Original Article

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Effect of Yiqi Bushen Shugan Huoxue Decoction on impaired endometrial receptivity associated with ovarian stimulation: A clinical trial, modular pharmacology, molecular docking, and experiment-based study

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SUMMARY: Endometrial receptivity plays a critical role in pregnancy, while controlled ovarian hyperstimulation (COH) — widely used for infertile patients — could impair endometrial receptivity and subsequent pregnancy outcomes. This study aims to explore the effect of Yiqi Bushen Shugan Huoxue Decoction (YBSHD) on impaired endometrial receptivity in patients with unexplained infertility (UI) undergoing COH and to determine the mechanism for it through modular pharmacology, molecular docking, and a murine model. First, we retrospectively studied 422 patients with UI who underwent COH to get pregnant. Results indicated that the live birth rate significantly increased in the YBSHD group. Second, a systematic network pharmacology analysis was performed to screen the ingredients and possible targets of YBSHD. The main targets concerning YBSHD and endometrial receptivity involved pathways including hormone regulation, inflammatory responses, and apoptosis. The active components of quercetin and kaempferol from YBSHD exhibited a strong binding affinity to key molecules, including BCL2, ESR1, IL6, IL1B, and TNF. Third, YBSHD improved endometrial receptivity in a murine COH model. Compared to the COH group, the number of embryo implantations and endometrial pinopodes significantly increased in the YBSHD group, indicating improved endometrial receptivity. YBSHD improved the local immune microenvironment in COH mice by regulating excessive hormone secretion, gene expression of inflammatory factors, and proportions of neutrophils and macrophages. Moreover, YBSHD inhibited apoptosis in the ovaries and uteruses of COH mice. In summary, YBSHD could increase the live birth rate in patients with UI, mainly because it can inhibit inflammation and cell apoptosis, thereby improving endometrial receptivity.

Keywords: endometrial receptivity, traditional Chinese medicine, modular pharmacology, inflammation, apoptosis

1. Introduction

Studies have found that the lifetime prevalence and period prevalence of infertility have reached 17.5% and 12.6%, respectively (*1,2*). Despite the development of *in vitro* fertilization embryo-transfer technology (IVF-ET), the clinical pregnancy rate and live birth rate remain around 30% worldwide (*3-5*). A major reason is the failure of the embryo to implant in a receptive uterus. Successful embryo implantation occurs during the synchronization of the embryo and the endometrium. However, controlled ovarian hyperstimulation (COH), commonly used for infertile women, may impair endometrial receptivity by altering the release of hormones and the window of implantation.

Various means of enhancing endometrial receptivity have been tested from bench to bedside, including aspirin (*6*), sildenafil (*7*), and endometrial scratching (*8*). However, the guidelines have yet to reach a definitive conclusion to date. Chinese herbal medicine has demonstrated unique advantages in dealing with poor endometrial receptivity in recent years. Current clinical

methods used to manage it include addressing a Kidney deficiency, Liver Qi stagnation, and a Spleen deficiency (*9,10*). The herbal formula Yiqi Bushen Shugan Huoxue Decoction (YBSHD), consisting of 18 precisely selected herbs, was formulated in line with traditional Chinese medicine (TCM) theory and the clinical experience of the present research team. Our clinical observations indicated that COH could adversely affect pregnancy outcomes, whereas emerging evidence suggests that YBSHD has the potential to enhance endometrial receptivity in infertile patients. Therefore, a clinical trial involving patients with unexplained infertility (UI) undergoing COH and a murine COH model were designed to explore the effect and mechanism of YBSHD on impaired endometrial receptivity caused by COH. Moreover, network pharmacology analysis and molecular docking were used to explore the mechanism of YBSHD. The flowchart for this study is shown in Figure 1. By establishing a comprehensive "YBSHD-active components-therapeutic targets-endometrial receptivity" network, the study has sought to provide scientific validation for the use of this traditional formulation in contemporary reproductive medicine.

2. Materials and Methods

2.1. A retrospective clinical trial

2.1.1. Subjects

Patients with UI who were seen at the Obstetrics and Gynecology Hospital of Fudan University between January 2018 and January 2022 were retrospectively identified. The study was approved by the ethics committee of this hospital (kyy2020-156), and this study was conducted in accordance with the Helsinki Declaration.

2.1.2. Criteria

Subjects were patients with UI who were unable to conceive after at least one year of sexual intercourse without contraception. Inclusion criteria included: 1) between 20 and 40 years of age; 2) body mass index (BMI) < 30 kg/m^2; 3) no tubal infertility according to hysterosalpingography or ultrasound; 4) no ovulation abnormalities; 5) the spouse's semen was within the normal range; 6) couples with a normal chromosome karyotype; 7) patients undergoing COH. Exclusion criteria included: 1) a congenital uterine malformation; 2) endometrial diseases such as intrauterine adhesions, endometrial polyps, thin endometrium, endometrial hyperplasia, endometritis, or endometrial tuberculosis; 3) submucosal fibroids, intramural fibroids, moderate or severe endometriosis, or adenomyosis; 4) endocrine and metabolic disorders; 5) autoimmune diseases; and 6) malignant tumors or other serious diseases. Patients with UI were selected by excluding those with evident causes of infertility or endometrial pathologies. Known confounders affecting endometrial receptivity were controlled for, enabling the evaluation of YBSHD's impact on COH-associated endometrial dysfunction in patients with UI.

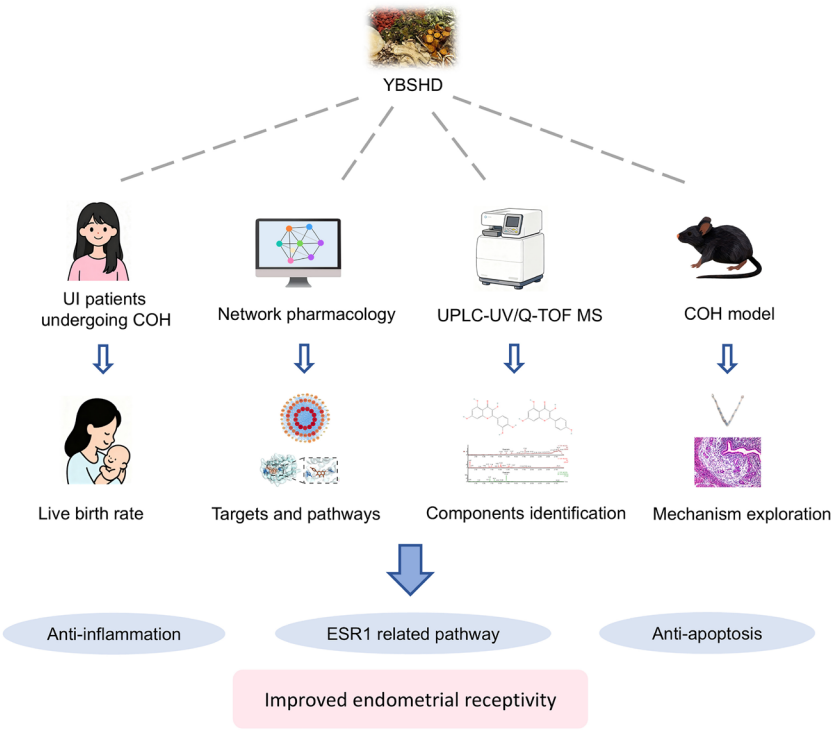

Figure 1. Flowchart of the study.

2.1.3. Intervention

Four hundred and twenty-two patients with UI were divided into a YBSHD group and a control group according to their medication regimen. The dosage regimen for COH for all patients was based on relevant clinical trials (*11,12*). Patients in the control group were administered letrozole 2.5 mg once a day for 5 consecutive days from the third to the fifth day of the menstrual cycle. Based on the control group, patients in the YBSHD group were administered both letrozole and YBSHD, the herbal components of which are listed in Supplementary Table S1 (*https://www.biosciencetrends.com/action/getSupplementalData.php?ID=278*), at the same time. YBSHD was administered orally at a dose of 150 ml twice a day for 14 days. For patients in all groups, 75 IU of human menopausal gonadotropin (HMG) was administered once daily for another 5 days after letrozole treatment. Follicles were monitored from the 10th to the 12th day of the menstrual cycle. Ten thousand IU of human chorionic gonadotropin (hCG) was injected when the dominant follicle reached 18 mm in diameter. Then, the patient was instructed to have sexual intercourse three times every other day. Dydrogesterone (10 mg, twice a day) was administered continuously for 10 days from the 15th to the 20th day of the menstrual cycle. Patients in both groups were treated for 3 consecutive menstrual cycles. The medication was discontinued if pregnancy was confirmed.

2.1.4. Outcomes

The primary outcomes included the clinical pregnancy rate and live birth rate. Definitions were based on the WHO guidelines and international consensus (*13,14*). Clinical pregnancy was defined as the presence of a gestational sac in vaginal ultrasound at six weeks of gestation. Live birth was defined as the delivery of a live fetus after 20 weeks of pregnancy. The evaluation of safety included liver and kidney function tests, routine blood tests, a routine stool examination, and routine urine tests.

2.2. Network pharmacology and fingerprint analysis

Methods for identifying the components and targets of YBSHD, prediction of the compound-target relationship network, protein-protein interaction (PPI) network and enrichment analysis, molecular docking, and fingerprint analysis of YBSHD are shown in the Supplementary Data (*https://www.biosciencetrends.com/action/getSupplementalData.php?ID=278*).

2.3. Animals and treatments

All C57BL/6 mice were purchased from Shanghai Jiesijie Experimental Animal Technology Co., Ltd. Female mice between the ages of 4 weeks and 6 weeks and male mice between the ages of 6 weeks and 8 weeks were used. The mice were housed in a specific pathogen-free environment (light/dark cycle, 12/12 h; ambient temperature, 20-25°C) and fed a regular chow diet ad libitum. All procedures conformed to ethical regulations and were approved by the Ethics Committee of the Hospital of Obstetrics and Gynecology, Fudan University. Mice were randomly divided into three groups (Ctrl group, COH group, and YBSHD group) based on weight. The workflow of the animal experiment is summarized in Figure 5A. The Ctrl and COH groups were gavaged with distilled water (0.2 ml/20 g bw/d) from day 1 to day 10. The YBSHD group was gavaged with an equal volume of YBSHD (29.3 mg/g bw/d) during the same period. Decoction products of YBSHD were obtained from the pharmacy of the Hospital of Obstetrics and Gynecology, Fudan University. They were concentrated and sterilized with a rotary evaporator and stored in a -20°C refrigerator for future use. Both the COH group and YBSHD group received intraperitoneal injections of PMSG (10 IU/0.1 ml, ProSpec Technogene, Israel) on day 7, followed by hCG (10 IU/0.1 ml, ProSpec Technogene, Israel) 48 h later. 0.1 ml of saline was given intraperitoneally to the Ctrl group simultaneously. Then, the female and male mice were mated at a ratio of 1:2 or 2:3 overnight. The morning of vaginal plug formation was counted as pregnant day (PD) 0.5. Mice were anesthetized with tribromoethanol (0.2 ml/10 g, Aladdin, China) and killed by cervical dislocation at PD 4.5 for tissue processing. Materials and methods for analysis of embryo implantation, H&E staining, scanning electron microscopy, ELISA of serum hormones, RT-qPCR, flow cytometric profiling of murine cells, and TUNEL staining are shown in the Supplementary Data (*https://www.biosciencetrends.com/action/getSupplementalData.php?ID=278*).

2.4. Statistical analysis

Methods for statistical analysis are shown in the Supplementary Data (*https://www.biosciencetrends.com/action/getSupplementalData.php?ID=278*).

3. Results

3.1. Effect of YBSHD on pregnancy outcomes in patients with UI undergoing COH

Potential subjects for the retrospective study were 656 patients with UI, 508 of whom met the criteria. Exclusions due to non-compliance with medical advice and loss to follow-up resulted in a final analysis of 214 controls and 208 patients receiving YBSHD, for a total of 422 patients (Supplementary Figure S1, *https://www.biosciencetrends.com/action/getSupplementalData.php?ID=278*). The demographic baseline characteristics

of the patients were analyzed and compared. The mean age was 31.4 ± 3.7 and 30.7 ± 4.0 for the control group and the YBSHD group (P = 0.06). Years of infertility were similar in the two groups (3.2 ± 2.1 versus 3.0 ± 1.9; P = 0.69). There were no significant differences between the two groups in terms of BMI (21.0 ± 5.7 versus 21.2 ± 4.5; P = 0.31).

The pregnancy outcomes were further analyzed in patients with UI. The clinical pregnancy rate was 26.17% in the control group and 32.69% in the YBSHD group, but the difference was not significant. The live birth rate was 21.03% in the control group. Compared to the control group, the live birth rate of 29.81% in the YBSHD group was significantly higher, suggesting that YBSHD effectively improves endometrial receptivity and promotes live births in patients with UI undergoing COH (Table 1). The safety of YBSHD was evaluated during the treatment and follow-up periods. Adverse reactions in the patients included nausea, vomiting, and mild abdominal pain. No serious drug adverse reactions were reported.

Table 1. Pregnancy outcomes of patients with UI

	Control group (n = 214)	YBSHD group (n = 208)	P value
Clinical pregnancy rate (%)	56 (26.17)	68 (32.69)	0.16
Live birth rate (%)	45 (21.03)	62 (29.81)	0.04

YBSHD, Yiqi Bushen Shugan Huoxue Decoction.

3.2. Identification of active ingredients and targets of YBSHD

The active ingredients of YBSHD and their corresponding targets were obtained using the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP) database (Supplementary Table S1, *https://www.biosciencetrends.com/action/getSupplementalData.php?ID=278*). The most common compounds sorted by topological degree were as follows: quercetin (in 9 herbs, degree=140), kaempferol (in 9 herbs, degree = 59), luteolin (in 4 herbs, degree = 55), β-sitosterol (in 7 herbs, degree=37), and stigmasterol (in 8 herbs, degree = 3) (Supplementary Table S2, *https://www.biosciencetrends.com/action/getSupplementalData.php?ID=278*). The target information was matched with the protein target information in the UniProt database, resulting in 224 compounds and 310 targets after deduplication. A total of 3327 compound-target relationships were visually depicted as a compound-target network by Cytoscape (Figure 2A).

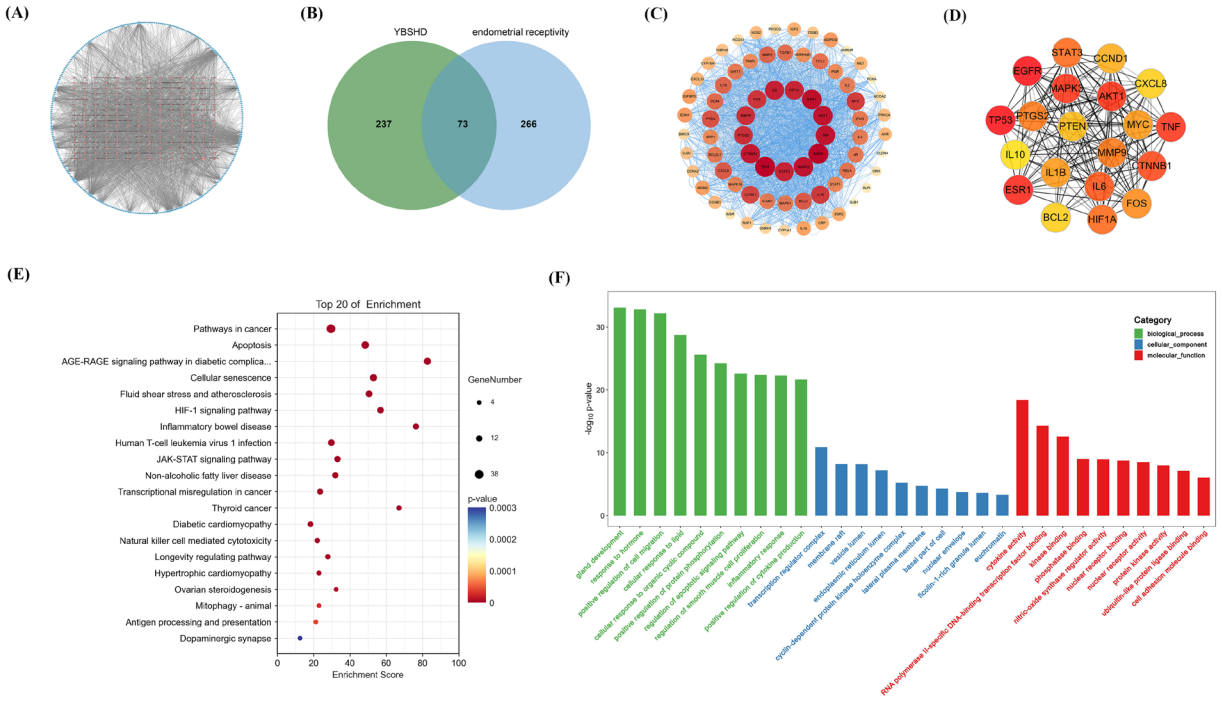

Figure 2. Network target analysis of YBSHD's effect on endometrial receptivity. (A) The network showed the compound-target relationship of YBSHD. Pink and blue nodes represent the compound and target genes of YBSHD, respectively. **(B)** The Venn diagram highlighted 73 overlapping targets between YBSHD and endometrial receptivity. **(C)** PPI network analysis of YBSHD targets and endometrial receptivity. The nodes represent target proteins, and the edges represent protein-protein associations. The node colors range from red to yellow, and sizes range from large to small, representing the degree of protein binding. **(D)** The top 20 targets network generated by Cytoscape. The nodes represent the core target genes, and the edges represent the interactions between targets. The node's color, which ranges from red to yellow, represents the degree of targets in descending order. **(E)** Bubble graph of the KEGG pathway for the top 20 core genes. **(F)** GO analysis showed therapeutic targets in biological processes, cellular components, and molecular functions.

3.3. Network analysis of YBSHD's effect on endometrial receptivity

Six hundred and seventy-eight targets related to endometrial receptivity were identified from the GeneCards, DrugBank, and OMIM databases, and 339 targets were finally determined based on relevance. A Venn diagram revealed 73 overlapping targets between the 339 endometrial receptivity-related targets and the 310 drug targets from YBSHD (Figure 2B). The 73 targets were imported into the String database to obtain a PPI network of YBSHD and endometrial receptivity, and the result was graphically depicted using Cytoscape (Figure 2C). The top 20 target genes, including TNF, ESR1, AKT1, HIF1A, BCL2, CXCL8, EGFR, TP53, MAPK3, CTNNB1, IL6, STAT3, PTGS2, MMP9, FOS, MYC, IL1B, CCNBD1, PTEN and IL10, were identified with the plugin cytoHubba (Figure 2D and Supplementary Table S3, *https://www.biosciencetrends.com/action/getSupplementalData.php?ID=278*).

3.4. Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) enrichment analysis

KEGG pathway and GO enrichment analysis were performed using the top 20 target genes identified. KEGG analysis indicated that the pathways associated with the regulation of YBSHD in endometrial receptivity mainly involve apoptosis of cells, JAK-STAT signaling pathway, ovarian steroidogenesis, mitophagy, antigen processing and presentation, *etc.* (Figure 2E). The biological processes involved in the GO enrichment analysis include inflammatory response, response to hormones, positive regulation of cell migration, regulation of apoptotic signaling pathways, positive regulation of cytokine production, and gland development. Cellular components involved in the GO enrichment analysis include transcription regulator complex, endoplasmic reticulum lumen, and nuclear envelope. The molecular functions involved include cytokine activity, phosphatase binding, nuclear receptor binding, nuclear receptor activity, cell adhesion and molecule binding (Figure 2F).

3.5. Molecular docking of quercetin and kaempferol

According to network pharmacology, the main ways for YBSHD to improve endometrial receptivity may be related to the response to hormone, regulation of apoptotic signaling pathway, and inflammatory response. Results also indicated that quercetin and kaempferol were the top two components in YBSHD in terms of degree and the number of single herbs containing these components. Therefore, quercetin and kaempferol were selected as the primary active components of YBSHD, and their potential binding affinity to those related targets (ESR1, BCL2, IL6, IL1B, and TNF) was evaluated using molecular docking (Figure 3). The general belief is that the absolute value of the docking score >5 kcal/mol denotes favorable binding, whereas an absolute value >9 kcal/mol implies exceedingly strong binding activity. Therefore, molecular docking further verifies the advantage of YBSHD compounds in acting on multiple targets and pathways. Quercetin and kaempferol from YBSHD demonstrated strong binding affinity with ESR1, BCL2, IL6, IL1B, and TNF (Table 2).

3.6. Analysis of the chemical composition of YBSHD

The above results confirmed the clinical efficacy of YBSHD in treating patients with UI. Network pharmacology suggested that YBSHD may act through multiple pathways, with quercetin and kaempferol (chemical structure shown in Figure 4A-B) likely serving as key components. Here, fingerprint analysis was used to identify the active ingredients of YBSHD. UPLC-UV/Q-TOF MS technology revealed the chromatographic fingerprint of YBSHD (Figure 4E). The mass spectrum of quercetin and kaempferol standards showed a detectable peak at a mass-to-charge ratio (*m/z*) of approximately 302 and 286, respectively (Figure 4C-D), which matches the molecular weight, suggesting that the mass spectra of the standards can serve as a reference for fingerprint analysis. The mass spectrum of YBSHD was compared to those of the two reference standards, and the results indicated that the chromatogram of YBSHD contained both quercetin and kaempferol peaks, confirming the presence of these two components in YBSHD.

3.7. Effect of YBSHD on endometrium and embryo implantation in COH mice

One of the pivotal indicators of decreased endometrial receptivity is the reduction in embryo implantation (*15*). Consistent with the clinical investigation of YBSHD in patients with UI undergoing COH, a COH mouse model was used to verify YBSHD's effect on endometrial receptivity. The implantation sites in each group were counted by injecting a Chicago sky blue dye solution intravenously into PD 4.5 mice. Results indicated that the mean number of implantation sites decreased significantly in the COH group compared to that in the Ctrl group, with observable uterine swellings and hydrops in the uterine horns in the COH group, suggesting a hyperstimulated state (Figure 5B). YBSHD treatment increased the number of embryo implantations (Figure 5F) and restored uterine morphology. There were no significant differences in the uterus index among the groups (Figure 5G).

Endometrial morphology was further observed using H&E staining. Compared to the Ctrl group, mice in the COH group had a significantly thinner endometrium and fewer glands, characterized by small glandular lumens with insufficient secretion and a more compact stroma

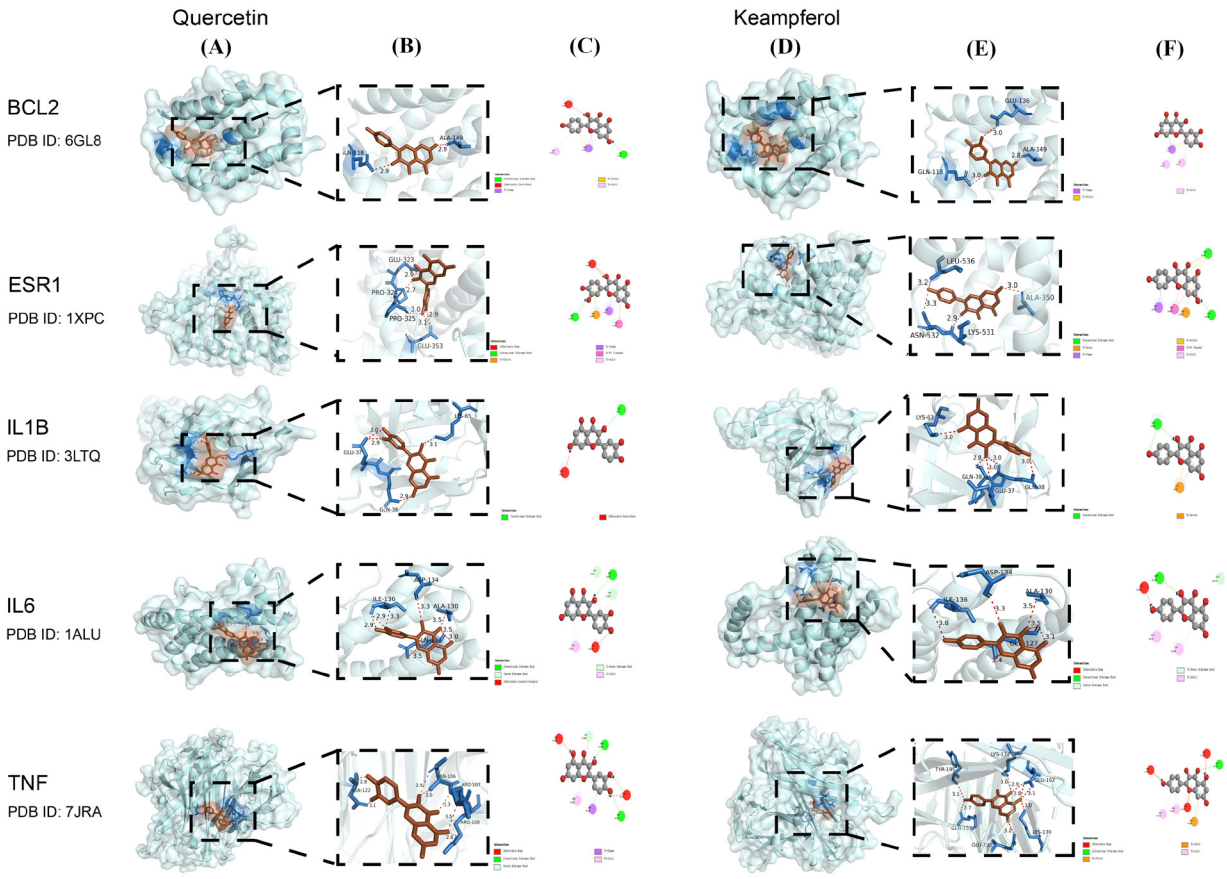

Figure 3. Molecular docking of quercetin and kaempferol with five gene proteins. Quercetin **(A)** / kaempferol **(D)** is located in the protein cavity pocket. Quercetin **(B)** / kaempferol **(E)** forms hydrogen bonds with key amino acid residues of proteins. Red dotted lines denote hydrogen bonds. Quercetin **(C)** / kaempferol **(F)** and protein 2D structure diagram. Green dotted lines represent hydrogen bonds. Pi-pi interaction is depicted with pink dotted lines, and pi-cation and pi-anion interactions are depicted with orange dotted lines.

Table 2. Molecular docking results of quercetin and kaempferol with five target genes

Component	Affinity (kcal/mol)				
	TNF	ESR1	IL6	IL1B	BCL2
Quercetin	-5.9	-7.2	-5.7	-5.0	-6.8
Kaempferol	-8.7	-8.3	-6.4	-6.3	-7.8

(Figure 5C-D). YBSHD administration thickened the endometrium and restored the number and size of uterine glands (Figure 5H-I). The enlarged lumen contained secretory substances, which displayed a secretory phase change. The stroma became loose and gradually differentiated into decidual cells after being treated with YBSHD. Pinopodes are a specific morphological marker of endometrial receptivity. The luminal surface of the endometrial epithelia in the COH mice was relatively smooth and flat, and the membrane projections were sparse with irregular arrangement and margin compared to the Ctrl group (Figure 5E). The reduction of pinopodes in COH mice indicated a lagged development of pinopodes. In mice treated with YBSHD, the dome-shaped bulge re-emerged on the endometrial epithelial surface and was covered with microvilli. An increased number of pinopodes confirmed improved endometrial receptivity in YBSHD mice (Figure 5J).

3.8. Effect of YBSHD on hormone receptors in COH mice

Estrogen and progesterone play a crucial role in the establishment of endometrial receptivity by regulating endometrial growth and development. Both hormones perform diverse functions mainly through binding to their corresponding receptors. Accordingly, the levels of expression of estrogen receptor 1 and progesterone receptor in the uterus were examined. Results confirmed that the expression of *Esr1* and *Pr* mRNA was significantly down-regulated in the COH model (Figure 5K-L). In contrast, YBSHD treatment significantly up-regulated the expression *of Esr1* compared to that in the COH group (Figure 5K). Leukemia inhibitory factor (LIF), one of the classic markers of endometrial receptivity, is also one of the downstream target proteins of ESR1. COH significantly decreased the expression of *Lif* mRNA, while YBSHD effectively increased its expression (Figure 5M), suggesting that YBSHD restored

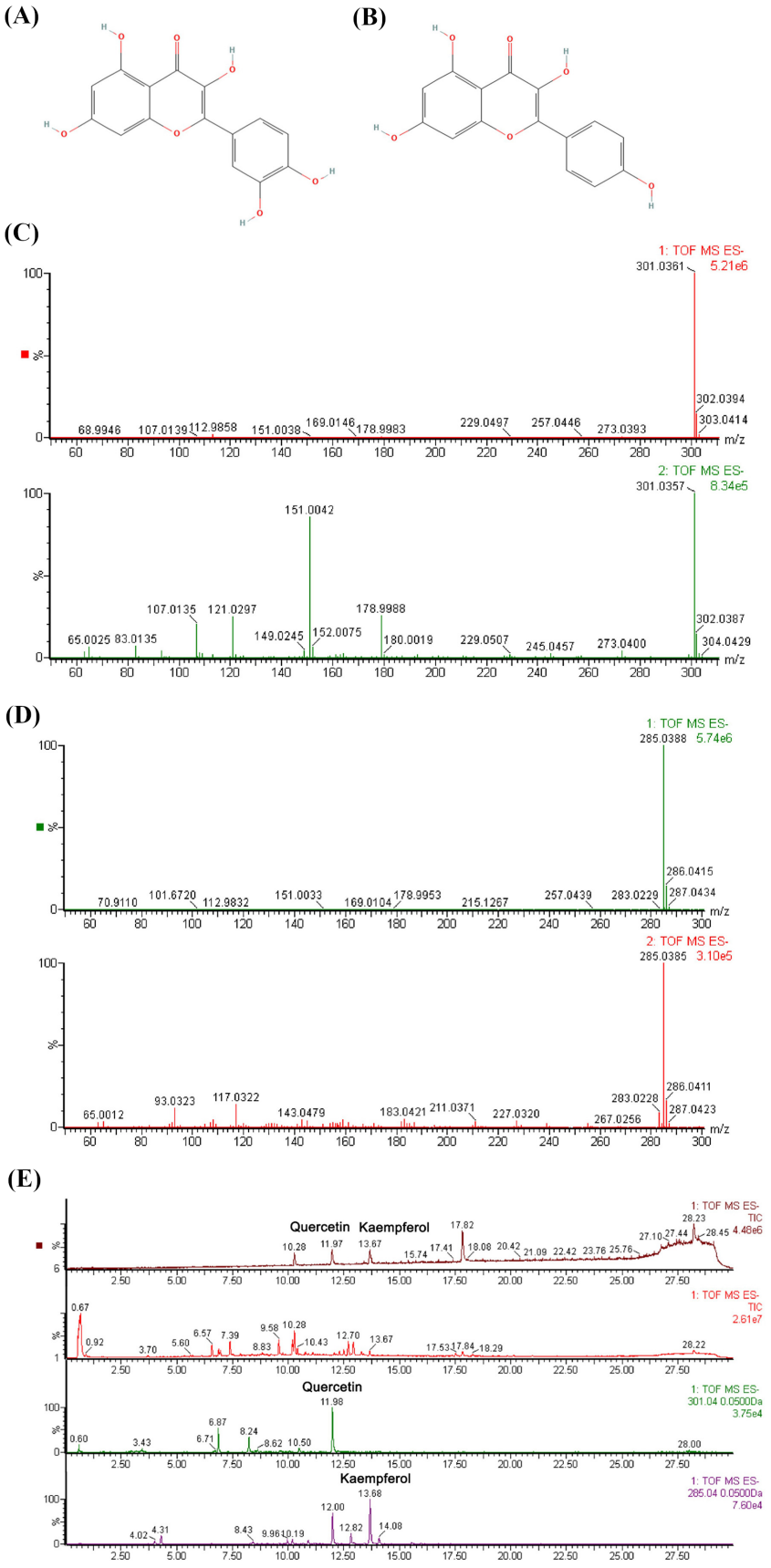

Figure 4. Fingerprint analysis of YBSHD using UPLC-UV/Q-TOF MS. Chemical structure of quercetin **(A)** and kaempferol **(B)**. Mass spectrum of the quercetin standard **(C)** and the kaempferol standard **(D)**. Primary and secondary mass spectrometry data are shown on the first and second lines, respectively. **(E)** Mass spectrum of YBSHD. The first line indicates the mass spectrum of the standards. The second line indicates the total ion chromatogram. Peaks for quercetin and kaempferol were detected in the third and fourth lines, respectively.

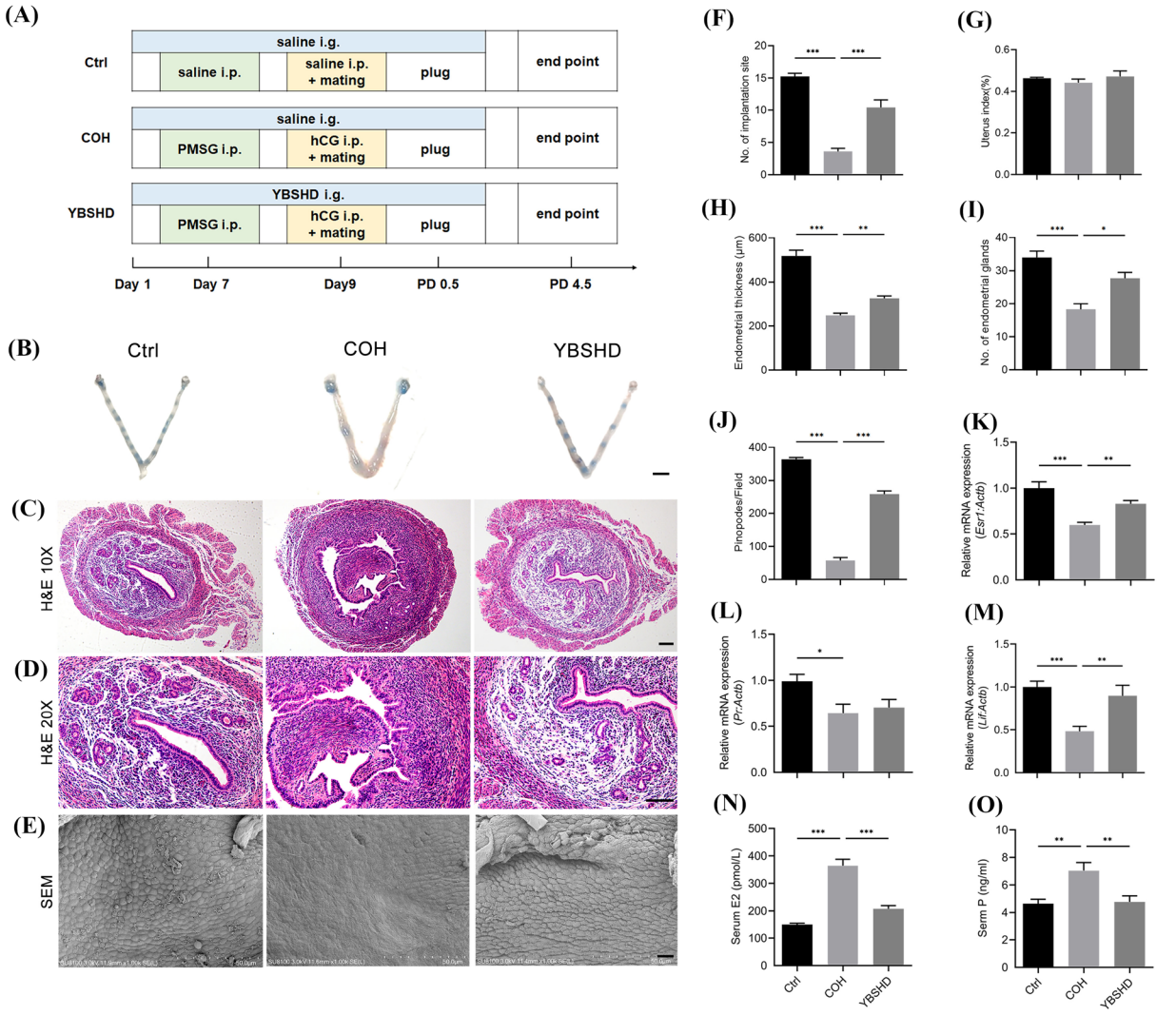

Figure 5. Improved implantation and endometrium were observed in PD 4.5 mice treated with YHSHD. (A) Schema of YBSHD treatment in a model involving COH mice. **(B)** Representative images of implantation sites dyed with Chicago sky blue in the PD 4.5 uterus among the three groups. **(C)** Representative H&E staining of endometrial thickness on PD 4.5 mice in individual groups (10 ×). **(D)** Representative images of H&E staining showed the changes in uterine glands and stroma in each group (20×). **(E)** Representative images of endometrial pinopodes according to SEM (1000×). Data depict the number of embryo implantation sites **(F)** and the uterine index **(G)** in mice treated as indicated. Quantitative results for uterine endometrial thickness **(H)** and glands **(I)** in PD 4.5 mice. **(J)** The average number of pinopodes per field in PD 4.5 mice. The expression of *Esr1* **(K)** and *Pr* **(L)** mRNA in the murine uterus was compared in different groups using PCR. **(M)** The expression of endometrial receptivity biomarker *Lif* was elevated by YBSHD compared to the level in COH mice. The effect of YBSHD on serum estrogen (E2) **(N)** and progesterone (P) **(O)** was assessed using ELISA. Data are expressed as the mean ± SEM. *$P < 0.05$ **$P < 0.01$, ***$P < 0.001$. Scale bar, **(B)** 500 μm, **(C)** 200 μm, **(D)** 50 μm, and **(E)** 100 μm.

endometrial receptivity.

3.9. Effect of YBSHD on murine estrogen and progesterone secretion in the COH model

During superovulation, exogenous gonadotropins not only promote follicular development but also change the hormone levels through a feedback regulation mechanism that affects the hypothalamic-pituitary-ovary axis (*16*). Therefore, the serum sex hormone levels in mice in each group were measured at PD 4.5. Results suggested that the average levels of serum estrogen were significantly elevated in the COH group compared to those in the Ctrl group. In contrast, YBSHD administration significantly down-regulated estrogen levels (Figure 5N). The serum levels of progesterone were also observed in different groups. The Ctrl group had an average of 4.64 ng/ml, while the COH group had an average of 7.04 ng/ml, which was 1.52 times higher than that in the Ctrl group. YBSHD significantly reduces the serum level of progesterone to the normal level (Figure 5O), indicating that it can ameliorate the supraphysiological level of the hormone caused by superovulation.

3.10. Effect of YBSHD on endometrial inflammation

Excessive hormones induced by COH may result in ovarian hyperstimulation syndrome (OHSS), which

is often followed by an inflammatory state (*17*). GO enrichment analysis implied that the role of YBSHD in endometrial receptivity may involve the regulation of hormones and inflammatory responses. Therefore, the GeneMANIA database was used to predict the key molecules that YBSHD regulates in the hormone-inflammatory network. As shown in Figure 6A, 76 genes were involved in the regulation of biological functions such as the response to steroid hormones, the regulation of hormone secretion, the response to peptide hormones, the regulation of inflammatory response, cell chemotaxis, and the production of molecular mediators in inflammatory responses. *Esr1*, but not *Pr*, was found to be down-regulated by YBSHD, pointing

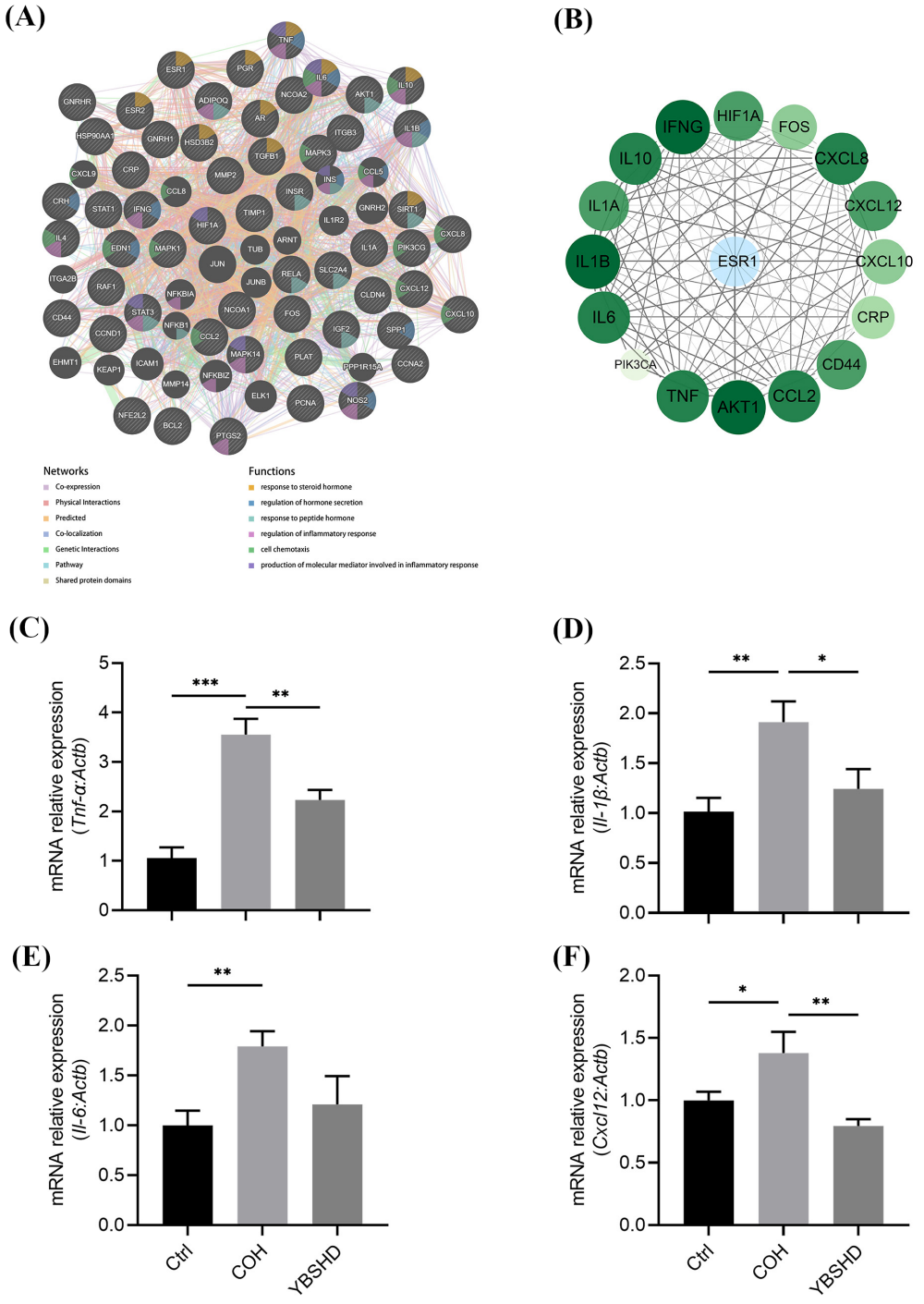

Figure 6. Regulation of YBSHD in the hormone-inflammatory network. (A) Network and function of YBSHD on hormone-inflammation regulation. **(B)** PPI analysis focused on the hormone-inflammatory network regulated by YBSHD. PCR revealed the expression of *Tnf-α* **(C)**, *Il-1β* **(D)**, *Il-6* **(E)**, and *Cxcl12* **(F)** mRNA in the murine uterus of different groups. Data are expressed as the mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

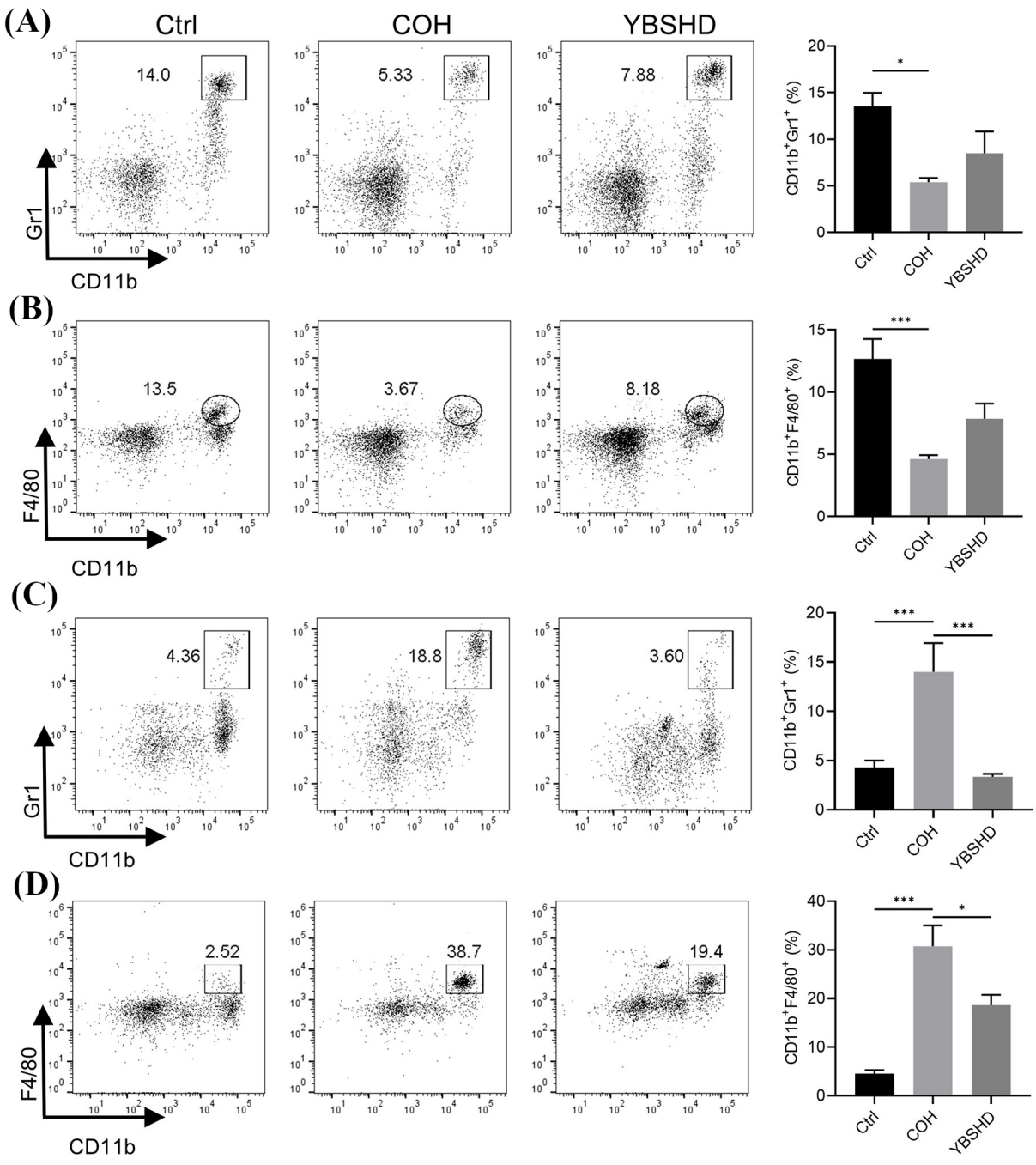

Figure 7. Flow cytometry analysis revealed changes in the uterine and blood immune cell population in COH mice treated with YBSHD. **(A)** The population of uterine $CD11b^+Gr\text{-}1^+$ neutrophils in mice. **(B)** The population of $CD11b^+F4/80^+$macrophages in the murine uterus was detected. **(C)** The population of $CD11b^+Gr\text{-}1^+$ neutrophils in the peripheral blood of mice. **(D)** The population of $CD11b^+F4/80^+$macrophage in the peripheral blood of mice. Data are expressed as the mean ± SEM. $^*P < 0.05$, $^{***}P < 0.001$.

towards the fact that *Esr1* and its downstream pathway regulate endometrial receptivity in COH mice after YBSHD treatment. GO enrichment analysis identified 17 key molecules, including ESR1 and inflammation-related genes (chemokines, pro-inflammatory and anti-inflammatory cytokines). The complex interaction between them was further depicted by PPI analysis (Figure 6B).

Given that COH may trigger an inflammatory response, the impact of YBSHD on local inflammatory mediators was further investigated. Results indicated that the expression of *Tnf-α* and *Il-1β* mRNA in the murine uterus of the COH group increased significantly. YBSHD significantly reduces the expression of *Tnf-α* and *Il-1β* (Figure 6C-D). A similar trend was seen in the expression of *Il-6*, but the difference was not significant (Figure 6E). The migration and interaction of immune cells during inflammation is known to be mediated by chemokines. The expression of the chemokine *Cxcl12* was found to be significantly higher in the COH group,

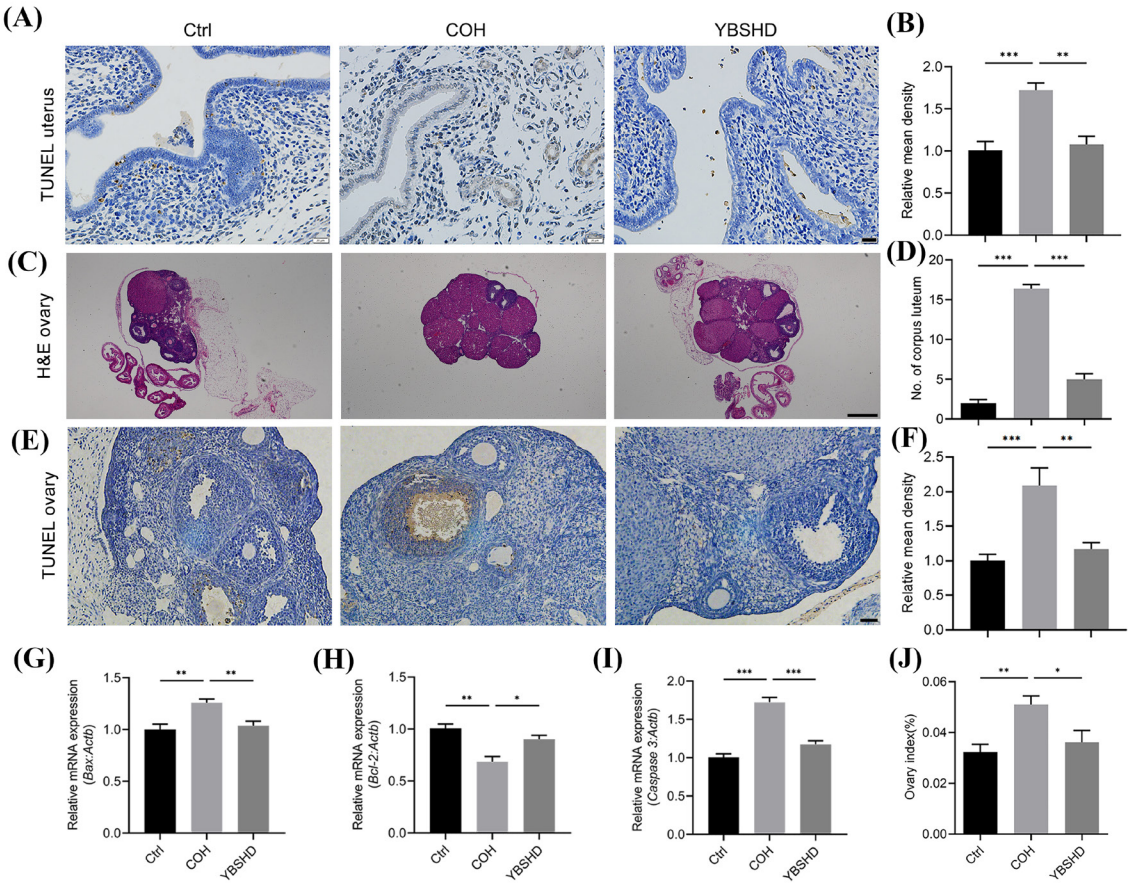

Figure 8. YBSHD affects apoptosis in the ovaries and uterus of COH mice. (A) TUNEL staining showed apoptosis in the murine uterus of different groups. **(C)** Morphology of a murine ovary characterized by H&E staining. **(D)** The number of corpora lutea in COH mice were compared after administration of YBSHD. **(E)** The effect of YBSHD on ovarian apoptosis is indicated by TUNEL staining. The quantified relative mean density of TUNEL staining in the ovaries **(B)** and uterus **(F)** of three groups. qPCR testing detected mRNA expression of the apoptosis molecules *Bax* **(G)**, *Bcl-2*(**H**), and *Caspase 3* **(I)**, regulated by YBSHD. **(J)** The ovary index was calculated in all mice of the groups. Data are expressed as the mean ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Scale bar, **(A)** 20 μm, **(C)** 500 μm, and **(E)** 50 μm.

and YBSHD significantly lowered its level (Figure 6F). As a result, YBSHD may ameliorate the inflammatory state and uterine immune environment through the regulation of inflammatory factors and chemokines.

Flow cytometry was performed to compare the myeloid cells between uterine and peripheral cells in PD 4.5 mice. Both the proportion of $CD11b^{+}Gr1^{+}$ neutrophils and $CD11b^{+}F4/80^{+}$ macrophages in peripheral blood significantly decreased in COH mice compared to Ctrl mice, while this was reversed by YBSHD (Figure 7A-B). In contrast, the proportion of $CD11b^{+}Gr1^{+}$ neutrophils and $CD11b^{+}F4/80^{+}$ macrophages from the murine uterus significantly increased in COH mice (Figure 7C-D). The opposite change in uterine and peripheral cells implied that peripheral neutrophils and macrophages may migrate to the uterus in response to inflammation. YBSHD treatment reversed the changes in the proportion of these two types of cells in the uterus and peripheral blood, possibly by downregulating the expression of the chemokine *Cxcl12* to inhibit migration from the peripheral blood to the uterus.

3.11. Effect of YBSHD on uterine and ovarian apoptosis

Apoptosis exists in embryo attachment and infiltration, while supra-physiological estrogen and the ensuing inflammation caused by COH may result in excessive apoptosis in the uterus, thereby compromising embryo implantation. KEGG and GO enrichment analysis indicated that YBSHD might regulate endometrial receptivity through modulation of apoptotic signaling pathways. The level of apoptosis in the murine uterus was subsequently quantified in all groups. Quantitative TUNEL assays revealed extensive apoptosis in both the luminal and glandular epithelium of the murine uterus induced by COH (Figure 8A-B), demonstrating that COH elicited marked endometrial apoptotic responses. The decrease in TUNEL-positive cells in endometrium treated with YBSHD suggested that this herbal formulation effectively suppresses COH-induced apoptosis.

Given the established impact of exogenous gonadotropins on mitochondrial dysfunction in granulosa and endometrial cells (*18*), key regulators of

the mitochondrial apoptotic pathway were examined in murine uterine tissues. PCR revealed significantly elevated expression of the pro-apoptotic Bcl-2 family member *Bax* in uterine tissues subjected to COH, and this was markedly attenuated by YBSHD (Figure 8G). Measurement of anti-apoptotic *Bcl-2* mRNA revealed significantly reduced expression in the COH group versus controls, while YBSHD treatment markedly restored its expression (Figure 8H). Significantly elevated levels of *Caspase 3* mRNA, a critical executor of apoptosis, were evident in the COH group. YBSHD administration significantly attenuated *Caspase 3* expression (Figure 8I), implying its protective role against COH-induced uterine apoptosis.

Considering the potential disruption that COH may cause in the hypothalamic-pituitary-ovarian axis, ovarian morphology in PD 4.5 mice was histologically evaluated (Figure 8C). COH induced pronounced ovarian enlargement (evidenced by elevated ovarian index) alongside follicular depletion, increased atretic follicles, and a ~3-fold accumulation of corpora lutea, which aligns with the expected ovarian hyperstimulation (Figure 8D&J). YBSHD treatment restored ovarian volume, lowered the ovarian index, and reduced excessive corpora lutea, confirming its protective role in ovarian hyperstimulation. TUNEL staining was also performed to assess YBSHD's impact on ovarian apoptosis in all groups (Figure 8E). Ovarian tissues in the Ctrl group exhibited virtually no TUNEL-positive cells. COH induced prominent TUNEL-positive signals in follicles, whereas YBSHD administration significantly reduced the relative mean density of TUNEL-positive cells (Figure 8F), indicating reduced ovarian apoptosis.

4. Discussion

In recent years, about 1/7 of couples worldwide have been affected by infertility (*19*), while UI accounts for about 30% to 60.3% (*20,21*). COH, a widely accepted recommendation in guidelines for the treatment of UI (*22,23*), can induce a supraphysiological increase in maternal estrogen levels. It may lead to the dislocation or deviation of the embryo and endometrial window period, thereby compromising embryo implantation and the pregnancy rate (*24,25*). Therefore, understanding the potential negative effects of COH on the endometrium and optimizing treatment strategies has become the focus of research to improve pregnancy outcomes. Through our inclusion and exclusion criteria, patients with UI undergoing COH were enrolled as a comparatively standardized baseline population for evaluation of endometrial receptivity. Improvement of the live birth rate in the YBSHD group suggests a potential benefit of YBSHD for patients with UI undergoing COH.

In recent years, network pharmacology has been used as a preliminary approach to reveal the potential mechanism of the active ingredients in TCM, and molecular docking is used to predict the binding affinity between the drug components and receptors (*26*). Network pharmacology and enrichment analysis indicated that YBSHD contains a variety of potential effective ingredients and may improve endometrial receptivity by regulating hormone response, inflammation, apoptosis, *etc*. PPI indicated that some molecules related to regulating hormone response, inflammation, and apoptosis were located in the core position, including ESR1, IL1B, IL6, TNF, and BCL2. UPLC-UV/Q-TOF MS technology indicated two active components of YBSHD, quercetin and kaempferol, which confirmed the predictions of network pharmacology. Molecular docking further confirmed the binding affinities of quercetin and kaempferol to TNF, ESR1, BCL2, IL1B, and IL6, implying a favorable binding affinity between the core components of YBSHD and targets of endometrial receptivity. Moreover, quercetin inhibited the inflammation and apoptosis in decidual cells stimulated with lipopolysaccharide (*27*), and it improved endometrial receptivity in diabetic mice (*28*). Kaempferol alleviated uterine and ovarian apoptosis in cypermethrin-exposed rats (*29*) and had anti-inflammatory action in a preeclampsia rat model (*30*). Quercetin and kaempferol increase ESR1 expression in rats with diminished ovarian reserve, and strong binding between these two compounds and ESR1 has been confirmed by surface plasmon resonance analysis (*31*). In line with these studies, our research demonstrated that YBSHD inhibits inflammation and cell apoptosis and it improves endometrial receptivity in COH mice. Thus, we hypothesize that the relative mechanisms are mediated by the regulation of ESR1 *via* quercetin and kaempferol. The role and mechanism of quercetin and kaempferol in endometrial receptivity will be investigated in a future study.

Previous animal experiments revealed that ovariectomized rats supplemented with high-dose estrogen had hydrops in the abdominal cavity and uterine horns, increased organ wet weight, and intestinal loop expansion (*32*). In the current study, YBSHD effectively alleviated the index and volume of murine ovaries elevated by COH. In addition, COH leads to estrogen secretion reaching its peak in the early luteal phase, which should have occurred in the mid-luteal phase. It causes a deviation in the window of implantation, which in turn affects the expression patterns of endometrium-related genes and proteins (*33,34*). The window of uterine receptivity opens only at lower estrogen levels but not at higher levels (*25,35*), which might explain the better embryo implantation in the YBSHD group as verified with Chicago sky blue dye, H&E staining, and electron microscopy, where no supraphysiological estrogen exposure occurred.

Ovarian stimulation can result in a reduction in ESR1 compared to natural cycles (*36,37*). In the current study, network pharmacology indicated that the reduction in

COH-induced supraphysiological estrogen by YBSHD may be mediated through ESR1. ESR1 is a key factor in the response of the endometrium to estrogen. In the early stages of pregnancy, estrogen activates the downstream signal network through ESR1, regulates the proliferation and differentiation of endometrial epithelial cells, and creates a suitable microenvironment for embryo implantation. The current study showed that YBSHD effectively up-regulates ESR1, activates the downstream target protein LIF (one of the biomarkers of endometrial receptivity), and inhibits excessive hormone levels, providing a stable endocrine environment for smooth embryo implantation.

Studies have found that patients with OHSS appear to be in an inflammatory process, accompanied by high levels of IL-1β, TNF-α, and IL-6 and high serum estrogen (*17*). Long-term estrogen supplementation can induce an M1-type inflammatory phenotype in mouse macrophages and promote the secretion of pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6 (*38*). Changes in inflammation-related molecules may accelerate the formation of a pro-inflammatory environment, leading to a disorder of the endometrial environment and affecting the failure of embryo implantation. Network pharmacology indicated a hormone-inflammatory interaction in the regulation of endometrial receptivity by YBSHD. We therefore analyzed the expression of genes related to inflammation in the mouse uterus and found that YBSHD significantly suppressed the expression of *Tnfα*, *Il1β,* and *Cxcl12* while slightly downregulating *Il6*. These findings substantiate the anti-inflammatory effect of YBSHD in COH mice.

Immune cells are essential for endometrial receptivity and embryo implantation. Increased M1-type macrophages have been found in the endometrium of patients with repeated implantation failure during the window of implantation, along with elevated proportions and cytotoxicity of uterine natural killer cells (*39*). An imbalance in macrophage polarization may disrupt the maternal-fetal interface during early pregnancy, fostering a pro-inflammatory microenvironment that compromises embryo implantation and pregnancy maintenance (*40*). Therefore, flow cytometry was performed to analyze the proportion of and phenotypic changes in immune cells in the murine uterus from each group. Results revealed that COH mice had an increased proportion of neutrophils ($CD45^+CD11b^+Gr1^+$) and macrophages ($CD45^+CD11b^+F4/80^+$) in the endometrium, indicating a disrupted endometrial immune microenvironment. Interestingly, peripheral blood indicated a decreased proportion of both neutrophils and macrophages in COH mice, suggesting an inverse correlation with their endometrial counterparts. We therefore speculate that some endometrial immune cells may have migrated from the peripheral circulation. A pro-inflammatory chemokine, CXCL12 binds to its receptor CXCR4 and recruits T lymphocytes and monocytes/macrophages that express the receptor to inflammatory sites, playing a classic chemokine role in immune responses (*41*). Elevated expression of *Cxcl12* was noted in the COH group, suggesting its potential involvement in the chemotaxis of peripheral immune cells. YBSHD significantly inhibited the expression of *Cxcl12* in the uterus and reduced the proportion of neutrophils and macrophages in the endometrium, indicating that YBSHD may improve the uterine immune microenvironment by regulating the local inflammatory response and immune cell subsets, which are conducive to embryo implantation.

A pro-inflammatory cytokine, TNF-α promotes apoptosis and participates in two patterns of cell death: apoptosis and necroptosis (*42,43*). TNF-α can induce apoptosis of follicular granulosa cells and endometrial epithelial cells during follicular atresia (*44,45*). We previously noted changes in the *Tnf-α* expression regulated by YBSHD, and network pharmacology suggested that YBSHD may affect the apoptotic pathway. Therefore, we further examined apoptosis in the murine uterus and ovary. TUNEL staining indicated an increase in the apoptosis of uterine and ovarian tissues as well as an increased number of ovarian atretic follicles in COH mice. The extent of apoptosis in the uterus and ovary decreased in the YBSHD group, and expression of the pro-apoptotic molecule *Bax* and the downstream execution molecule *Caspase 3* was downregulated, while expression of the anti-apoptotic molecule *Bcl2* was upregulated. These results indicate that YBSHD may not only alleviate the inflammatory response caused by COH but also reduce cell apoptosis.

The current study has shown that YBSHD alleviates the reduced endometrial receptivity caused by COH through the mechanism of 'anti-inflammatory-anti-apoptotic-hormone regulation', which provides new insight into improving pregnancy outcomes and optimizing the COH regimen. However, this study has several limitations. While patients with UI were selected to minimize confounders related to endometrial receptivity, the heterogeneity of UI should be considered. In addition, there may have been possible selection bias in the retrospective study. Although a COH mouse model was used to match the clinical scenario of patients with UI undergoing COH, murine studies cannot fully simulate the dynamic changes in human tissue. The mechanism by which YBSHD improves endometrial receptivity and regulates ESR1, LIF, and its downstream signaling pathways has not been fully explored. In the future, its efficacy needs to be verified through multi-center randomized trials and the synergistic effect and molecular mechanism of specific components of YBSHD need to be analyzed, addressing the limitations of COH therapy and opening up a new avenue for integrated traditional Chinese and Western medicine to bring about successful pregnancy outcomes in patients with UI.

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