 1,385 patients identified. The GZFL formula plus Western medicine significantly improved the ovulation rate (risk ratios [RR] 1.24; 95% confidence intervals [CI] 1.15–1.34) and pregnancy rate (RR 1.53; 95% CI 1.38 to 1.69) than the Western medicine alone. Adjuvant treatment with the GZFL formula also significantly decreased the serum FSH (mean difference [MD] -0.48 U/l; 95% CI -0.80 to -0.15), total testosterone (standard mean difference [SMD] -1.07; 95% CI -1.71 to -0.44), LH level (MD -2.19 U/l; 95% CI -3.04 to -1.34), and HOMA-IR (MD -0.47; 95% CI -0.60 to -0.34). However, there was no significant difference in the miscarriage rate (RR 0.89; 95% CI

0.36–2.20) and serum estradiol level (SMD 0.34; 95% CI -0.25 to 0.94) between two groups.

Conclusions: The GZFL formula as adjuvant therapy can improve the ovulation and pregnancy rates in women with PCOS. Its beneficial effects may correlate with reducing FSH, total testosterone, and LH and ameliorating insulin resistance. However, more well-designed RCTs with larger samples and multicenter trials are required to confirm the current findings due to uncertainty of the evidence.

Systematic review registration: PROSPERO identifier, CRD42022354530.

KEYWORDS

Guizhi Fuling capsule/pill, insulin resistance, meta-analysis, polycystic ovary syndrome, pregnancy, sex hormone

Introduction

Polycystic ovary syndrome (PCOS) is a hormonal disorder common among reproductive-age women. The pooled mean prevalence of PCOS was 21.27% using different diagnostic criteria (1). Women with PCOS are more likely to develop certain long-term health sequelae including type 2 diabetes, metabolic syndrome, and endometrial cancer (2). Apart from hormonal imbalance and metabolic problems, fertility reduced in ovulatory women with PCOS is also a big concern (3). Alterations in oocyte competence are considered potential causative factors for subfertility in women with PCOS (4). Management of fertility reduced in ovulatory women with PCOS include lifestyle changes, pharmacological ovulation induction, reproductive technologies, or laparoscopic ovarian drilling (5). However, achievement of successful fertility in women with PCOS remains a major concern (6).

Traditional Chinese medicine (TCM) has been used to treat gynecological diseases including PCOS. The Guizhi Fuling (GZFL) formula was firstly described in Jingui Yaolue of the Han dynasty. This prescription includes *Semen Persicae*, *Ramulus Cinnamomi*, *Poria*, *Radix Paeoniae Alba*, and *Cortex Moutan*. This classical formula exhibits the effects of activating blood and dissolving blood stasis according to TCM theory. The combination of the GZFL capsule/pill has been widely applied for treatment of PCOS (7, 8). A previous meta-analysis published in Chinese (9) has concluded that the GZFL formula combined with Western medicine was superior to the Western medicine in improving the ovulation and pregnancy rate in women with PCOS. However, the impact of the GZFL formula on sex hormone level and insulin resistance was not well-characterized in this meta-analysis.

No previous meta-analysis published in English literature has specially focused on the add-on effect of the GZFL formula for management of reduced fertility potential in women with PCOS. To address this knowledge gap, we conducted a systematic review and meta-analysis of randomized controlled trials (RCTs) to evaluate the add-on effect of the DZFL formula for treatment of infertility associated with PCOS.

Methods

Literature search

The current study was performed and reported based on the guidelines of Preferred Reporting Items for Systematic Reviews and Meta-Analyses (10). Our study was registered in the PROSPERO database (CRD42022354530). Two reviewers independently searched PubMed, Embase, Cochrane Library, Wanfang, VIP, SinoMed, and China National Knowledge Infrastructure databases until 09/12/2022. Keywords for the literature search included the following (Supplemental Text S1): "Gyejibokryeong-Hwan" OR "Guizhi Fuling" OR "Gui zhi Fu ling" AND "polycystic ovary syndrome" OR "polycystic ovarian syndrome" OR "PCOS" AND "randomized controlled trial" OR "random." Reference lists of retrieved studies and reviews were also manually searched to identify any additional eligible studies.

Study selection

Studies satisfying the following criteria were included: 1) patients with a clinical diagnosis of PCOS; 2) study design:

RCTs; 3) GZFL formula regardless of capsule, pill, or decoction plus Western medicine versus the same Western medicine alone as intervention; and 4) the primary endpoint was the ovulation rate, pregnancy rate, and miscarriage rate. The secondary endpoints included the serum follicle-stimulating hormone (FSH), total testosterone, luteinizing hormone (LH), estradiol level, and homeostasis model assessment insulin resistance (HOMA-IR). Exclusion criteria included the following: 1) modified GZFL formula as intervention; 2) GZFL formula combined with any complementary therapy as intervention; 3); any different treatment except for the GZFL formula between two groups; 4) patients with Cushing's syndrome or congenital adrenal hyperplasia, and 5) duplicate publication or suspected plagiarism.

Data extraction and quality assessment

The following data were collected by two independent reviewers from the selected trials: name of the first author, publication time, number of patients, mean age or age range, type/dosage of GZFL, detailed Western therapy, duration of intervention, duration of follow-up, outcome measures, and quality assessment information. The Cochrane Collaboration risk-of-bias tool was applied to evaluate the methodological quality of eligible trials, which assesses randomization generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessors, incomplete outcome data, selective outcome reporting, and whether to enroll patients according to TCM syndrome. Any disagreement between the two reviewers was settled by consensus or asked for the third reviewer.

Data analysis

All data were analyzed using Review Manager version 5.1 and STATA 12.0 (STATA Corp LP, College Station, TX, USA). The effect sizes were summarized by pooling weight mean difference (WMD) or standard mean difference (SMD) with a 95% confidence interval (CI) for the continuous outcome data. For the binary outcome data, we pooled the risk ratios (RR) with 95% CI for the GZFL formula plus Western medicine versus the Western medicine alone. Heterogeneity across trials was examined using the I^2 statistic and Cochrane Q test. A random-effect model was selected when there was significant heterogeneity (I^2 statistic ≥50% and/or p-value <0.1 of the Cochrane Q test); otherwise, we selected a fixed-effect model. To investigate the robustness of the pooling effect size, we conducted a leave-out one trial sensitivity analysis. Subgroup analysis was conducted according to the types of Western medicine, course of treatment, and form of prepared GZFL formula. Begg's test (11) and Egger's test (12) were used to assess publication bias. In the case of significant publication bias, the trim-and-fill analyses were used to correct the pooling effect size. The GRADE method was used to summarize the certainty of evidence.

Results

Search results and study characteristics

The literature search identified 776 articles, of which 538 articles were left after exclusion of duplicates. After reviewing the titles and abstracts, 490 articles were excluded and then 48 articles were left for full-text evaluation. Finally, 16 trials (13–28) were included in the meta-analysis after applying the predefined criteria for inclusion and exclusion. A flowchart of the study selection is shown in Figure 1.

The main features of the included trials are shown in Table 1. These eligible trials were published between 2008 and 2022. All the included trials were conducted in China and published in Chinese. A total of 1,385 women with PCOS were identified. The duration of treatment varied from three to six menstrual cycles. For each course of treatment, the GZFL formula was administered at a dosage of 2.79 to 12 mg daily except for the menstrual period. Clomiphene was administered at a dosage of 50 to 100 mg daily from the 5th to 10th days of menstruation. Ethinylestradiol/cyproterone acetate (ECA) 2 mg/0.035 mg daily was administered from the 5th day of menstruation for 21 consecutive days. Supplemental Figure S1 and Figure S2 summarize the risk of bias of included trials. According to the

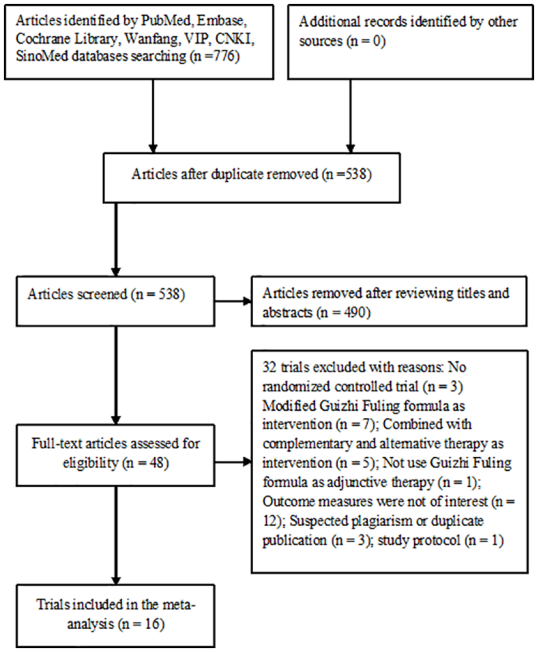

FIGURE 1
Flowchart showing the trial selection process.

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TABLE 1 Main features of clinical trials included in the meta-analysis.

Author/ year	Sample size	Age (years)	Main interventions		Course of treatment	Follow-up duration	Outcome measures
			Outcome measures	Outcome measures			
Zhao HB 2008 (13)	GZFL:34 Con: 34	28-34	GZFL capsule 2.79 g/day + clomiphene citrate 50 mg/day × 5	Clomiphene citrate 50 mg/day × 5	3 months		Pregnancy, ovulation, LH, FSH, TT, HOMA-IR
Shi SQ 2010 (14)	GZFL:24 Con: 17	Not reported	GZFL capsule 2.79 g/day + clomiphene citrate 50 mg/day × 5	Clomiphene citrate 50 mg/day × 5	3 months		Pregnancy, ovulation, TT
Ye HJ 2012 (15)	GZFL:30 Con: 30	GZFL: 27.7 ± 3.6 Con: 27.8 ± 3.8	GZFL capsule 2.79 g/day + ECA 2 mg/0.035 mg/day + clomiphene citrate 50 mg/day × 5	ECA 2 mg/0.035 mg/day + clomiphene citrate 50 mg/day × 5	3 menstrual cycles		Pregnancy, ovulation, LH, TT
Shao JY 2016 (16)	GZFL:44 Con:44	GZFL: 26.1 ± 5.16 Con: 25.3 ± 4.54	GZFL capsule 2.79 g/day + clomiphene citrate 50 mg/day × 5	Clomiphene citrate 50 mg/day × 5	3 months		Pregnancy, ovulation, LH, FSH, TT, Estradiol
Zhang LY 2016 (17)	GZFL:55 Con: 55	GZFL: 30.8 ± 6.9 Con: 29.8 ± 6.6	GZFL pill 12 g/day + ECA 2 mg/ 0.035 mg/day + metformin 1.5 g/ day	ECA 2 mg/0.035 mg/ day + metformin 1.5 g/ day	3 menstrual cycles		Pregnancy, ovulation, miscarriage, LH, FSH, TT, Estradiol, HOMA-IR
He WJ 2017 (18)	GZFL:45 Con: 32	GZFL: 27.49 ± 6.11 Con: 27.85 ± 6.18	GZFL pill 12 g/day + ECA 2 mg/ 0.035 mg/day	ECA 2 mg/0.035 mg/ day	3 menstrual cycles		Pregnancy, ovulation, LH, TT
Tian Y 2017 (19)	GZFL:54 Con: 53	GZFL: 29.8 ± 4.5 Con: 29.3 ± 4.1	GZFL capsule 2.79 g/day + ECA 2 mg/0.035 mg/day + metformin 1.0 g/day	ECA 2 mg/0.035 mg/ day + metformin 1.0 g/ d	3 menstrual cycles		Pregnancy, ovulation, LH, FSH, TT, Estradiol, HOMA-IR
Wang ZY 2017 (20)	GZFL:52 Con: 52	GZFL: 28.02 ± 3.81 Con: 27.21 ± 4.17	GZFL capsule 2.79 g/day + ECA 2 mg/0.035 mg/day	ECA 2 mg/0.035 mg/ day	3 months	3 months	Pregnancy, ovulation, LH, FSH, Estradiol
Cui YJ 2018 (21)	GZFL:28 Con: 28	GZFL: 28.0 ± 3.8 Con: 28.3 ± 2.5	GZFL tablet 2.88 g/day + ECA 2 mg/0.035 mg/day	ECA 2 mg/0.035 mg/ day	3 menstrual cycles	6 months	Pregnancy, ovulation, TT
Luo J 2018 (22)	GZFL:54 Con: 54	GZFL: 30.23 ± 2.19 Con: 29.65 ± 2.47	GZFL pill 12 g/day + ECA 2 mg/ 0.035 mg/day + clomiphene citrate 50 mg/day × 5	ECA 2 mg/0.035 mg/ day + clomiphene citrate 50 mg/day × 5	3 menstrual cycles	12 months	Pregnancy, miscarriage, LH, FSH, TT, estradiol

(Continued)

TABLE 1 Continued

Author/ year	Sample size	Age (years)	Main interventions		Course of treatment	Follow-up duration	Outcome measures
			Outcome measures	Outcome measures			
Wu ZW 2018 (23)	GZFL:40 Con:40	GZFL: 28.34 ± 3.59 Con: 28.16 ± 3.64	GZFL capsule 2.79 g/day + ECA 2 mg/0.035 mg/day	ECA 2 mg/0.035 mg/ day	6 menstrual cycles	12 months	Pregnancy
Cui Y 2019 (27)	GZFL:79 Con:78	GZFL: 28.97 ± 2.92 Con: 29.03 ± 3.08	GZFL pill 6–12 g/day + pioglitazone 15 mg/day	Pioglitazone 15 mg/day	3 menstrual cycles	6 months	Pregnancy, ovulation
Zhang Y 2019 (24)	GZFL:28 Con:28	GZFL: 25-40 Con: 24-38	GZFL capsule 2.79 g/day + ECA 2 mg/0.035 mg/day + metformin 1.0 g/day	ECA 2 mg/0.035 mg/ day + metformin 1.0 g/ day	3 menstrual cycles		Pregnancy, ovulation
Zhao XH 2019 (25)	GZFL:44 Con: 44	GZFL: 31.7 ± 3.6 Con: 31.4 ± 3.6	GZFL capsule 2.79 g/day + ECA 2 mg/0.035 mg/day	ECA 2 mg/0.035 mg/ day	3 months		Pregnancy, ovulation, LH, Estradiol
Zhao SY 2019 (26)	GZFL:53 Con: 53	GZFL: 28.54 ± 5.02 Con: 27.93 ± 3.21	GZFL pill 12 g/day + clomiphene citrate 100 mg/day	Clomiphene citrate 100 mg/day	4 menstrual cycles	12 months	Pregnancy, ovulation, FSH, TT, Estradiol
Liu W 2022 (28)	GZFL:45 Con: 46	GZFL: 28.76 ± 2.30 Con: 28.14 ± 2.52	GZFL capsule 2.79 g/day + ECA 2 mg/0.035 mg/day + clomiphene citrate 50 g/day × 5	ECA 2 mg/0.035 mg/ day + clomiphene citrate 50 g/day × 5	3 menstrual cycles		Pregnancy, ovulation, LH, TT, Estradiol

GZFL, Guizhi Fuling; Con, control; ECA, ethinylestradiol/cyproterone acetate; LH, luteinizing hormone; FSH, follicle-stimulating hormone; TT, total testosterone; HOMA-IR, homeostasis model assessment insulin resistance.

Cochrane Collaboration risk-of-bias tool, most of the trials were classified as suboptimal methodological quality with an unclear risk of bias. Only two trials (15, 28) enrolled the patients based on the TCM syndrome differentiation.

Ovulation rate

Fourteen trials (13–21, 24–28) reported the effect of the GZFL formula as an adjuvant therapy on the ovulation rate. As shown in Figure 2, the GZFL formula plus Western medicine significantly improved the ovulation rate (RR 1.24; 95% CI 1.15–1.34) compared with the Western medicine alone in a random-effect model, with significant heterogeneity (I^2 = 39.7%, p = 0.063). Leave-out one trial sensitivity analysis showed that the pooled RR of the ovulation rate ranged from 1.21 to 1.26 (all p-values < 0.05). Table S1 describes the results of subgroup analysis. Both the Begg's test (p = 0.002) and the Egger's test (p = 0.002) suggested the likelihood of publication bias. However, the "trim-and-fill" analysis showed that the corrected pooling RR of ovulation rate was 1.27 (95% CI 1.17–1.38).

Pregnancy rate

All the included trials reported the effect of the GZFL formula as an adjuvant therapy on the pregnancy rate. As shown in Figure 3, the GZFL formula plus Western medicine significantly improved the pregnancy rate (RR 1.53; 95% CI 1.38–1.69) compared with the Western medicine alone, without significant heterogeneity (I^2 = 32.8%, p = 0.10). Leave-out one trial sensitivity

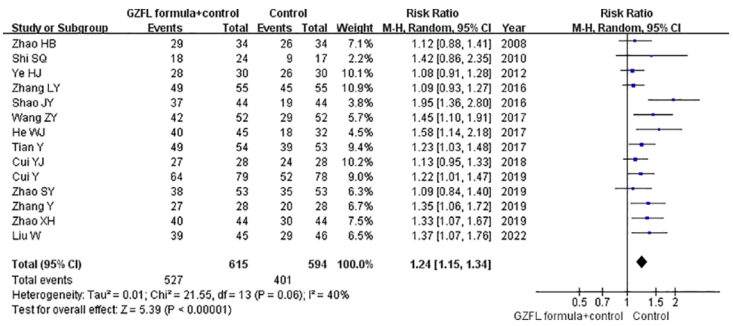

FIGURE 2
Forest plots showing the pooled ovulation rate comparing the GZFL formula plus Western medicine with the Western medicine alone.

analysis showed that the pooled RR of pregnancy rate ranged from 1.49 to 1.60 (all p-values < 0.05). Table S2 summarizes the results of subgroup analysis. The Begg's test (p = 0.006) and the Egger's test (p < 0.001) indicated the likelihood of publication bias. However, the "trim-and-fill" analysis suggested that the corrected pooling RR of pregnancy rate was 1.33 (95% CI 1.24–1.41).

Miscarriage rate

Two trials (17, 22) reported the effect of the GZFL formula as an adjuvant therapy on the miscarriage rate. As shown in Figure S3, there was no significant difference on miscarriage rate (RR 0.89; 95% CI 0.36–2.20; I^2 = 0.0%, p = 0.390) between the GZFL formula plus Western medicine and the Western medicine alone in a fixed-effect model.

Follicle-stimulating hormone

Seven trials (13, 16, 17, 19, 20, 22, 26) reported the effect of the GZFL formula on the serum FSH level. As shown in Figure 4, a random-effect model meta-analysis indicated that the GZFL formula plus Western medicine significantly reduced the serum FSH level (MD -0.48 U/l; 95% CI -0.80 to -0.15) compared with the Western medicine alone, with significant heterogeneity (I^2 = 86.0%, p < 0.001). Leave-out one trial sensitivity analysis indicated that the pooled MD of FSH ranged from -0.38 to -0.56 (all p-values < 0.05).

Luteinizing hormone

Nine trials (13, 15–20, 22, 28) reported the effect of the GZFL formula on the serum LH level. As shown in Figure 5, a random-effect model meta-analysis showed that the GZFL formula plus Western medicine significantly reduced the serum LH level (MD -2.19 U/l; 95% CI -3.04 to -1.34) compared with the Western medicine alone, with significant heterogeneity (I^2 = 94.0%, p < 0.001). Leave-out one trial sensitivity analysis indicated that the pooled MD of LH ranged from -2.02 to -2.50 (all p-values < 0.05).

Total testosterone

Eleven trials (13–19, 21, 22, 26, 28) reported the effect of the GZFL formula on the serum level of total testosterone. As shown in Figure 6, a random-effect model meta-analysis suggested that

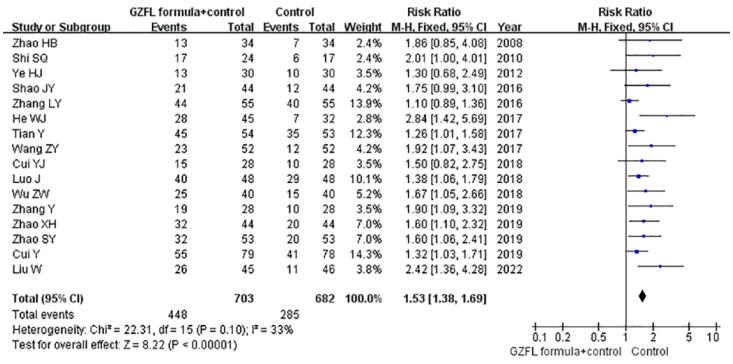

FIGURE 3
Forest plots showing the pooled pregnancy rate comparing GZFL formula plus Western medicine to the Western medicine alone.

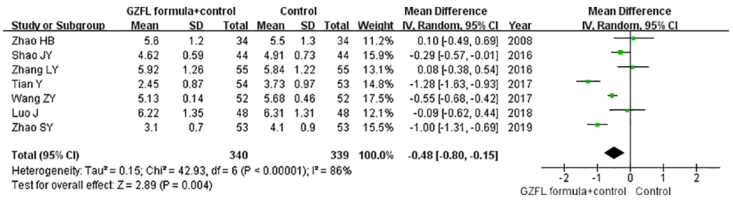

FIGURE 4
Forest plots showing the pooled serum follicle-stimulating hormone level comparing the GZFL formula plus Western medicine with the Western medicine alone.

the GZFL formula plus Western medicine significantly reduced the serum total testosterone level (SMD -1.07; 95% CI -1.71 to -0.44) compared with the Western medicine alone, with significant heterogeneity (I^2 = 95.0%, p < 0.001). Leave-out one trial sensitivity analysis indicated that the pooled SMD of total testosterone ranged from -0.81 to -1.18 (all p-values < 0.05). The Begg's test (p = 0.043) and the Egger's test (p = 0.099) indicated the likelihood of publication bias. However, the "trim-and-fill" analysis suggested that the corrected pooling SMD of serum total testosterone level was unchanged.

Estradiol

Nine trials (14, 16, 17, 19, 20, 22, 25, 26, 28) reported the effect of the GZFL formula on the serum estradiol level. As shown in Supplemental Figure S4, a random-effect model meta-analysis showed that there was no significant difference in serum estradiol level (SMD 0.34; 95% CI -0.25 to 0.94; I^2 = 94.0%, p < 0.001) between the GZFL formula pus Western medicine and Western medicine groups. Leave-out one trial sensitivity analysis indicated that the pooled SMD of estradiol ranged from 0.11 to 0.45 (all p-values > 0.05).

Homeostasis model assessment insulin resistance

Three trials (13, 17, 19) reported the effect of the GZFL formula on HOMA-IR level. As shown in Supplemental Figure S5, a random-effect model meta-analysis indicated that the GZFL formula plus Western medicine significantly reduced the HOMA-IR level (MD -0.47; 95% CI -0.60 to -0.34) compared with the Western medicine alone, with substantial heterogeneity (I^2 = 70.0%, p = 0.03). Leave-out one trial sensitivity analysis indicated that the pooled SMD of HOMA-IR ranged from -0.43 to -0.57 (all p-values < 0.05).

GRADE quality of evidence

The quality of evidence is summarized in Supplemental Table S3. The overall certainty of evidence was very low to moderate.

Discussion

This systematic review and meta-analysis evaluated the add-on effect of the GZFL formula for treating reduced fertility potential in women with PCOS. The main findings of our study were that the GZFL formula in combination with Western medicine significantly improved the ovulation and pregnancy rates in women with PCOS. The GZFL formula as adjuvant therapy could improve approximately 24% and 53% of the ovulation rate and pregnancy rate, respectively, when compared with the Western medicine alone. Moreover, adjuvant treatment with the GZFL formula also significantly reduced the serum FSH, total testosterone, and LH levels as well as HOMA-IR.

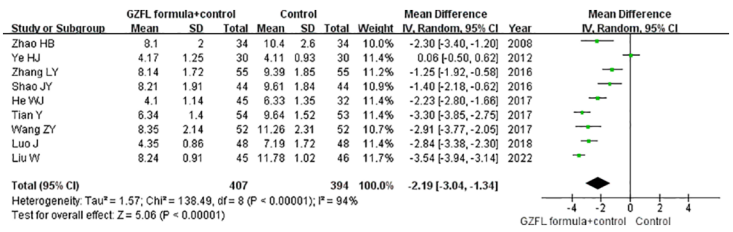

FIGURE 5
Forest plots showing the pooled serum luteinizing hormone level comparing the GZFL formula plus Western medicine with the Western medicine alone.

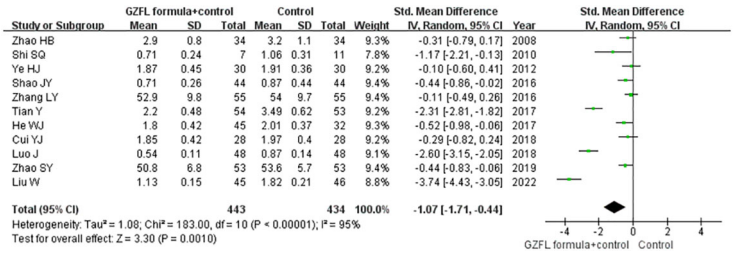

FIGURE 6
Forest plots showing the pooled serum total testosterone level comparing the GZFL formula plus Western medicine with the Western medicine alone.

Considering these findings all together, the GZFL formula as add-on therapy to Western medicine can achieve additional beneficial effects in women with PCOS. It should be noted that adjuvant treatment with the GZFL formula appeared to have no clear effect on the miscarriage rate. However, the certainty of evidence was very low to moderate mainly due to the unclear risk of bias and significant heterogeneity of the included trials.

Our subgroup analysis showed that the add-on effects of the GZFL formula on the ovulation and pregnancy rate were stronger in the studies with more than three menstrual cycles' treatment. The GZFL capsule appeared to exert better effects on the ovulation and pregnancy rates than the GZFL pill in the subgroup analysis, suggesting that the preparation of GZFL may affect its clinical effect. Regarding the types of Western medicine used, the add-on effect of GZFL appeared to be stronger in the patients who administered ECA or clomiphene citrate alone.

The pathological physiological manifestations of the PCOS are characterized by dysfunction of the hypothalamus–pituitary–ovarian axis and the gonadotropin-releasing hormone secretion, which can result in the secretion of serum FSH, LH, testosterone, and estrogen levels. Our meta-analysis indicated that the GZFL formula combined with Western medicine has more beneficial effects in reducing the serum levels of FSH, LH, and testosterone than Western medicine alone. In addition, the GZFL formula also had additional beneficial effects in reducing insulin resistance. A preclinical study showed that the GZFL formula could ameliorate insulin resistance in PCOS-insulin resistance rat through regulating intestinal flora to control inflammation (29). Moreover, the GZFL formula also inhibited granulosa cell autophagy and promoted follicular development to attenuate ovulation disorder in PCOS-insulin resistance rats (30).

Clomiphene citrate, ECA, metformin, and pioglitazone are used for the treatment of PCOS in the included trials. For patients with ovulatory infertility, clomiphene citrate has long been the gold standard for ovulation induction. Clomiphene citrate remains the first-line pharmacological therapy for infertility associated with PCOS (31). ECA suppresses the male sex hormones (androgens). However, use of ECA could increase the risk for venous thromboembolic complications (32).

Whether the add-on GZFL formula to Western medicine increases the adverse events is a big concern. Only one trial (27) reported adverse events including rash, headache, and insomnia. There was no significant difference in adverse events between the GZFL formula and control group. Adding the GZFL formula to Western medicine appeared to not increase the adverse events in this trial. However, we were unable to draw a firm conclusion about the safety of the GZFL formula combined with Western medicine. Future RCTs are warranted to investigate whether the GZFL formula as adjuvant therapy to Western medicine increases the adverse events.

The current systematic review and meta-analysis had important clinical implications. Adding the GZFL formula to Western medicine could significantly improve the ovulation and pregnancy rates. Regarding the preparation of the GZFL formula, the effect of the GZFL capsule on the ovulation and pregnancy rates appeared to be stronger than the GZFL pill. More than three menstrual cycles' treatment could exert better effects than that with less than three menstrual cycles. In addition, the add-on effect of the GZFL formula was more pronounced in combination with ECA or clomiphene citrate alone. Based on the theory of TCM, the GZFL formula is more suitable for patients with Qi stagnation and blood stasis syndrome. However, TCM syndrome differentiation was not considered in the majority of included trials. Future trials should consider the TCM syndrome differentiation in the process of patient selection.

Our systematic review and meta-analysis had certain limitations. First, a major concern is the methodological flaws of the analyzed trials. Only six trials clearly report the method of randomization. Nevertheless, all the included trials did not mention the allocation concealment and blind method. Second, majority of the included trials did not take into account the TCM syndrome differentiation in their diagnostic procedures, which could have resulted in potential selection bias of patients. Third, there was significant heterogeneity in the pooling serum hormone level and HOMA-IR. Different patients' characteristics, course of treatment, types of GZFL formula, and regimens of Western medicine may contribute to the observed heterogeneity. Fourth, all included original RCTs were written in Chinese, which gives difficulty for the readers to evaluate the

quality of original trials. Finally, results of stratified analysis were potentially unreliable because of the small number of trials included in the subgroups.

Conclusions

The GZFL formula as adjuvant therapy to Western medicine can improve the ovulation and pregnancy rate in women with PCOS. The beneficial effects of the GZFL formula may correlate with reducing serum FSH, total testosterone, LH, and ameliorating insulin resistance. However, more well-designed RCTs with larger samples and multicenter trials are required to confirm the current findings due to the uncertainty of 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 author.

Author contributions

Study conception/design and interpretation of data: WM; literature search, data extraction, quality assessment, and statistical analysis: AR and NT; writing of the manuscript: LE; revising of the manuscript: WM. All authors read and approved the final version of manuscript.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.995106/full#supplementary-material

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The mutational analysis of mitochondrial DNA in maternal inheritance of polycystic ovarian syndrome

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Introduction: Polycystic Ovarian Syndrome (PCOS) is a globally prevalent condition that leads to infertility in women. While environmental factors contribute to PCOS, maternal genetics also play a significant role. Currently, there is no definitive test for identifying predisposition to PCOS. Hence, our objective is to discover novel maternal genetic risk factors for PCOS by investigating the genomes of patients from Pakistan.

Methods: We utilized Next-Generation Sequencing (NGS) to sequence the complete mitochondrial DNA of three PCOS patients. Subsequently, we employed MitoTIP (Mitochondrial tRNA Informatics Predictor) and PON-mt-tRNA tools to identify variations in the mitochondrial DNA. Our analysis focused on the genes MT-RNR1, MT-RNR2, MT-ATP6, MT-TL2, and MT-CYTB, which displayed common variations in all three genomes. Additionally, we observed individual variations. The D-loop region exhibited the highest frequency of mutations, followed by the non-coding regions of RNR1 and RNR2 genes. Moreover, we detected frameshift mutations in the mitochondrially encoded NADH Dehydrogenase 2 (MT-ND2) and mitochondrially encoded NADH Dehydrogenase 5 (ND5) genes within individual genomes.

Results: Our analysis unveiled six regions with common variations in the mitochondrial DNA of all three PCOS patients. Notably, the MT-RNR1, MT-RNR2, MT-ATP6, MT-TL2, and MT-CYTB genes exhibited these variations. Additionally, we identified individual variations in the mitochondrial DNA. The D-loop region displayed the highest mutation frequency, followed by the non-coding regions of RNR1 and RNR2 genes. Furthermore, frameshift mutations were detected in the MT-ND2 and ND5 genes within individual genomes.

Conclusion: Through our study, we have identified variations in mitochondrial DNA that may be associated with the development of PCOS and have the potential to serve as predisposition tests. Our findings highlight the presence of novel mutations in the MT-RNR1, MT-RNR2, MT-ATP6, MT-TL2, and MT-CYTB genes, as well as frameshift mutations in the MT-ND2 and ND5 genes. Pathogenicity analysis indicated that most variants were likely to result in benign cysts. However, the frameshift mutations in the ND2 gene were associated with a high risk of complications and pathogenicity in PCOS. This is the first report identifying these mutations and their association with PCOS, contributing to our understanding of the genetic factors underlying the condition.

KEYWORDS

mitochondrial DNA, mutations, PCOS, genome, sequence analysis, pathogenicity

Introduction

Polycystic ovarian syndrome (PCOS) is a complex endocrine disorder that affects up to 8%–13% of women in their reproductive age (1). PCOS is a growing concern worldwide, with increasing incidence rates reported (2). The disorder is characterized by the presence of numerous ovarian cysts visible through ultrasound inspection (3). PCOS is associated with various symptoms such as menstrual irregularities, hormonal dysfunction, dermatological issues, psychological problems, high cancer risk, and metabolic disorders (4). Various gynecological issues are associated with PCOS, including anovulatory infertility (5), variations in oocyte competency (OC), which can lead to subfertility (6, 7), endometrial dysfunction, and abnormal trophoblast invasion and placentation (8), which can increase the risk of miscarriage and pregnancy complications in women with PCOS (5). PCOS women who have hyperandrogenic conditions are also at a higher risk of developing pervasive developmental disorders (PDDs) (9). Hormonal dysfunction is considered a key feature of PCOS (8, 10), while insulin resistance and hyperandrogenism have also been reported, leading to decreased folliculogenesis and an increased risk of comorbidities and androgenic alopecia (11). The most prominent impact of PCOS on women's lives is menstrual irregularities and ovarian cancer. PCOS is a multifactorial disorder, with several risk factors contributing to its etiology, including obesity, neuroendocrine status, environment or lifestyle, and genetic makeup (3). Variations in mitochondrial DNA (mtDNA) are increasingly recognized as a genetic cause (12, 13). Both nuclear and mitochondrial genetic variations have been associated with PCOS pathogenesis (14). Nuclear genes, including calpain 10 (CAPN10), cytochrome family P450, insulin (INS) gene, androgen receptor (AR), fat mass obesity (FTO) gene, and follicle-stimulating hormone receptor (FSHR) gene, have been shown to be associated with PCOS (15).

The mitochondrial genome is considered more vulnerable to oxidative damage and has a high mutation rate due to the lack of protective histones, inefficient DNA repair mechanisms, and its proximity to the electron transport chain (ETC), where oxygen-derived free radicals are frequently generated (16). Hence, the mitochondrial genome is considered a Pandora's box of pathogenic mutations (16). This study was designed to screen the whole mitochondrial DNA (WMTDNA), comprising 37 mitochondrial genes using next-generation sequencing and to predict *in silico* the resultant common variations for pathogenicity. *In silico* analysis was used to evaluate the pathogenicity of mutations and their impact on subjects.

Material and methods

Ethical statement

The experimental procedures were approved by the Ethical Committee of the Institution and Board of Advanced Studies and Research at Hazara University, Mansehra (21300), and Pakistan under notification number F.No.73/HU/ORIC/IBC/2017/400.

Consent, recruitment of patients, and families

The Rotterdam criteria have been used for diagnosing PCOS patients (10). According to these criteria, a patient must have two of the following three symptoms: hyperandrogenism (biochemical or clinical), oligo- or anovulation, and polycystic ovary morphology (PCOM), as determined by ultrasound inspection.

After obtaining informed consent and a physical examination by a gynecologist, selected patients were interviewed about their family history and other details, and pedigrees were constructed to trace the maternal inheritance pattern of their disorder. Saliva samples were then collected from each patient. Patients with hyperprolactinemia, thyroid and adrenal diseases, 21-hydroxylase deficiency, and androgen-secreting tumors were excluded because these disorders mimic the symptoms of PCOS. Finally, three fully expressed syndromic patients were selected and subjected to whole mitochondrial genome sequence (WMGS) analysis to draw the genetic portrait of mitochondrial mutational hotspots associated with maternally inherited PCOS.

Variant calling and identification of homoplasmic and heteroplasmic mutations were carried out. After obtaining detailed family histories and information about deceased members, the pedigrees of the three families were constructed (see Figure 1). The mutations identified in WMGS were assessed in other family members of the probands through Sanger sequencing.

DNA extraction, NGS analysis, and identification of variants

DNA was extracted from saliva samples using the phenol-chloroform method (17). Nanodrop quantification and gel electrophoresis were performed to determine the quantity and quality of the isolated DNA. Samples were then carefully labeled and stored at −20°C. The labeled samples were sent to a commercial company for DNA sequence analysis. Online DNA analysis tools like National Center for Biotechnology Information (NCBI) Blast, Universal Protein Resource (Uniprot), and UGENE were used to conduct further alignment and investigations. The resulting nucleotide sequences were compared to the revised Cambridge Reference Sequence (rCRS). We performed Sanger sequencing for validation of the identified common variations for D-loop, ATP6, MT-TL2, and CYTB.

In the UGENE (http://ugene.net/) editor, two nucleotide sequences were aligned, and variations were checked. Multiple sequence alignment was performed using integrated multiple sequence comparison by log expectations (MUSCLE) on UGENE.

Score-based evaluation of tRNA variants for pathogenicity and validation by *in silico* predictive tools

Mitochondrial tRNA Informatics Predictor (MitoTIP) and PON-mt-tRNA were utilized to determine the pathogenicity of mitochondrial variations. MitoTIP was employed to assess the pathogenicity status of genetic variations, while the PON-mt-tRNA, a multifactorial probability-based prediction approach, was used to classify the studied and identified mitochondrial variants. Some variants were reported to be deleterious, some benign, one novel frameshift, and one pathogenic based on the results from both MitoTIP and PON-mt-tRNA. The difference in the degree of predicted variants is attributed to the fact that both tools operate on distinct algorithms/principles and consider diverse factors (18–20).

Results

The current study focused on the mitochondrial genome of patients with maternally inherited PCOS. To achieve this, three patients were selected for the study, each from a different family with a history of maternally inherited PCOS. Furthermore, the experiment focused on analyzing the WMTDNA of these patients to identify any mutations or genetic variations that may be associated with the development of PCOS.

Clinical evaluations

At the onset of the condition, the average age range was 30–35 years. Among these patients, two were unmarried and one was married but having infertility issues. One PCOS patient was suffering from diabetes due to insulin resistance, and the other two had catamenia, dermatological, metabolic, and hormonal issues. Other family members of these three patients also suffered from PCOS. Most of the symptoms, such as obesity, dermatological problems, catamenia, hirsutisms, and hormonal issues, are common in the three PCOS patients. The detailed clinical data of the PCOS patients are given in Table 1.

Common mitochondrial DNA mutations were identified in the coding and noncoding region of the three PCOS patients in the present study

The analysis of the whole mitochondrial genome has enabled us to identify a set of mutations present in three familial subjects with PCOS. The mutations that were identified in all three genomes are presented in Table 2. Specifically, eight mutations at eight different positions in six genes were identified as common among the three PCOS patients. Of these, two mutations were located in the D-loop

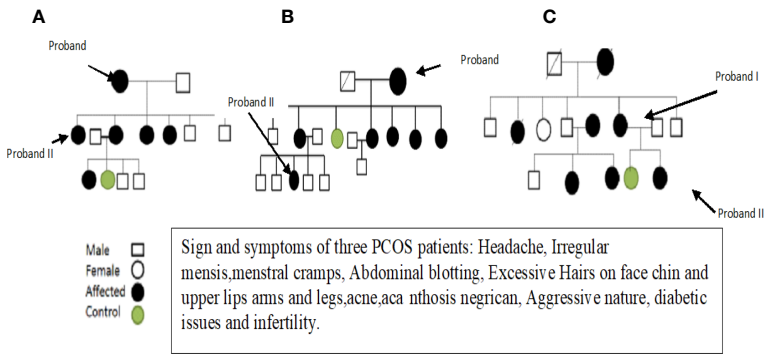

FIGURE 1
The family pedigree of patients 1 **(A)**, 2 **(B)**, and 3 **(C)** based on the information provided.

TABLE 1 Clinical manifestations of three PCOS patients selected for WMTDNA analysis.

Clinical characteristics	Patient 1	Patient 2	Patient 3
Remarks	Patient	Patient	Patient
Candidate's age	30	28	35
Family history	+	+	+
Marriage history	Married	Unmarried	Unmarried
Catamenia problems	+	+	+
Irregular menses	+	+	+
Metrorrhagia	+	+	+
Amenorrhea	+	–	–
Diabetic problem	+	–	–
Insulin resistance	+	–	–
Dermatological problems	+	+	+
Hirsutism	+	+	+
Infertility problems	+	–	–
Obesity	+	+	+
Elevated abdominal circumference	+	+	+
Hormonal issues	+	+	+

"+" Presence of PCOS symptoms in PCOS patients; "–" Absence of PCOS symptoms in PCOS patients.

region, one in RNR1, one in RNR2, one in ATP6, one in MT-TL2, and one in CYTB, while four were missense variants, two were identified in intergenic regions, and one was a noncoding transcript. To further validate these findings, the mutations were revalidated in other PCOS patients through Sanger sequencing of the individual positions, as depicted in Figure 2.

Mutations were identified in the whole mitochondrial genome sequence of the three PCOS patients in the present study

The nucleotide sequence analysis has identified 36 mutations in PCOS patient 1, including 11 variants in the D-loop region, seven variants in the *RNR1* and *RNR2* genes, nine synonymous mutations, and nine missense mutations (Figure 3).

Similarly, the nucleotide sequence analysis has identified 38 mutations in PCOS patient 2, including 14 variants in the D-loop region, five variants in the *RNR1* and *RNR2* genes, nine synonymous mutations and eight missense mutations, and one stop-loss and one frameshift (Figure 4).

Furthermore, the WMTDNA nucleotide sequence analysis has identified 19 mutations in PCOS-positive patient 3, including five variants in the D-loop region, four variants in the *RNR1* and *RNR2* gene, five synonymous mutations and three missense mutations, and one stop-loss and one frameshift (Figure 5).

Pathogenicity status of the studied mutations

The pathogenicity of the identified variants was evaluated using different tools. Results revealed that among all the common

TABLE 2 Common mitochondrial DNA mutations identified in the coding and noncoding regions of three PCOS patients in the present study.

Sr. No.	Mutation	Gene name	Complete name	Location
1	73A>G	D-loop	Displacement loop	Intergenic region
2	263A>G	D-loop	Displacement loop	Intergenic region
3	1438A>G	RNR1	12S ribosomal RNA	Noncoding transcript
4	3106CN>C	RNR2	16S ribosomal RNA	Missense variant
5	8860A>G	ATP6	ATP synthase F0 subunit 6	Missense variant
7	12308A>G	MT-TL2	Mitochondrial transfer RNA leucine 2	Missense variant
8	15326A>G	CYTB	Cytochrome *b*	Missense variant

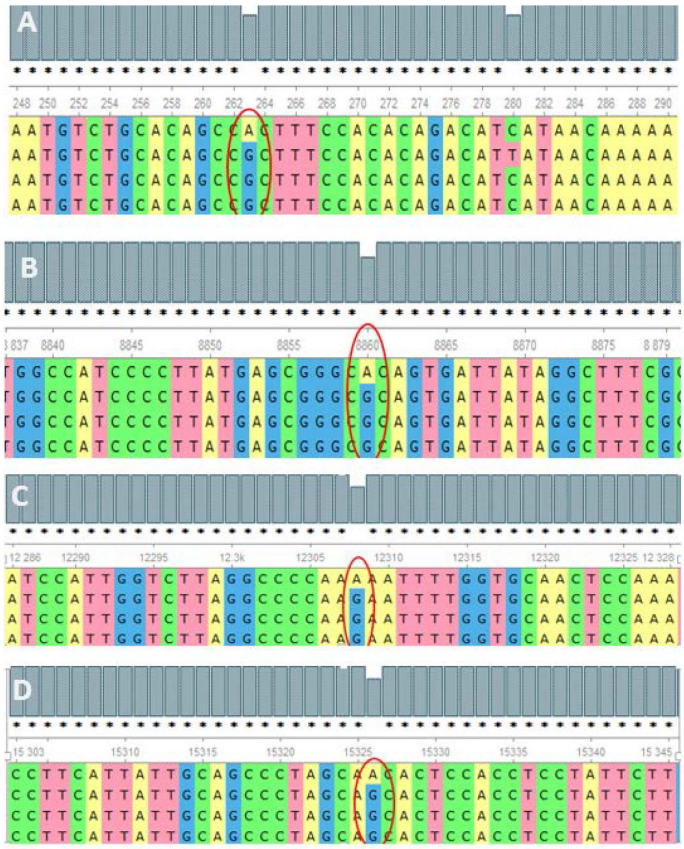

FIGURE 2
Alignment of the sequence resulted in nucleotides from maternally inherited PCOS-positive patients with rCRS Accession No. NC-012920.1 exhibiting mutations (encircled) at positions **(A)** 263A>G (D-loop), **(B)** 8860A>G (MT-ATP6), **(C)** 12308A>G (MT-TL2), and **(D)** 15326A>G MT-CYTB.

mutations identified in the three whole mitogenomes of PCOS patients after NGS analysis, mutations reported in RNR1 at positions 709A>G, 750A>G, 1438A>G, and 1393 G>A were found to be resulting in translation defects that were pathogenic. MT-12S rRNA variations are possibly associated with disruption of mitochondrial function. The mutations found in RNR2 at positions 2706A>G, 2831G>A, 3106CN>C, and 1888G>A. These mutations are present at the 530 loops of the ribosome, and they affect the codon–anticodon interaction at the A site (acceptor site). These mutations lead to the improper movement of the small subunit

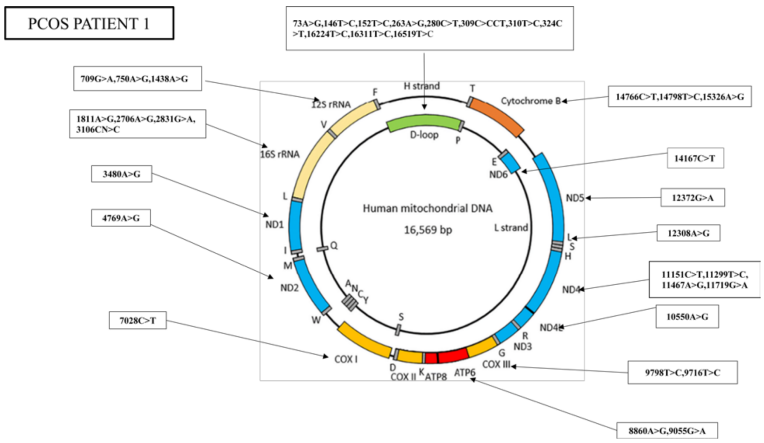

FIGURE 3
Whole mitogenome variants identified in PCOS patient 1. Mitochondria consist of two strands; an outer heavy strand and an inner light strand. Overall, 36 mutations were identified in the whole mitochondrial genome: 11 in D-loop, three in RNR1, four in RNR2, one in ND1, one in ND2, one in COX1, two in ATP6, two in –COX3, one in ND4L, four in ND4, one in ND5, one in ND6, and three in CYTB.

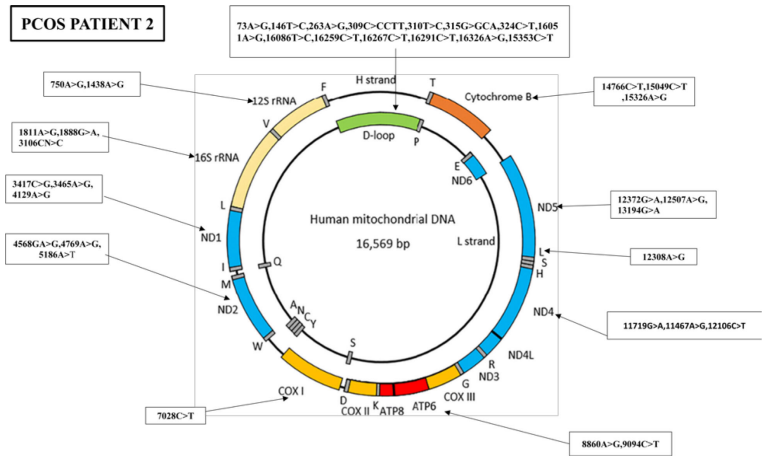

FIGURE 4
Mutations identified in the whole mitochondrial DNA sequence of PCOS-positive patient 2. Overall, 38 mutations were identified in the whole mitochondrial genome of PCOS patient 2: 14 mutations were found in D-loop, two in RNR1, three in RNR2, three in ND1, three in ND2, one in COX1, two in ATP6, three in ND4, three in ND5, one in MT-TL2, and three in CYTB.

(SSU) head during translocation and finally the translocation (Table 3).

ML probability of pathogenicity: average probability of pathogenicity predicted by 20 machine learning (random forests) predictors

In addition, a mutation in the *MT-ATP6* gene at position 8860A>G with a score of 0.1 was declared as tolerated with a score of 0.003. A mutation in mitochondrial transfer RNA leucine 2 at position 12308A>G with a score of 11.8509 was declared likely neutral with a score of 0.41. A mutation at position 11719G>A in the *ND4* gene is a synonymous variant and pose no impact on the amino acid type.

Discussion

Overall, the present study aimed to investigate the mitochondrial genome of patients with maternally inherited PCOS. Three patients, each from a different family with a history of maternally inherited PCOS, were selected for the study. The study focused on analyzing the WMTDNA of these patients to identify any mutations or genetic variations that could be associated with the development of PCOS. By analyzing the mitochondrial genome of these patients, we aimed to gain insights into the potential genetic factors that contribute to the development of this disorder, specifically those that are passed down maternally.

PCOS is a common endocrine disorder that affects reproductive-age women worldwide and is the leading cause of ovulatory dysfunction and infertility (21). PCOS is characterized by hyperandrogenism, anovulation, and polycystic ovaries, along with

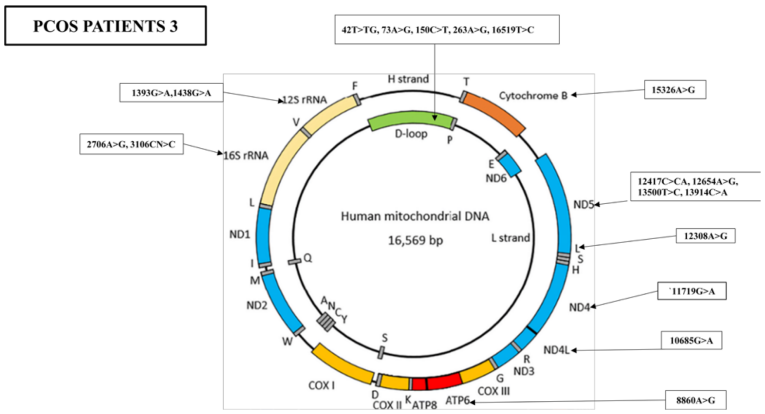

FIGURE 5
Mutations identified in the whole mitochondrial DNA sequence of PCOS patient 3. Overall, 19 mutations were identified in the whole mitochondrial genome: five mutations were found in D-loop, two variants in RNR1, two variants in RNR2, one in ATP6, one in ND4, one in ND4L, one in TL2, four in ND5, and one in CYTB.

TABLE 3 Pathogenicity prediction of genetic variants detected in PCOS patients.

Sr. No.	Genes	Mutation	rsID	SIFT; PolyPhen	MitoTIP score	Pon-mt-tRNA	Comment
1	MT-RNR1	1438A>G	rs2001030	–	–	–	Noncoding transcript exon variant
2	MT-RNR2	3106CN>C	rs368669629	–	–	–	Noncoding transcript exon variant
3	MT-CYTB	15326A>G	rs2853508	0.28 tolerated; 0.009 benign	–	–	
4		11719 G>A					Synonymous variant
5	MT-ATP6	8860A>G	rs2001031	0.1; 0.003 tolerated	–	–	Missense variant
6	MT-TL2	12308A>G	rs2853498	–	11.8509 (Q3)	0.41	

insulin resistance, obesity, and metabolic disorders (1). Several predisposing risk factors, including genetic, neuroendocrine, lifestyle/environmental, and obesity, have been linked to PCOS development (3). Moreover, mtDNA mutations are also considered to contribute to the pathogenesis of PCOS. MI mutations that were potentially associated with PCOS-IR were as follows: mt-tRNA$^{Leu\ (UUR)}$ A3302G and C3275A mutations; mt-tRNA^{Gln} T4363C and T4395C mutations; mt-tRNA$^{Ser(UCN)}$ C7492T mutation; mt-tRNA^{Asp} A7543G mutation, mt-tRNA^{Lys} A8343G mutation, mt-tRNA^{Arg} T10454C mutation, and mt-tRNA^{Glu} A14693G mutation (22). They utilized a whole mitochondrial genome sequencing analysis for three maternally inherited PCOS familial subjects. Their findings revealed several variations in mtDNA. Patient 1 had 11 variants in the d-loop region, including 73A>G, 146T>C, 152T>C, 263A>G, 280C>T, 309C>CCT, 310T>C, 324C>T, 16224T>C, 16311T>C, and 16519T>C. The variant 73A>G, 146T>C, and 152T>C have been previously reported to be associated with gastric colon and oral cancer, PCOS, and breast cancer (23–25). Women with PCOS may be more susceptible to some cancers due to their abnormal metabolic and hormonal conditions.

Prolonged hormone stimulation is known to be associated with the development of endometrial, ovarian, and breast cancers in women (24, 26). In this study, we identified several mtDNA variants in the three maternally inherited PCOS familial subjects that have been previously reported to be associated with various types of cancer and other diseases. Variant 263A>G was found to be linked with perilesional skin and skin tumors and PCOS (27). Variants 310T>C and 324C>T have been associated with epithelial ovarian cancer (28), while 16224T>C and 16311T>C have been linked to perilesional skin and skin tumors (27). Variant 309C>CCT has been associated with the etiology of malignant melanoma (29). One variant in the *COX1* gene, which has been reported to be associated with PCOS, esophageal cancer, congenital contract, and obesity, was found in patient 1 (23, 30, 31). Similarly, variant 11719G>A has been linked to breast cancer, obesity, and PCOS (24, 31). Moreover, one variant reported in transfer RNA leucine 2 at nucleotide position 12308A>G has been associated with breast cancer, colorectal cancer, prostate and kidney cancer, Alzheimer's disease, and cardiomyopathy (24, 25, 32, 33).

Our investigation identified three variants in the *CYTB* gene, including 14766C>T and 14798T>C, which have not been previously reported in association with PCOS (27). Women with a predisposition to PCOS may experience metabolic and hormonal issues such as insulin resistance and hyperandrogenism, which can lead to weight gain and eventually obesity. Obesity, in turn, can exacerbate the symptoms of PCOS, resulting in further metabolic complications and reproductive abnormalities. Our study also identified two mitochondrial variants in the *ATP6* gene, namely 8860A>G and 9055G>A. These variants have been previously associated with hypertrophic cardiomyopathy (25) and nonsyndromic hearing loss (28). The vascular characteristics of arterial walls involved in the atherogenic process may be directly influenced by androgen excess in PCOS, as reported by Wu et al. (34) in 2020. Furthermore, mutations were discovered in both the coding and noncoding regions of mitochondria in selected patients who suffered from various issues such as skin problems, hormonal imbalances, diabetes, menstrual problems, and infertility. Other family members were also identified as having this disorder (Table 1).

Fourteen variants were identified in the D-loop of patient 2, including 73A>G, previously found to be associated with gastric colon and oral cancer (24), 146T>C with oral cancer (23) and PCOS, 263A>G in association with PCOS (24), 310T>C with malignant melanoma (26), and 513G>GCA with nodular sclerosing Hodgkin lymphoma (Mitomap). Variants not previously reported in the literature with any disease but identified in our study that may be associated with PCOS are 16051A>G, 16086T>C, 16259C>A, 16267C>T, 16291C>T, 16326A>G, and 16353C>T.

In PCOS patient 2, two variants were identified in RNR1, including 750A>G, which has been reported to be associated with brain tumors (28), obesity (31), and PCOS (24), while 1438A>G is associated with obesity (31), type 2 diabetes, Parkinson's disease, and PCOS. In RNR2, three variants were reported, including 1811A>G, which has been associated with congenital cataracts (30) and PCOS (24). However, 1888G>A and 3106CN>C have not been reported in previous literature with any disease. Three variants were identified in ND1, including 3417C>G, 3465A>G, and 4129A>G, with no previous reports in association with any disorder, making them novel findings in this investigation. Similarly, three variants were reported in ND2, including the 4769A>G (frameshift) variant previously associated with esophageal cancer and PCOS (24), while 4569GA>G and 5186A>T (stop loss) variants have not been reported in previous studies with any disease. In the *ATP6* gene, two variants were

identified at position 8860A>G, reported to be associated with hypertrophic cardiomyopathy and PCOS, while 9094C>T is associated with primary ovarian insufficiency (35). In PCOS patient 2, a single variant was found in transfer RNA leucine 2, previously associated with breast cancer (24), and colorectal and kidney cancer (33). Three variants were identified in the cytochrome *b* gene, including 15326A>G, previously associated with PCOS (27), while 14766C>T and 15049C>T mutations have not been reported in any previous studies.

Five variants were identified in the mitochondrial D-loop region of PCOS patient 3, including 73A>G, 150C>T, 263A>G, and 16519T>C, previously associated with PCOS and gastric, colon, and oral cancers. In addition, 42T>TG was also identified. Two variants were identified in RNR1, including 1393G>A with no previous reports and 1438A>G with a reported association with obesity (31), type 2 diabetes, Parkinson's disease, and PCOS (27). However, 3106CN>C has not been reported in previous literature with any disease.

One variant was identified in ND4L at position 10685G>A, and four variants were identified in ND5 at positions 12417C>CA, 12654A>G, 13500T>C, and 13914C>A. One variant was identified in ND6 at position 14305G>A, and one variant was identified at position 15326A>G, both associated with PCOS (27). We identified mutations in both the coding and noncoding regions of mitochondria, which may have phenotypic effects on patients. PCOS patient 3 also suffered from skin issues such as acne and hirsutism, hormonal problems, diabetes, menstrual problems, frequent urination, and some psychological issues. Other family members were also found to be suffering from this disorder (refer to Table 1). The identified mutations and their biological effects in our current study and in other previous studies have been summarized in Table 4.

Conclusions

In conclusion, studying the mutation spectrum of the entire mitochondrial genome is a valuable tool for investigating various maternally inherited genetic disorders in humans, including PCOS. Mitochondrial dynamics, including the mutational spectrum, can be explored to elucidate the etiology of such genetic diseases, given that mitochondria have all the mechanisms for energy transduction in many cells and organs. Homoplasmic variations can have catastrophic consequences, while heteroplasmic mutations may have a lesser impact. This study identified mutations in the *D-loop*, *MT-RNR1*, *MT-RNR2*, *MT-ATP6*, *MT-TL2*, and *MT-CYTB* genes that are most likely associated with the etiology of maternally inherited PCOS in Pakistan. To help patients receive appropriate treatment, genetic testing for the condition and public awareness efforts should be implemented. To validate the association of these mutations with PCOS, it is recommended to predict the defects and replicate the sequence analysis with a larger sample size in other parts of the world. Furthermore, the paternal inheritance pattern should be studied in PCOS patients from Pakistani families. Overall, this study highlights the potential of mitochondrial genetic variations as a novel biomarker for PCOS diagnosis and management. Further research is needed to establish a causal relationship between these mutations and the development of PCOS.

TABLE 4 Mitochondrial DNA mutations identified in our stud of the WMTDNA of PCOS patients and their association with other diseases.

Sr. No.	Mutation	Gene name	Biological effect in the current study	Biological function	References
1	73A>G	D-loop	PCOS	Breast cancer; PCOS; gastric, colon, and oral cancers	Czarnecka et al. (24) and Zhuo et al. (27)
2	263A>G	D-loop	PCOS	Perilesional skin and skin tumor, PCOS	Zhuo et al. (27) and Durham et al. (36)
3	1438A>G	MT-RNR1	PCOS	PCOS	Zhuo et al. (27) and Wang et al. (31)
4	3106CN>C	MT-RNR2	PCOS, dermatological issues	PCOS, dermatological issues	In the current study
5	8860A>G	MT-ATP6	PCOS	Hypertrophic cardiomyopathy, PCOS	Zhuo et al. (27) and Grasso et al. (32)
6	11719G>A	MT-ND4	PCOS	Breast cancer, obesity, PCOS	Czarnecka et al. (24) and Zhuo et al. (27)
7	12308A>G	MT-TL2	PCOS	Breast, colorectal, and kidney cancers; Alzheimer's disease; cardiomyopathy	Czarnecka et al. (24), Weigl et al. (37), and Booker et al. (33)
8	15326A>G	MT-CYTB	PCOS	PCOS	Zhuo et al. (27)

Eight different mutations at a different positions in six genes, these mutations are already reported in association with different diseases.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Ethical Committee of the Institution and Board of Advanced Studies and Research at Hazara University, Mansehra (21300), Pakistan under notification numberF.No.73/HU/ORIC/IBC/2017/400. The patients/participants provided their written informed consent to participate in this study.

Author contributions

SB, MK, GA, ST, and MZ designed the study and wrote the manuscript. ST and MZ supervised the manuscript. SB, TN, QU, MK, AU, MF, SG, MN, and GA helped in the collection of data resources and editing of the final version of the manuscript. Moreover, data analysis and collection were completed by SB, TN, and MF. 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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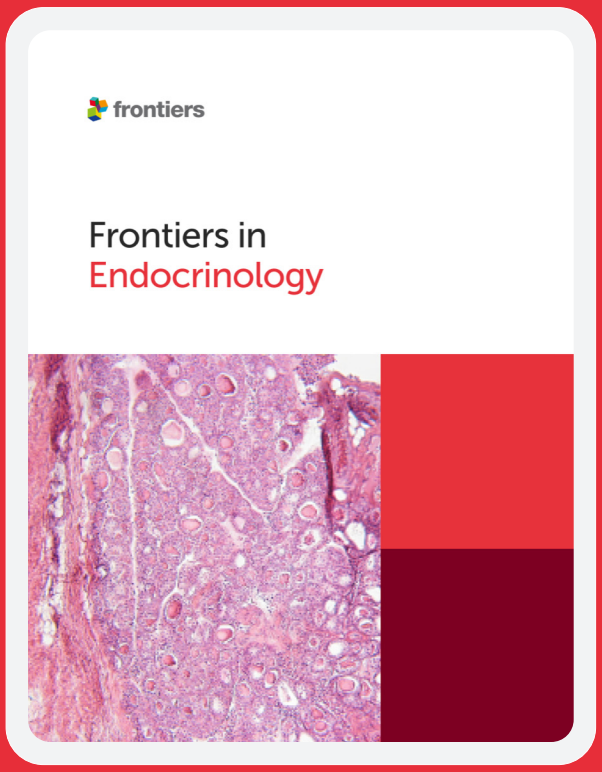

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