Original Article

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Effect of Chinese patent medicine Kunling Pill on endometrial receptivity: A clinical trial, network pharmacology, and animal-based study

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SUMMARY Although pregnancy success rates are raised with assisted reproductive technology, it still cannot meet clinical demands. Kunling Pill (KLP), a traditional Chinese medicine, is widely used in various gynecological disorders, particularly in improving fertility and pregnancy rates. However, the underlying mechanism of how KLP affects pregnancy outcomes remains unclear. This study aimed to explore the effects and mechanisms of KLP on endometrial receptivity. Firstly, a retrospective trial was conducted to validate the efficacy of KLP on repeated implantation failure (RIF) patients. The result indicated a significant increase in the proportion of live birth in KLP group (30.56%) compared to the control group (16.89%). Secondly, network pharmacology methods predicted the active components and network targets of KLP. Endometrial receptivity is closely associated with the activation of inflammatory factors, predicting the function of KLP on the immune system. The estrogen and apoptotic signaling pathways were also highlighted in the gene ontology enrichment analysis. Thirdly, a decreased endometrial receptivity model was established by controlled ovarian hyperstimulation (COH) in female C57BL/6 mice, divided into the COH and KLP groups. Normal female mice are as control group. *In vivo*, KLP administration could increase endometrial thickness and the number of endometrial glands and pinopodes. In the endometrium, KLP supplementation upregulated the expressions of estrogen receptor α, progesterone receptor, endothelial nitric oxide synthase, and integrin αVβ3 in the murine uterus and reduced serum levels of estrogen and progesterone. KLP regulated the uterine immune cells and inhibited cell apoptosis in the ovary *via* Bcl-2/Bax/caspase-3 pathway. In conclusion, KLP administration raised the live birth rate in RIF patients to optimize medication regimens, mainly because KLP ameliorated impaired endometrial receptivity.

Keywords Kunling Pill, endometrial receptivity, implantation, apoptosis

1. Introduction

The success of *in vitro* fertilization (IVF) has increased dramatically (*1*); however, failures have become inadmissible for both providers and patients. Growing evidence highlights the importance of receptive endometrium in IVF and impaired endometrial receptivity is one of the key factors in low pregnancy rates and repeated implantation failure (RIF) (*2-4*). Moreover, as controlled ovarian hyperstimulation (COH) is commonly used in assisted reproductive technology (ART) nowadays, the adverse impact of COH on endometrial receptivity has become a major issue for patients. Small and undifferentiated endometrial glands were found in patients undergoing minimal stimulation with clomiphene citrate (*5*). Thus, the management of endometrial receptivity is a difficult and critical issue.

Receptive endometrium depends on the endocrine milieu, maternal immune system, hematologic factors, anatomic factors, *etc.* (*6*). Firstly, the proliferation and differentiation of the endometrium are regulated by steroid hormones, such as estrogen and progesterone, which are produced mainly by ovaries. Sex steroids act *via* their receptors to initiate the specific genes

and mediators' expression in the endometrium at the molecular level. Supraphysiologic estrogenic milieu during COH could result in desynchrony in the development of different cells and aberrations in the transcriptome of the endometrium, finally impairing endometrial receptivity (*7-8*). Secondly, there is a large amount of immune cell infiltration in the human endometrium at the window of implantation (WOI), including macrophages, T cells, and natural killer cells (*9*). Single-cell RNA sequencing confirms that endometrial immune profile disorders are associated with RIF at WOI (*3*), providing a deeper insight into the endometrial microenvironment disorder of RIF. Successful embryonic implantation requires a normal microenvironment. Integrin αVβ3 and endothelial nitric oxide synthase (eNOS) are of vital predictive values for endometrial receptivity in women with unexplained infertility, correlating to implantation failure (*10-11*). Although research has focused on improving endometrial receptivity, further research is needed to improve pregnancy outcomes for subfertile couples (*1,12*).

Traditional Chinese medicine (TCM) prescriptions have been widely accepted, and research has focused on their applications and mechanisms for treating infertility and gynecopathy in recent years in China (*13-15*). Kunling Pill (KLP), a Chinese patent medicine, is available to treat women with infertility and recurrent spontaneous abortion. KLP is composed of 31 kinds of Chinese herbs, and a total of 169 active ingredients were identified (*16*). KLP can help promote angiogenesis, regulate the balance of serum hormones, improve oocyte quality and diminished ovary reserve, and enhance endometrial receptivity (*16-18*). However, the underlying mechanism of how KLP affects RIF patients remains unclear.

This study was performed to determine the effect and possible mechanism of KLP on RIF. The effect of KLP on RIF patients was analyzed based on a retrospective study. Next, network pharmacology and a COH animal model were conducted to determine its possible mechanisms.

2. Materials and Methods

2.1. A clinical trial

2.1.1. Population

A retrospective study was carried out to evaluate the effect of KLP on patients with RIF at the Obstetrics and Gynecology Hospital of Fudan University between January 2021 and October 2022. The study received approval from the hospital's Ethics Committee (No. 2021-154-X1) and was conducted according to the Declaration of Helsinki 2002. The flowchart indicates the recruitment of patients (Figure 1).

RIF patients were selected following inclusion criteria: 1) at least three previous failures of IVF-ET therapy; 2) age 20 - 40 years old; 3) regular menstrual cycle; 4) negative autoimmune antibodies; 5) hysteroscopy or hysterosalpingogram indicating patency of at least one fallopian tube and normal cavity

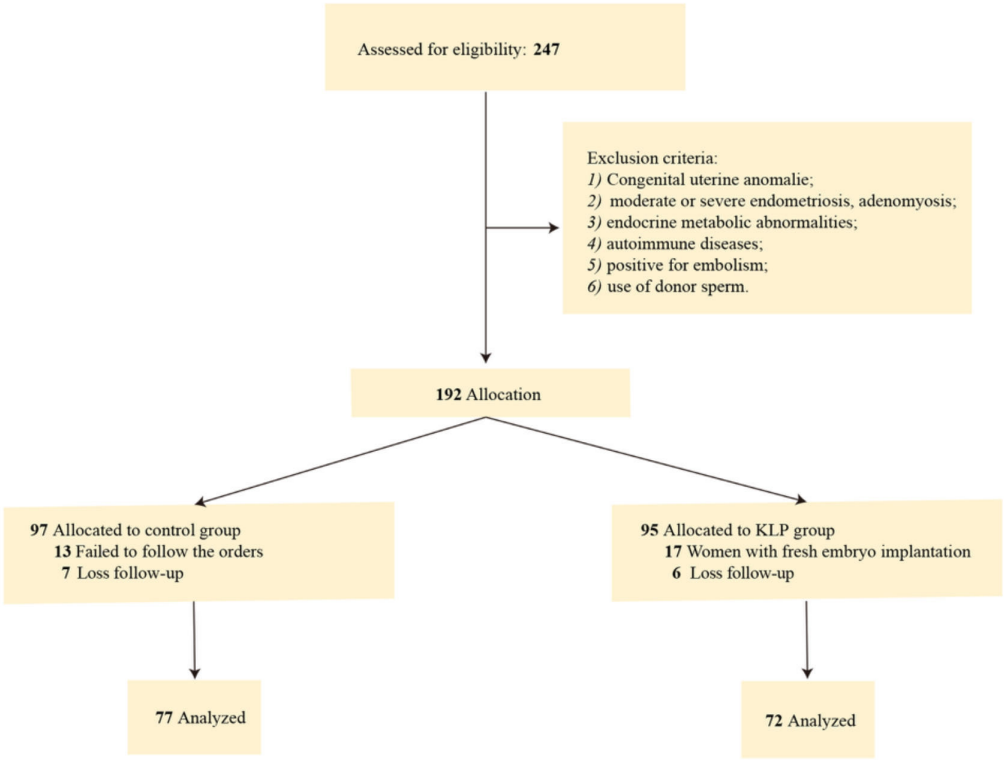

Figure 1. Flowchart of patients included in this study

morphology; 6) normal semen quality of male partner; 7) normal chromosomal karyotype of the couple; 8) voluntary participation in the study, willingness to take medication and follow up according to the treatment protocol. The exclusion criteria were: 1) congenital uterine anomalies; 2) moderate or severe endometriosis, adenomyosis; 3) endocrine metabolic abnormalities, such as polycystic ovary syndrome, diabetes mellitus, insulin resistance and hypothyroidism; 4) autoimmune diseases such as systemic lupus erythematosus, antiphospholipid syndrome, dry syndrome and scleroderma; 5) positive for embolism; 6) use of donor sperm.

2.1.2. Design and treatment

Patients were randomized into the control and KLP groups for embryo transfer in IVF cycles. For the control group, once the ovulation was confirmed by ultrasound and serum progesterone was examined, patients were given a trimodality therapy including estradiol valerate (DELPHARM Lille S.A.S., Lille, Lys-Lez-Lannoy Cedex, France) 1 mg bid po + progesterone injection (Zhejiang Xianju Pharmaceutical Co., Ltd., Xianju, Zhejiang, China) 60 mg qd im + dydrogesterone (Abbott Biologicals B.V., Veerweg, Zuid-Holland, Netherlands) 10 mg tid po. For the KLP group, patients were given KLP (Tasly Pharmaceutical Co., Ltd., Tianjin, China) 15 pills bid po from day 2 or day 3 of the menstrual cycle. Once the ovulation was confirmed, the following procedure was the same as the control group except for KLP supplementation. The embryo transfer of the two groups was conducted on day 4 of the trimodality therapy; KLP was stopped on the day before embryo transfer.

2.1.3. Outcome

The primary outcome measure of live birth was defined as the delivery of newborns ≥ 20 weeks of gestation. Positive pregnancy test (positive for urine human chorionic gonadotrophin (hCG) test), clinical pregnancy (presence of gestational sac on ultrasound at 6 weeks of gestation), and ongoing pregnancy (heartbeat activity confirmed by ultrasound at 8 weeks of gestation) were considered as secondary outcomes.

2.2. Network pharmacology

2.2.1. Selection of active ingredients by pharmacokinetic absorption, distribution, metabolism, and excretion prediction

Relevant components contained in KLP were collected on the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP) (*https://tcmsp-e.com/*). We introduced drug likeness (DL) ≥ 0.18 and oral bioavailability (OB) ≥ 30% as screening conditions to collect the relevant compound components for preliminary screening. The potential targets of different compounds were further obtained and collated in the TCMSP database.

The UniProt database (*https://www.uniprot.org/*) was searched to obtain the validated human protein targets using "Homo sapiens" and "Reviewed" as screening criteria. Gene targets of each compound in the TCMSP database were matched with protein targets in the UniProt database to obtain the target information. The compound-target relationship network was analyzed by Cytoscape 3.8.2 (*http://cytoscape.org/*).

2.2.2. Target prediction and protein-protein interaction (PPI) network

"Endometrial receptivity" related disease targets were collected from GeneCards (*https://www.genecards.org/*) database. The intersection between KLP and endometrial receptivity targets was further imported into the String database (*http://string-db.org/*). Screening conditions of "Homo sapiens" and "confidence level > 0.4" were used to construct the PPI network. Cytoscape software was applied for re-editing the network in a color-based mode. The core 20 target genes were screened by the cytohuba plugin of Cytoscape after that.

2.2.3. Functional enrichment analysis and biological network construction

The obtained top 20 target genes were enriched for gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) pathway analysis by the Metascape platform (*https://metascape.org/gp/index.html*). KEGG enrichment analysis was used to identify core signaling pathways in endometrial receptivity regulated by KLP. GO enrichment analysis includes molecular function (MF), cellular component (CC), and biological process (BP) analysis. The pathways were regarded to be significantly enriched when $p < 0.05$. The Hiplot platform (*https://hiplot.com.cn/*) and Oebiotech platform (*https://cloud.oebiotech.cn/task/*) were adopted to visualize GO and KEGG pathway analysis in this study.

2.3. Reagents

Pregnant mare serum gonadotropin (PMSG) and hCG were purchased from ProSpec Technogene (Ness-Ziona, Central District, Israel) for COH treatment. KLP was obtained from Tasly Pharmaceutical Co., Ltd. and was dissolved in saline. DNase I, collagenase type IV, and Chicago sky blue dye were bought from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Estradiol (E2) (Senxiong Biotech, Shanghai, China) and progesterone (P) (BioVendor, Brno, Moravian, Czech Republic) enzyme-linked immunosorbent assay (ELISA) kit were used for serum hormone measurement. Tissue RNA Purification

Kit and Color Reverse Transcription Kit were purchased from EZBioscience (Roseville, MN, USA). Hieff qPCR SYBR Green Master Mix was bought from Yeasen (Shanghai, China). Antibodies of estrogen receptor α (ERα) (Santa Cruz Biotechnology, Dallas, Texas, USA), eNOS (Abcam, Cambridge, Cambridgeshire, UK), integrin αVβ3 (Novus Biologicals, Centennial, CO, USA), and progesterone receptor (PR) (Santa Cruz Biotechnology) were used for immunohistochemistry (IHC). Live/Dead Cell Stain Kit and RPMI 1640 were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). BV605-conjugated anti-mouse CD3e or Gr1, PE-conjugated anti-mouse CD8, APC-conjugated anti-mouse CD62L or F4/80, APC-Cy7-conjugated anti-mouse CD45, FITC-conjugated anti-mouse CD4 or CD11b, PerCP-Cy5.5-conjugated anti-mouse CD44, PE-Cy5.5-conjugated anti-mouse CD3 antibodies, and Fc receptor blocker were purchased from Biolegend (San Diego, CA, USA), Thermo Fisher Scientific, or BD Pharmingen (San Diego, CA, USA).

2.4. Animals and treatments

8-week-old female and male C57BL/6 mice were purchased from the Laboratory Animal Facility of the Chinese Academy of Sciences (Shanghai, China). Mice were habituated to housing conditions for seven days on a 12 h light and dark cycle following the Chinese Council for Animal Care guidelines. The animal experiments were approved by the experimental animal ethics committee of Fudan University (No. 202210034S).

Mice were divided into three groups: control (Ctrl), COH, and KLP; n =15 for each group. In the KLP group, mice were intragastrically administered 0.91 g/kg body weight (BW) KLP daily from the day of oestrus (*18*). Mice in control and COH groups received equivalent volumes of saline. After seven days of gavage and before mating, mice in COH and KLP groups were first treated with 5 IU/mouse PMSG by intraperitoneal injection and were injected with 5 IU/mouse hCG 48 h later. Mice in the Ctrl group accepted an equivalent volume of saline injection. After the final injection, all female and male mice were mated at a ratio of 2:1 overnight. The next morning vaginal plugs were examined to indicate successful mating (day 0.5 of pregnancy, PD 0.5). Pregnant female mice were sacrificed on PD 4.5 for sample collection.

2.5. Analysis of embryo implantation

Chicago sky blue dye solution (0.1 g/kg-BW) was intravenously injected on PD 4.5 to visualize implantation sites. Mice were sacrificed 10 minutes later, and the intact uterus was separated to evaluate the blue and enclosed implantation sites within the uterine wall.

2.6. Histology and immunostaining

Ovarian and uterine tissues were fixed in 4% formaldehyde for 24 h and embedded in paraffin. Sections (4 μm thick) were deparaffinized, ehydrated, and stained with hematoxylin and eosin (H&E). For immunostaining, sections were brought to boil using a microwave for antigen retrieval and quenched using 3% hydrogen peroxide (H_2O_2). Specimens were blocked for 30 min and then incubated overnight at 4℃ with eNOS, ERα, PR, and integrin αVβ3 primary antibodies, respectively. The next day, samples were incubated with a biotinylated secondary antibody for 50 min after being rinsed in buffer and followed by 3,3'-diamino-benzidine staining. The slides were then photographed under a light microscope (Nikon Instruments, Inc., Shanghai, China). The quantitative analysis of eNOS, ERα, PR, and αVβ3 was determined using Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA).

2.7. Scanning electron microscopy

Uterine samples were fixed in 2.5% glutaraldehyde and post-fixed with 1% osmium tetroxide. After several washes in the buffer, tissues were dehydrated and subjected to critical point drying (Quorum, Laughton, East Sussex, UK). The samples were then mounted on the specimen holder, sputter coated with palladium gold using an ion sputter coater (Hitachi, Tokyo, Japan). Endometrial pinopodes were finally visualized under a scanning electron microscope (Hitachi).

2.8. Measurement of hormone concentration

Blood samples were quickly collected by cardiac puncture after isoflurane anesthesia and solidified at room temperature. Serum was prepared to determine hormone concentration by centrifugation at 3,000 rpm for ten min. According to the manufacturer's protocol, serum E2 and P concentrations were examined using ELISA kits.

2.9. RT-qPCR

Total tissue RNA was isolated from pregnant mice using Tissue RNA Purification Kit following the manufacturer's instructions. Total RNA was used to synthesize cDNA using the Color Reverse Transcription Kit. Quantitative real-time PCR was performed with qPCR SYBR Green Master Mix on a QuantStudio 6-Flex (Applied Biosystems, Waltham, MA, USA). The relative gene expression was compared to β-actin and calculated using $2^{-\Delta\Delta Ct}$ methods. The primers were synthesized by Sangon (Shanghai, China), and the sequences are shown in Table S1 (*http://www.ddtjournal.com/action/getSupplementalData.php?ID=151*).

2.10. Flow cytometric profiling of immune cells

Mice uterine fragments were prepared for single-cell

suspensions followed by continuous digestion in RPMI 1640 containing collagenase type IV (Sigma-Aldrich, Inc.) and DNase I (Sigma-Aldrich, Inc.) at 37°C in a gentle shaker. Once the fragments were dissociated (30 min), the cell suspension samples were passed through a 70 μm filter mesh and then resuspended in phosphate buffer solution (PBS) to count for further procedure. Single-cell suspensions were stained for 30 min at room temperature in the dark using a Live/Dead Cell Stain Kit and blocked with anti-CD16/CD32 mAb. Then the cell suspensions were stained with PE-anti-CD8, PE-Cy5.5-anti-CD3, PerCP-Cy5.5-anti-CD44, BV605-anti-CD3e, BV605-anti-Gr1, FITC-anti-CD4, FITC-anti-CD11b, APC-anti-CD62L, APC-anti-F4/80, APC-Cy7-anti-CD45. Flow cytometry was performed on CytoFLEX (Beckman Coulter, Brea, CA, USA), and data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

2.11. TUNEL staining

Apoptosis in ovarian sections was detected by TUNEL Cell Apoptosis Detection Kit (Servicebio, Wuhan, Hubei, China). Paraffin sections were deparaffinized with xylene and rehydrated in graded alcohol series. The samples were washed in PBS and incubated with prepared Proteinase K working solution for 30 min at 37℃. After being quenched with 3% H_2O_2 for 20 min to inactivate the endogenous peroxidases, the samples were incubated with 50 μL Equilibration Buffer for 10 min and 56 μL TdT buffer at 37°C for 1 h, respectively. The slides were then rinsed with PBS and incubated with Streptavidin–HRP conjugate at 37°C for 30 min. Finally, samples were stained with diaminobenzidine and counterstained with hematoxylin. The slides were finally photographed and analyzed using Image-Pro Plus 6.0 software (Media Cybernetics).

2.12. Statistical analysis

Continuous variables were presented as mean ± standard deviation (SD), and the differences were compared by *t*-test or one-way ANOVA if normally distributed. Data that did not conform to normality were subjected to the Mann-Whitney or Kruskal-Wallis test. All the above analyses were two-sided, and a p value < 0.05 was considered statistically significant. All statistical analyses were conducted using GraphPad software (San Diego, CA, USA).

3. Results

3.1. KLP improved the pregnancy outcomes of RIF patients

A total of 247 patients were included, of whom 192 met the eligibility criteria, and 149 completed the follow-up. The demographic baseline characteristics of these individuals are presented in Table 1. Baseline characteristics of the Ctrl and KLP groups included age, body mass index (BMI), and the number of embryos previously transferred. No difference in baseline characteristics was found between the two groups. Both positive pregnancy test rates and clinical pregnancies had been improved in the KLP group versus the Ctrl group. We noted that all patients who achieved ongoing pregnancies resulted in live births. The KLP group showed ~2-fold higher ongoing pregnancy and live birth rates compared to the Ctrl group (Table 2).

3.2. Targets prediction and components identification of KLP

We use TCMSP to analyze the components of KLP. Two hundred and seventy-three active ingredients were predicted with the standard DL ≥ 0.18 and OB ≥ 30% in KLP. The most common active ingredients include β-sitosterol (in 14 herbs), stigmasterol (in 11 herbs), quercetin (in 10 herbs), and kaempferol (in 6 herbs). All the active ingredients were further screened for the corresponding targets in the TCMSP database and matched with 295 targets of the UniProt database. The target genes associated with the active compounds in KLP were analyzed by Cytoscape software. The compound-target network of KLP contains 273 compounds and 295 targets.

3.3. Network target analysis of KLP on endometrial receptivity

Based on the GeneCards and OMIM databases, 339 out of 678 target genes related to endometrial receptivity were collected and screened. Matching the 295 candidate targets of KLP on endometrial receptivity, 68 overlapping genes were considered hub targets in the drug-disease network. To further reveal the protein-protein interaction

Table 1. Descriptive baseline characteristics of patients with RIF

	Control group (n = 77)	KLP group (n = 72)
Age (years old)	33.4 ± 3.8	32.2 ± 4.2
BMI (kg/m^2)	21.2 ± 3.1	22.0 ± 2.7
No. of embryos previously transferred	4.3 ± 2.8	4.7 ± 3.1

KLP: Kunling Pill; BMI; body mass index.

Table 2. Pregnancy outcomes of patients

	Control group	KLP group
Positive pregnancy test rate (%)	25/77 (32.47)	29/72 (40.28)
Clinical pregnancy rate (%)	21/77 (27.27)	26/72 (36.11)
Ongoing pregnancy rate (%)	13/77 (16.89)	22/72 (30.56)*
Live birth rate (%)	13/77 (16.89)	22/72 (30.56)*

KLP: Kunling Pill. *p < 0.05.

relationship of these targets, the PPI network, which contains 68 nodes and 1150 edges, was constructed by the STRING database and re-edited by Cytoscape software (Figure 2A). The top 20 target genes including TP53, ESR1, EGFR, MAPK3, AKT1, TNF, STAT3, CTNNB1, IL6, HIF1A, MYC, FOS, PTGS2, MMP9, CCND1, IL1B, HSP90AA1, PTEN, CXCL8 and IL10 were screened using the cytohuba plug-in (Figure 2B, Table 3).

3.4. KEGG and GO enrichment analysis

Sixty-eight target genes were performed to explore the possible pathways of KLP in improving endometrial receptivity *via* the KEGG pathway and GO enrichment analysis. A total of 1445 significant GO terms were obtained. We found that the BP enrichment results were mainly related to gland development, response to the hormone or growth factor, regulation of apoptotic signaling pathway, cytokine production, and inflammatory response. MF processes were related to signaling receptor regulator activity, nitric-oxide synthase regulator activity, nuclear steroid receptor activity, integrin binding, hormone activity, *etc.* (Figure 3A). A total of 170 signaling pathways in KEGG enrichment analysis were screened out. The most significantly enriched pathways were presented in Figure 3B. The top-ranked pathways involved the estrogen signaling pathway, apoptosis, chemokine signaling pathway, progesterone-mediated oocyte maturation, cytokine-cytokine receptor interaction, p53 signaling pathway, ovarian steroidogenesis, *etc*.

3.5. Effect of KLP on PD4.5 murine embryo implantation and endometrium

Firstly, we observed embryo implantation in COH murine model after KLP treatment. As shown in Figure 4A, the implantation sites at PD4.5 can be scored visually by Chicago sky blue dye. COH model resulted in impaired implantation compared to the Ctrl group. In contrast, the KLP group ameliorated the compromised endometrial receptivity by increasing the counting of implantation sites to complete recovery (Figure 4B, $p < 0.001$). Murine endometrial morphology was then observed by optical microscope and scanning electron microscope. H&E staining showed that the uterine endometrium of the COH mice was thinner than control ones (Figures 4C-4D), with decreased endometrial glands and compact stroma (Figures 4E-4F). The orally given KLP thickened the endometrium and increased the endometrial glands of COH mice. The glandular lumen enlarged and contained secretory material. Vacuoles, which served as the dissolved ground substance of the glycogen particles, began to progress toward the glandular lumen. Meanwhile, the endometrial stroma became loose and edematous in KLP treated group. Pinopode formation at the top of the endometrial epithelium is well documented as a marker of endometrial receptivity. Compared to the Ctrl group, the absence of pinopodes in the COH group indicated damage to endometrial receptivity

Table 3. Top 20 target genes of KLP

Rank	Gene	Protein name	Score
1	*TP53*	Cellular tumor antigen p53	60
2	*EGFR*	Epidermal growth factor receptor	59
3	*ESR1*	Estrogen receptor	58
4	*MAPK3*	Mitogen-activated protein kinase 3	56
4	*TNF*	Tumor necrosis factor	56
4	*AKT1*	RAC-alpha serine/threonine-protein kinase	56
7	*CTNNB1*	Catenin beta-1	55
8	*HIF1A*	Hypoxia-inducible factor 1-alpha	54
8	*IL6*	Interleukin-6	54
8	*STAT3*	Signal transducer and activator of transcription 3	54
11	*FOS*	Proto-oncogene c-Fos	51
11	*MYC*	Myc proto-oncogene protein	51
11	*MMP9*	Matrix metalloproteinase-9	51
11	*PTGS2*	Prostaglandin G/H synthase 2	51
15	*CCND1*	G1/S-specific cyclin-D1	49
15	*IL1B*	Interleukin-1 beta	49
17	*PTEN*	Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase PTEN	48
18	*HSP90AA1*	Heat shock protein HSP 90-alpha	45
19	*CXCL8*	Interleukin-8	44
20	*RELA*	Transcription factor p65	42

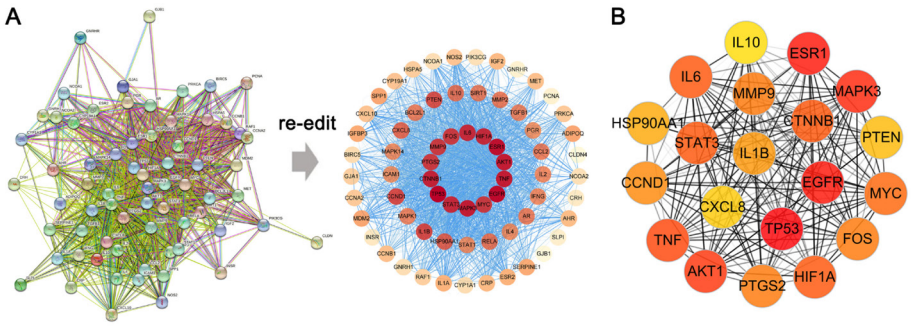

Figure 2. Identification of KLP's components and targets on endometrial receptivity. (A) PPI network showed the protein relationship between KLP and endometrial receptivity. The network was re-edited by Cytoscape. The node's color is marked from red to yellow according to the degree value in descending order. **(B)** Cytoscape showed the top 20 target genes. The higher the degree value, the deeper the color.

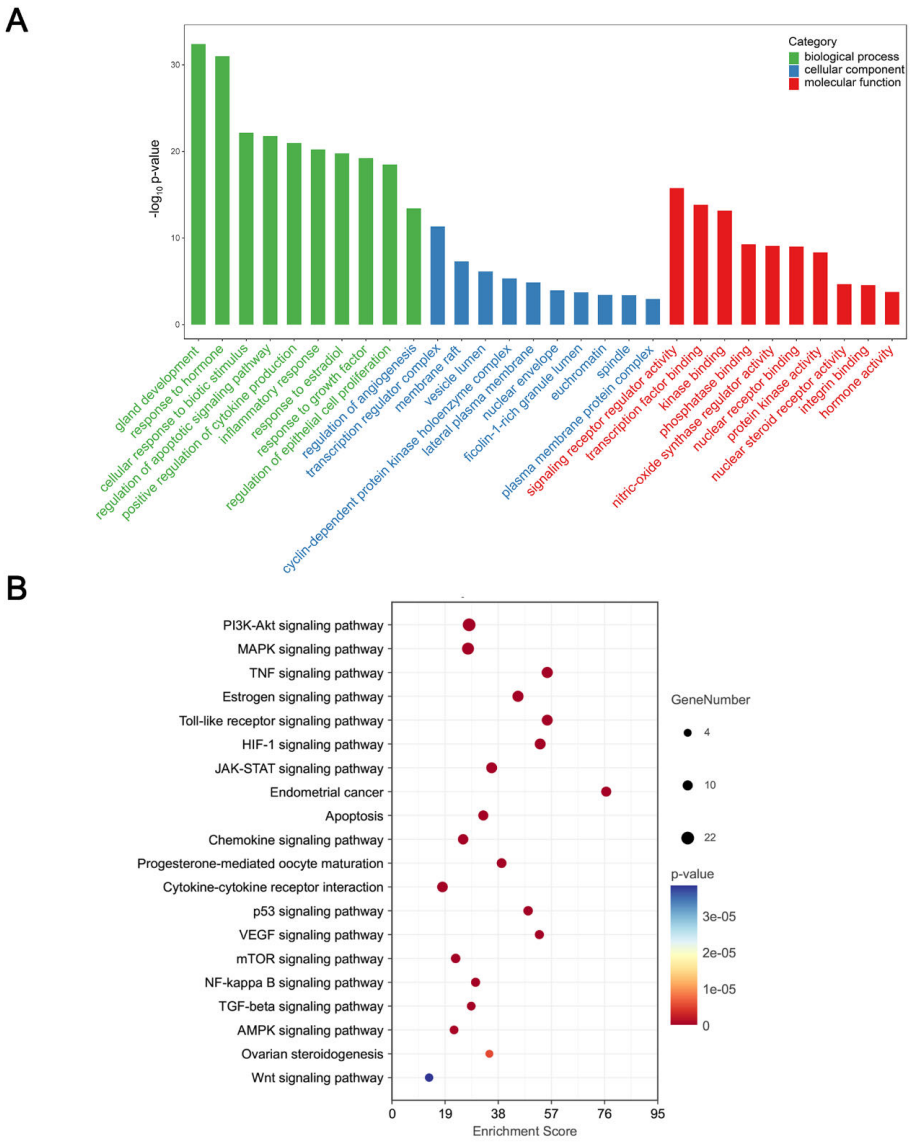

Figure 3. Enrichment analysis of KLP on endometrial receptivity. (A) The biological process, cellular component, and molecular function of GO analysis were shown. **(B)** KEGG pathway analysis showed the top 20 enrichment pathways.

caused by the intervention of COH (Figure 4G). After KLP treatment, pinopode morphology and coverage were significantly improved, consistent with increased quantities (Figure 4H, $p < 0.001$).

3.6. Effect of KLP on mice estrogen and progesterone activity

The serum hormone of estrogen was compared among different groups in PD4.5 mice. COH evokes supra-physiological serum levels of E2 and P, while KLP significantly attenuated their levels (Figures 5A-5B). The hormone receptors were further analyzed to explore changes in hormone signaling activation. Compared to the Ctrl group, RT-qPCR results suggested that *Erα* and *Pr* mRNA expression in the uterus was significantly downregulated in the COH mice (Figures 5C-5D), while KLP reversed it. A similar pattern was seen in protein level confirmed by IHC as KLP significantly repaired the decreased expression of ERα induced by COH intervention (Figures 5E-5F). We also examined progesterone activity as it collaborates with estrogen in successful implantation during WOI. IHC revealed that COH resulted in a significant drop in PR level, and KLP slightly elevated it (Figures 5G-5H).

3.7. Effect of KLP on endometrial receptivity and angiogenic activity in COH mice

Angiogenesis plays a crucial role in embryo implantation. We examined eNOS expression and found that eNOS was downregulated in the COH group and upregulated in the KLP group significantly, which suggests a potential target for KLP in angiogenesis (Figures 6A-6B). Integrin αVβ3 is a commonly used marker in assessing endometrial receptivity. We found that COH intervention

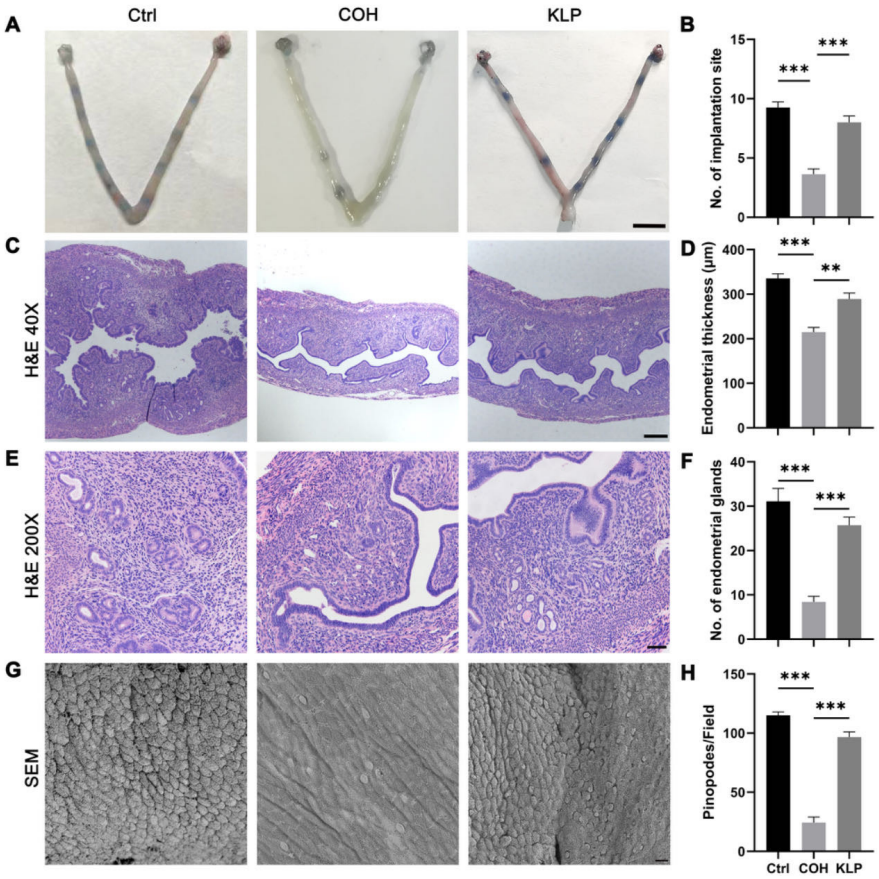

Figure 4. KLP treatment improved the successful implantation and endometrial development on PD 4.5 in COH mice. (A) Representative uteri with implantation sites determined by Chicago sky blue (blue bands) at PD 4.5. **(B)** The mean number of implantation sites on PD 4.5 in individual groups. **(C)** Representative H&E staining showed changes in murine endometrial thickness (40 ×). **(D)** The average endometrial thickness in each group. **(E)** Representative H&E staining showed changes in the morphology of uterine glands and stroma (200×). **(F)** The average number of uterine glands in each group. **(G)** The endometrial pinopode was observed using SEM (1000 ×). **(H)** Quantitation of pinopodes per field. Data are presented as the mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$. Scale bar, **(A)** 5 mm, **(C)** 200 μm, **(E)** 50 μm, and **(G)** 50 μm.

downregulated its expression, and KLP reversed it significantly (Figures 6C-6D), which indicates that KLP may improve endometrial receptivity by regulating the expression of integrin αVβ3.

3.8. Effect of KLP on immune cell populations of the murine uterus

To determine the cellular distribution and differentiation of uterine immune cells after KLP administration on COH mice, flow analysis of single cells in uterine tissue identified the proportion of T cells, myeloid cells of neutrophils, and macrophages in three groups. The ratio of $CD4^+/CD8^+$ T cells demonstrated a significant drop by COH intervention and a partial rise by KLP treatment (Figures 7A-7B). T cell activation status was further assessed using the classic CD44 and CD62L markers. Around 51% of cells in the Ctrl group had a $CD44^+CD62L^-$ effector phenotype. We found a 61% higher count of $CD44^+CD62L^-$ T cells after the COH process, with a drop to 52% by KLP supplementation (Figures 7C-7D). The proportion of uterine myeloid cells was also analyzed. Compared to the Ctrl group, $CD11b^+Gr1^+$ neutrophils increased in the COH group, and KLP supplementation could lower the proportion significantly (Figures 7E-7F). The functions of macrophages are crucial for establishing and maintaining pregnancy. The flow cytometry implicated a significant rise in $CD11b^+F4/80^+$ macrophage induced by COH, and KLP decreased the frequency (Figures 7G-7H).

3.9. Effect of KLP on ovarian cell apoptosis

Given that KLP could regulate serum E2 and P levels, we speculated that KLP might affect ovarian function. The ovary morphology in three groups was first studied by H&E staining (Figure 8A). Compared to the Ctrl group, mice in the COH group had ovaries with poor follicles and reduced corpus luteum (Figure 8B). Treatment with KLP reversed this damage, as more follicles and less corpus luteum were observed (Figure 8B). Moreover, TUNEL staining distinguished the apoptotic granulosa cells (Figure 8C). The mean density of apoptotic cells rose significantly in the COH group compared to the Ctrl group (Figure 8D). Treatment with KLP could significantly recover the apoptosis induced by COH in

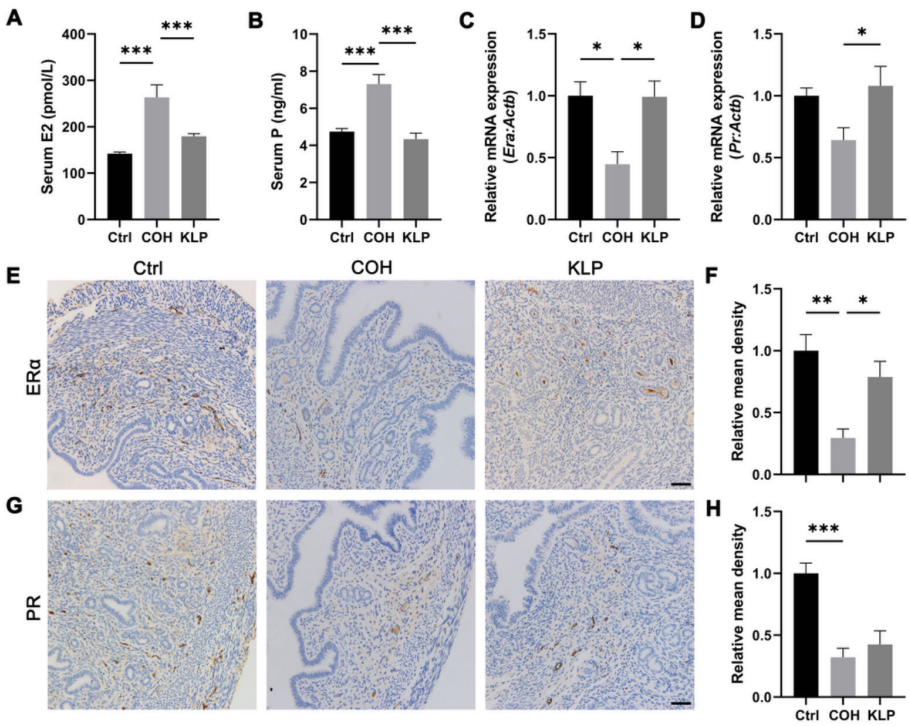

Figure 5. KLP treatment altered ovarian hormone activity impaired by COH. (A-B) ELISA determined serum levels of **(A)** estrogen and **(B)** progesterone. **(C-D)** mRNA expression of hormone receptor **(C)** *Erα* and **(D)** *Pr* in murine uterus. **(E-F)** Representative IHC staining pattern and quantitative analysis of ERα in murine uterus. **(G-H)** Representative IHC staining pattern and quantitative analysis of PR in murine uterus. Data are presented as the mean ± SEM. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. Scale bar, 50 μm.

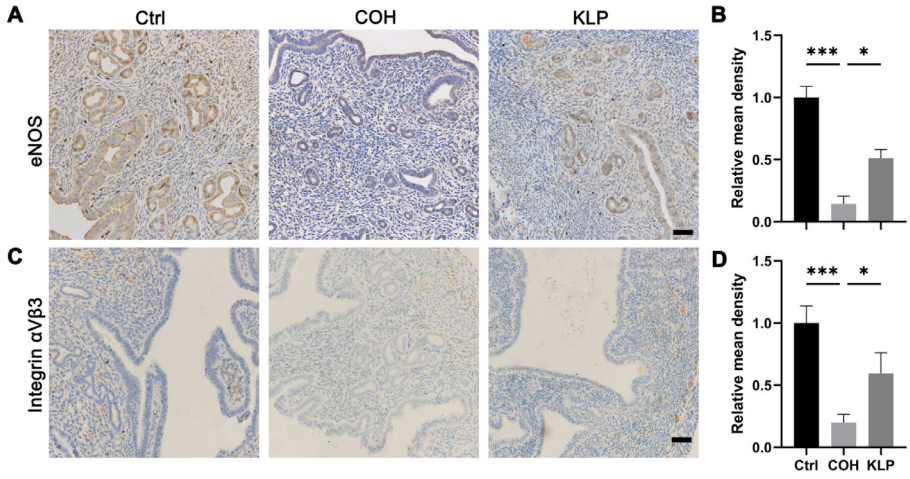

Figure 6. KLP ameliorated endometrial receptivity and angiogenic activity in COH mice. (A) Representative IHC staining of eNOS in the murine uterus from each group. **(B)** The expression of eNOS was quantified as relative mean density. **(C-D)** Representative IHC images and quantification of integrin αVβ3 in the murine uterus from each group. Data are presented as the mean ± SEM. **p* < 0.05, ****p* < 0.001. Scale bar, 50 μm.

the murine ovary. Thus, we further checked the related gene expression concerning apoptosis. mRNA expression of anti-apoptotic *Bcl-2* was downregulated in the COH model compared to the Ctrl group, and KLP significantly reversed the expression (Figure 8E). Mice in the model and Ctrl groups reached significant differences in apoptotic *Bax* and *Caspase 3* gene expression (Figures 8F-8G). After KLP administration, the expression of *Bax* and *Caspase 3* was significantly lower than that in the COH group (Figures 8F-8G), demonstrating the potential role of KLP in inhibiting apoptosis induced by COH intervention.

4. Discussion

In the present study, our results validated KLP as a

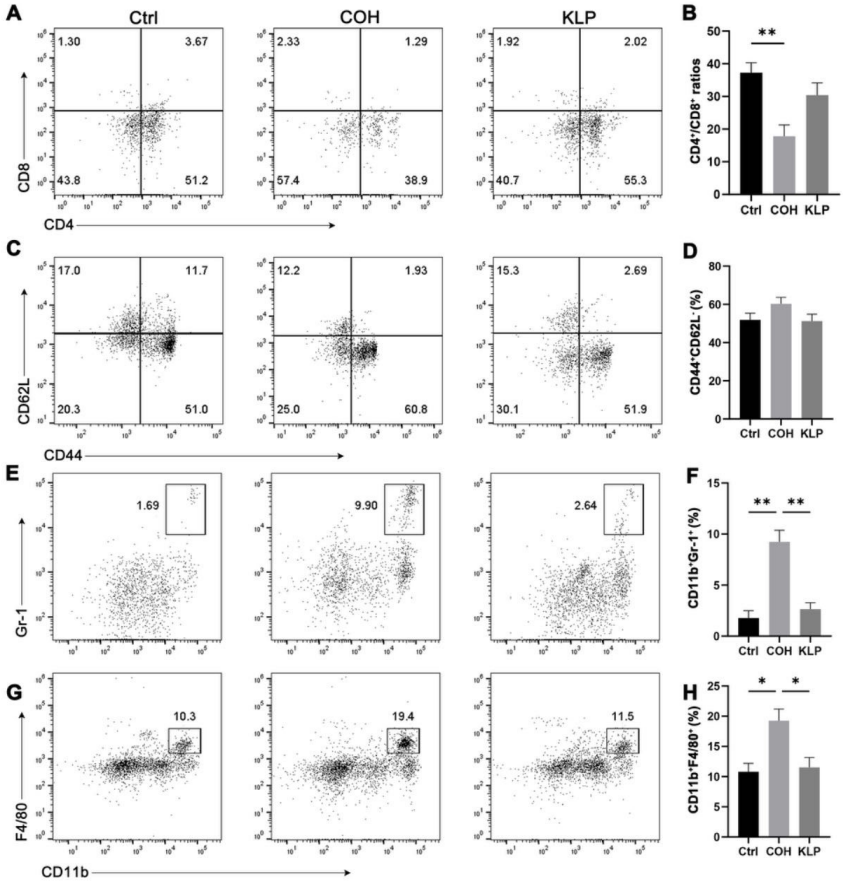

Figure 7. KLP affected uterine immune cell profiles of COH mice based on flow cytometry analysis. (A-B) The population of uterine $CD4^+$ T cell and $CD8^+$ T cell in mice. Data are plotted as $CD4^+/CD8^+$ ratio in T cells. **(C-D)** T cell activation status was detected using CD44 and CD62L markers. Data depict the percentages of $CD44^+CD62L^-$ T cells. The percentages of **(E-F)** neutrophil ($CD11b^+Gr\text{-}1^+$) and **G-H)** macrophage ($CD11b^+F4/80^+$) in the uterus. Data are presented as the mean ± SEM. *$p < 0.05$, **$p < 0.01$.

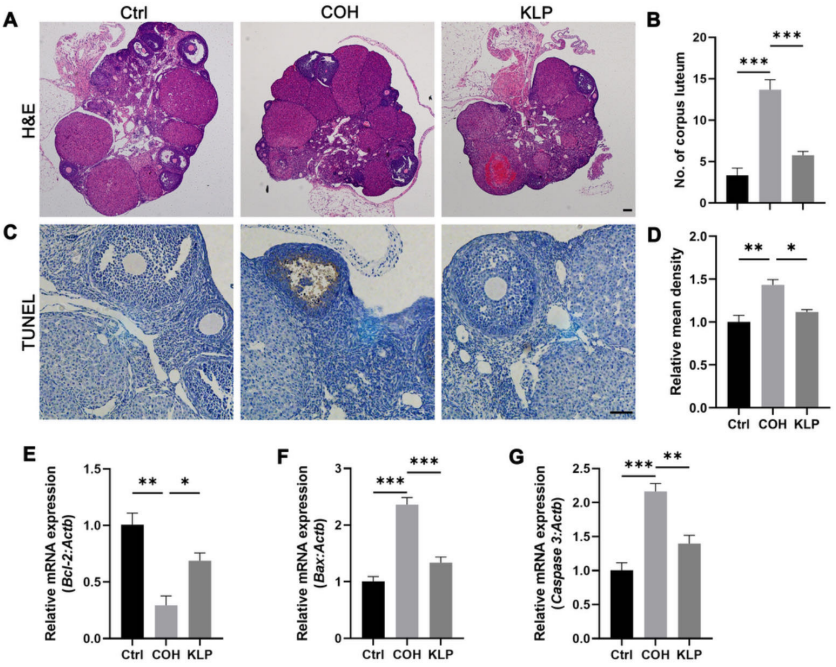

Figure 8. KLP inhibited ovarian apoptosis induced by COH. (A) Representative H&E staining showed ovarian morphology in each group. **(B)** The number of corpus luteum in the murine ovary was calculated. **(C)** Detection of apoptotic cells in the ovary by TUNEL staining. **(D)** TUNEL-positive cells were quantified as relative mean density. Expression of apoptosis-related genes **(E)** *Bcl-2,* **(F)** *Bax,* and **(G)** *Caspase 3* were examined by RT-qPCR. Data are presented as the mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Scale bar, **(A)** 100 μm and **(C)** 50 μm.

beneficial assisting therapy for RIF firstly. Secondly, an integrated strategy was performed to explore and identify the components of KLP by integrating network pharmacology. Finally, we demonstrated that KLP improved impaired endometrial receptivity *via* Bcl-2/Bax/caspase-3 pathway in a COH model.

Successful pregnancy is a physiological process, which seems to be a silent "war" between the embryo and the mother (*2*). Approximately 12.7% of reproductive-age women seek infertility treatment each year, which is increasing yearly (*19*). Although success rates are raised with ART, it still cannot meet clinical demands. TCM, a complementary and alternative medical system, has been utilized widely in China. Given that KLP is a Chinese patent medicine for infertility, our clinical study in RIF patients indicated an increased pregnancy rate and live birth rate under a trimodality therapy with KLP application (30.56%), compared to the trimodality treatment (16.89%).

The composition of TCM is complex; all kinds of Chinese medicine often have some similar chemical components, so the search for the main effective components will help to analyze the key points of Chinese medicine in treating diseases and provide direction for the follow-up molecular mechanism. Network pharmacology, a biological system analysis approach, was utilized to uncover the relationship between multi-component medicines and the network target of diseases (*20-21*). The topological analysis found twenty key targets of KLP, including TP53, ESR1, EGFR, MAPK3, AKT1, TNF, STAT3, CTNNB1, IL6, HIF1A, MYC, FOS, PTGS2, MMP9, CCND1, IL1β, HSP90AA1, PTEN, CXCL8, and IL10. These 20 targets are closely related to endometrial receptivity. Studies have shown that endometrial receptivity is closely associated with inflammatory factors, such as IL6, IL1β, and CXCL8, predicting the function of KLP on the immune system. In GO enrichment analysis, the estrogen and apoptotic signaling pathways were highlighted. In brief, these results indicated a combination of mechanisms of KLP in suppressing inflammation, which was validated by flow cytometry analysis *in vivo*.

According to the research results of network pharmacology, we conducted an *in vivo* experiment at the animal level to verify the results from the bioinformatics analysis. KLP could ameliorate a pseudodecidual-like effect on murine uterine endometrium. Morphological changes carried out by KLP supplementation that coincident with the secretory phase reveal a receptive state of mice endometrium. Evaluation of sex steroids indicates that KLP may regulate endogenous hormones corresponding to the receptive endometrium. In our research, over secretion of E2 and P was induced by PMSG and hCG, which act as exogenous gonadotropin in COH mice. KLP helps in homeostasis by restoring hormones to normal levels.

There are two types of ER and PR in mice, ERα/β and PR-A/B. ERα and PR-A are the main receptors in the establishment of endometrial receptivity in knock-out mice (*22-24*). The maximal ERα and PR expression occurred in the menstrual cycle's mid-to-late proliferative phase (*25*). It was noted that elevated progesterone correlated with the under-expression of ERα in glandular epithelium (*26*). In our study, we detected a similar pattern where ERα decreased in COH mice, along with a high level of progesterone concentration. KLP supplementation raised the expression of endometrial ERα and PR, demonstrating the ameliorating effect of KLP on impaired endometrial receptivity through hormone receptors signals, consistent with the results of network pharmacology. In a normal pregnancy mice model, the expression of eNOS was found to be stood out on gestation day 5 and was higher in implantation sites compared to that inter-implantation sites (*27*). In idiopathic recurrent spontaneous miscarriage patients, eNOS down-regulation is associated with vascular dysfunction and impaired endometrial perfusion, possibly making the endometrium unreceptive (*28*). In cases of unexplained infertility, integrin αVβ3 is significantly lower in endometrium and has great predictive value for endometrial receptivity among biomarkers in the uterine fluid (*29-30*). *Cyperus Rotundus* L., one of the main components of KLP, enhances the adhesion of trophoblastic cells onto endometrial Ishikawa cells by upregulating leukemia inhibitory factor-dependent integrin αVβ3 expression (*31*). Our study showed that KLP could promote eNOS and integrin αVβ3 expression in mice endometrium, confirming the potential effect of KLP on ameliorating impaired endometrial receptivity.

GO enrichment analysis suggested that inflammatory response may be related to the efficacy of KLP. Since immune cells were involved in the inflammatory response during the peri-implantation period (*32*), flow cytometry was used to detect the proportion changes of uterine immune cells. A decrease in the ratio of $CD4^+$/$CD8^+$ T cells by COH intervention revealed a relative increase in cytotoxic $CD8^+$ T cells, which was partially reversed by KLP supplementation. Proportions of $CD11b^+Gr1^+$ neutrophils and $CD11b^+F4/80^+$ macrophage were significantly reduced in the KLP group implicating the immune regulation effect of KLP.

Apoptotic signaling pathways may play a role in the regulation of KLP, as indicated by KEGG and GO enrichment analysis. Therefore, we assessed the apoptosis of the murine ovary by TUNEL staining and qPCR. KLP ameliorated COH-induced ovarian apoptosis *via* upregulating *Bcl-2* expression and downregulating the expression of *Bax* and *Caspase 3*. High-level estrogen binds to phosphodiesterase 3A and induces apoptosis by blocking the endoplasmic reticulum-mediated translation of BCL-2 and other anti-apoptotic proteins (*33*). We speculated that KLP might partially exert its role through an anti-apoptotic action in COH mice by reducing the excessive hormone levels.

In conclusion, the present study demonstrated that KLP administration raised the live birth rate in RIF patients, improved the morphology of COH mice endometrium, and regulated the expression of endometrial receptivity biomarkers, serum hormones, and immune cells, which were due to that KLP ameliorated the impaired endometrial receptivity *via* Bcl-2/Bax/caspase-3 pathway.

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