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ORIGINAL RESEARCH

Mechanism of YJKL Decoction in Treating of PCOS Infertility by Integrative Approach of Network Pharmacology and Experimental Verification

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Purpose: Currently, there is still no clear treatment for polycystic ovary syndrome (PCOS). YJKL has better therapeutic effects and lower toxic side effects for PCOS type infertility. This study aims to clarify the potential mechanism of YJKL Decoction in the treatment of PCOS based on network pharmacology and experiments verification.

Patients and Methods: Network pharmacology and experimental validation approach were used to investigate the bioactive ingredients, critical targets and potential mechanisms of YJKL Decoction against PCOS. Firstly, we use network pharmacology methods to collect core targets, and then validate their effects on diseases through experiments.

Results: Five core targets were screened, Threonine kinase 1 (AKT1), Cellular tumor antigen p53 (TP53), Tumor necrosis factor (TNF), Albumin (ALB) and Vascular endothelial growthfactor A (VEGFA). KEGG analysis showed that YJKL treatment for PCOS mainly include AGE-RAGE signaling pathway in diabetic complications, TNF signaling pathway and HIF-1 signaling pathway. The molecular docking results showed that compounds have higher affinity with targets. Finally, experimental results had shown that YJKL Decoction had an better therapeutic effects in the treatment of PCOS.

Conclusion: Based on a systematic network pharmacology approach and experimental verification, our results comprehensively illustrated the active ingredients, potential targets, and molecular mechanism of YJKL for application to PCOS and helps to illustrate mechanism of action on a comprehensive level.

Keywords: traditional Chinese Medicine, YJKL, polycystic ovary syndrome, PCOS, molecular docking, experimental validation

Introduction

Infertility is a common fertility disorder that can be caused by various factors. These include problems with ovulation, and organic issues within the body.[1] One specific condition that frequently leads to infertility is PCOS. PCOS is a complex reproductive endocrine syndrome characterized by multiple clinical manifestations, such as hyperandrogenism and persistent anovulation. Approximately 10% of women of childbearing age are affected by PCOS. Due to its disordered endocrine state and ongoing anovulation, many patients with PCOS also experience fertility issues, with infertility rates being particularly high in this group.[2] It is estimated that natural abortion rates among PCOS patients are around 41%.[3]

PCOS not only causes female infertility, but also has broader effects on the body. It can lead to infrequent menstruation, amenorrhea, and hirsutism. Moreover, PCOS can impact the endometrium, glucose and lipid metabolism, and cardiovascular system. Women with PCOS are at a higher risk of developing endometrial cancer, breast cancer,

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obesity, coronary heart disease, atherosclerosis, and diabetes. In addition to these physical health concerns, PCOS can also have psychological effects on patients, due to problems such as irregular periods, infertility, acne, obesity, and hirsutism.[4]

Given the consequences of PCOS, it is crucial to understand its pathogenesis and advancements in treatment. Improving the pregnancy rate among patients with ovulatory dysfunction infertility in PCOS is of great importance, as is reducing the miscarriage rate and lowering overall treatment costs. There is currently no better treatment method for this disease. For example, taking metformin is highly susceptible to drug resistance and has extremely high toxic side effects. Therefore, finding a safe and effective treatment method is very important. Due to the fact that traditional Chinese medicine treatment fundamentally addresses the root cause of the disease and has very few toxic side effects. Therefore, we will focus on traditional Chinese medicine and hope to find a better treatment method.

Traditional Chinese Medicine (TCM) has been widely utilized to treat diseases in clinical practice. Academician Xiao Peigen has expressed the importance of modernizing TCM by combining traditional elements with modern science and technology. This approach aims to develop new medications that not only retain the distinctive characteristics of TCM but also effectively treat complex diseases.[5] TCM treatment places emphasis on evidence-based practices.[6] Additionally, TCM offers advantages such as low toxicity, long-lasting curative effects, and affordability, making it a promising and comprehensive clinical treatment option. However, the complex relationships between the mechanisms of action, targets, components, and pathways of herbal medicine, as well as the unknown material basis of these medicines, have hindered the development of TCM in disease treatment.

One example of TCM's effectiveness is the use of the Yi-Jing-Tang (YJT) Decoction to regulate menstruation by addressing heart, liver, and spleen deficiencies. YJT Decoction is primarily used to treat perimenopausal syndrome, which refers to a series of symptoms caused by fluctuations or decreases in sexual hormones in women before and after menopause. It mainly involves dysfunction of the autonomic nervous system, accompanied by neuropsychological symptoms.[7] In our research, we have enhanced the formulation of YJT Decoction and modified its treatment indications in the initial stage. We will temporarily name it YJKL. I hope to have certain therapeutic effects on PCOS.

Network pharmacology is an emerging discipline that aims to uncover the molecular connections between drugs and diseases, effectively addressing the limitations of traditional pharmacological research methods.[8] Its focus on systematically elucidating scientific puzzles aligns with the core philosophy of TCM, which encompasses the characteristics of being multi-component, multi-target, and multi-pathway.

In this study, we aimed to investigate the molecular mechanism of YJKL Decoction in treating infertility caused by PCOS. We have been employed network pharmacology, molecular docking, and experimental validation to explore its mechanism. The detail of workflow of this study is shown in Figure 1.

Materials and Methods

Chemicals and Reagents

Preparation of YJKL

The YJKL raw materials used in this study were as follows: Rehmannia glutinosa (production license number: 20180384, batch number: 20230601), Angelica sinensis (production license number: 20180384, batch number: 20221201), Atractylodes macrocephala (production license number: 20160298, batch number: 20230601), Dioscorea (production license number: 20160265, batch number: 20230401), Paeonia lactiflora (production license number: 20180377, batch number: 20210901), Hippophae rhamnoides (production license number: 20160298, batch number: 20210701), Moutan cortex (production license number: 20180384, batch number: 20230801), Coastal glehnia root (production license number: 20180377, batch number: 20211001), Eucommia ulmoides (production license number: 20160298, batch number: 20230301), Codonopsis (production license number: 20160265, batch number: 20230401), Bupleurum chinense (production license number: 20180384, batch number: 20230603), Cyperus rotundus (production license number: 20160265, batch number: 20221002), and Perilla (production license number: 20180377, batch number: 20200301).

The raw materials of traditional Chinese medicines were weighed and mixed. They were soaked in water equivalent to 8–10 times the total weight of the herbs for 20–30 minutes. They were then boiled for 20–30 minutes, filtered, and the

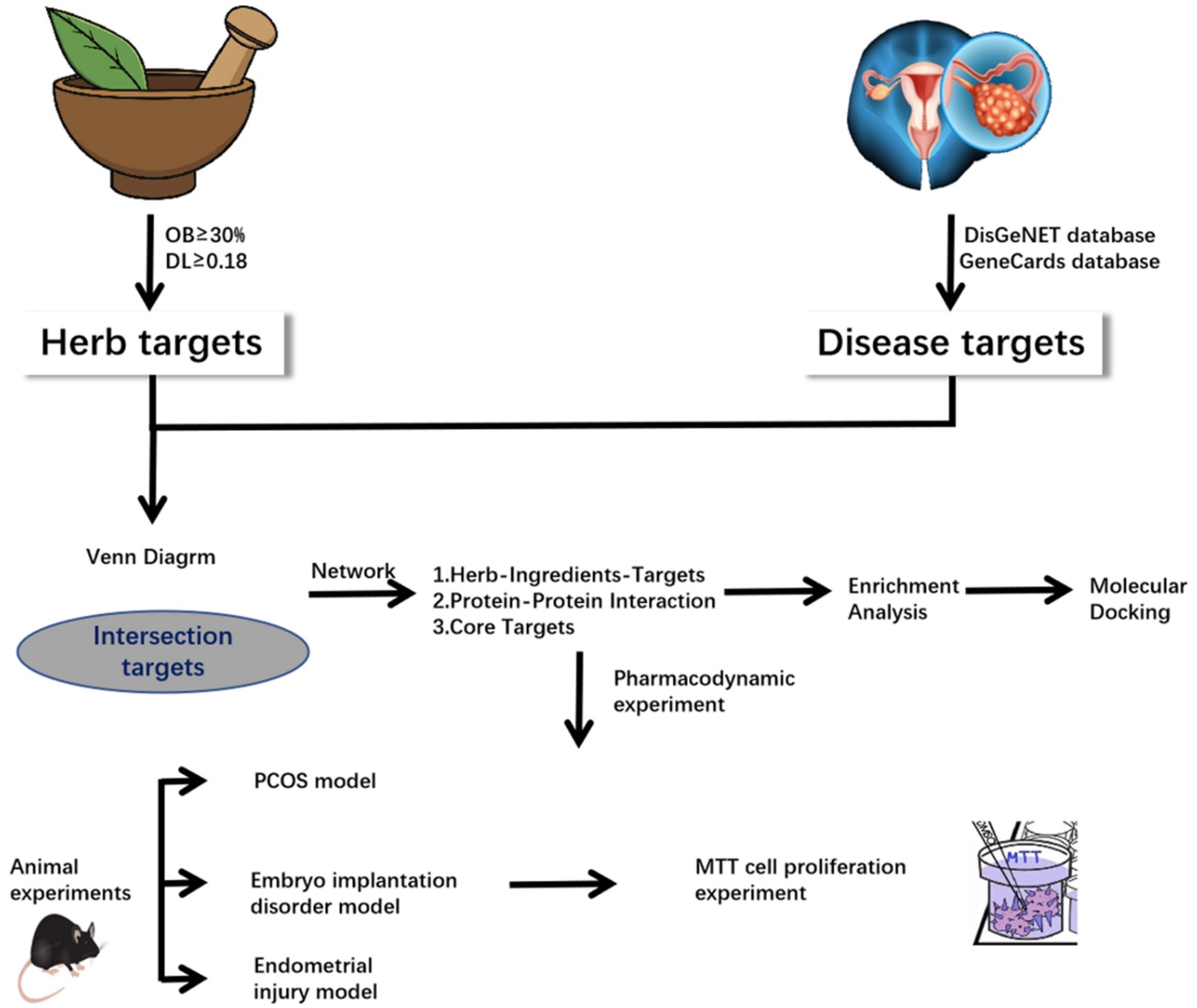

Figure 1 Detailed workflow of present study.

filtrate was collected. The filter residue was mixed with water equivalent to 6–8 times the weight of the medicinal material, boiled for 20–30 minutes, and filtered again. The filtrates were combined and the resulting medicinal solution was concentrated to a raw drug content of 2 g/mL to obtain the Chinese medicine composition.

Reagent

Dehydroepiandrosterone (DHEA) (Macklin, Shanghai, China). Glycerol (National Pharmaceutical Group, Shanghai, China). Penicillin and streptomycin Hyclone (Logan, UT, USA). Roswell Park Memorial Institute (RPMI) 1640, Dulbecco's Modified Eagle Medium (DMEM), and Fetal bovine serum (FBS) were purchased from Procell (Wuhai, China). Reverse transcription test kit and qRT-PCR test kit (Novoprotein, Shanghai, China). HIF-1α(1:1000, affinity), AKT (1:1000, abmart), P-AKT (1:1000, affinity)

Cell and Animal

Human umbilical vein endothelial cells (HUVEC) were obtained from the cell bank of the Typical Culture Preservation Committee of the Chinese Academy of Sciences (Shanghai, China). HUVEC was obtained from the Cell Bank of the Typical Culture Preservation Committee of the Chinese Academy of Sciences. They were cultured in DMEM

supplemented with 10% FBS and 0.1% penicillin and streptomycin at 37°C with 5% CO2. The cells were used between 2–5 passages.

All mice used in this study were purchased from Anhui Experimental Animal Center and Sibeifu Biotechnology Co., Ltd. The animal study was approved by the Ethics Committee of Anhui Medical University (protocol code 82230030) for studies involving humans and (protocol code LLSC20231914) for studies involving animals, and the welfare of experimental animals follows the 3R principle. Fifty specific-pathogen-free (SPF), C57BL/6J mouse (aged 3 weeks). The mice were provided free access to food and water and were group-housed in the animal laboratory of Anhui Medical University in an environment with a controlled temperature of 20–25°C and a relative humidity of 40–60% on a 12 h/12 h light/dark cycle. Subcutaneous injection of 0.2mL/day DHEA (soluble in glycerol, 70mg/kg) was administered to all mice except the Normal Control group for 20 days to establish models. After the establish of modeling, mice were treated with orally administered medication at a concentration of 25.0g/kg/day of raw medicine for 12 days.

Screening the Active Ingredients and Targets of YJKL Decoction

The active ingredients of YJKL Decoction were identified using the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP) (https://tcmspw.com/tcmsp.php). Criteria for selection included drug-like properties ≥ 0.18 and bioavailability ≥ 30%.[9] The targets of the active ingredients were obtained from the TCMSP database and converted to gene names using the Universal Protein (UniProt) (https://www.uniprot.org) database.[10] A relationship network among the traditional Chinese medicine, active ingredients, and target genes of YJKL Decoction was constructed using Cytoscape 3.8.2 software.

Collecting the Therapeutic Targets of PCOS

Therapeutic targets for PCOS were obtained by searching the Disease GeneNetwork (DisGeNet) (https://www.disgenet.org) database and the GeneCards (https://www.genecards.org) database using "polycystic ovary syndrome" as the index keyword.[11,12] The search was limited to "Homo sapiens".

Construction of YJKL-Active Compound-Target Network

The active compounds in YJKL and their potential targets were imported into Cytoscape software 3.8.2 to construct a network showing the interaction between the herb formulation, active compounds, and target genes.[13] Nodes in the network represented herbs, bioactive compounds, and their related targets, while edges represented the interactions between nodes. The "degree" value of a node indicated the number of links connected to it, with higher degree values indicating more important targets.

Establishment of a Protein-Protein Interaction (PPI) Network

Intersection targets between herbs and diseases were identified using Venn 2.1.0 software (www.liuxiaoyuyuan.cn), and the overlapping gene symbols were used to import the intersection targets into the STRING database (https://cn.string-db.org).[14] The condition was limited to "Homo sapiens" and a high confidence score with a correlation degree of ≥0.4 was set to construct a PPI network of common targets.[15]

Screening of Core Targets

We downloaded the TSV files and imported them into Cytoscape 3.8.2 software to visualize the data. Cytoscape was used to create ingredient-target networks and generate network pharmacology images. We then used the cytoNCA plugin in Cytoscape to determine the topological characteristics of the network, such as degree, betweenness, proximity, and eccentricity. We used the degree value as a threshold to screen the main targets.[16]

Gene Ontology (GO) Enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analysis

To explore the biological function of potential targets in PCOS, we utilized the MetaScape database (https://metascape.org/gp/index.html#/ma-in/step1) to perform GO analysis and obtain KEGG data (http://www.bioinformatics.com.cn/).[17] GO analysis screens for biological processes (BP), cellular components (CC), and molecular functions (MF).[18] KEGG enrichment analysis helps identify important signaling pathways involved in biological processes. We uploaded the GO and KEGG data to the Bioinformatics platform for visual analysis. GO and KEGG terms with a p-value < 0.01 were selected for further investigation.

Molecular Docking

We retrieved the three-dimensional structures of the proteins from the Protein Data Bank (PDB) database (https://www.rcsb.org/) and the structures of the small molecule ligands from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The AutoDock Tools 1.5.7 software was used to preprocess the receptors and ligands. We then performed molecular docking using AutoDock Vina.[19] The final docking conformation was selected based on its strong affinity and visualized using PyMOL 2.3.

Molecular Dynamics (MD)

Simulation MD simulations were conducted using the Standard Dynamics Cascade module of Discovery Studio 2019 software to assess the stability of ligand molecules in protein complexes. We selected the complexes with the highest fractions after molecular docking for simulation.[20] The system was modeled using an extended simple point charge water model. The entire system was placed in a solvent chamber with periodic boundaries filled with water molecules. To maintain electrical neutrality, Cl^- and Na^+ ions were used. We initially minimized the energy of the system using the steepest descent method.[21] The system was then equilibrated using the NPT (constant number, volume, and temperature of particles) and NPT (constant number, pressure, and temperature of particles) ensembles. Trajectories were generated to obtain the final protein-ligand complex model for MD simulation.[22]

Isolation of Primary Mouse Ovarian Granulosa Cells (MOGC)

Aged 6–8 weeks female C57BL/6 mice were housed in a SPF level animal laboratory. After quarantine, the mice were subcutaneously injected with horse chorionic gonadotropin (PMSG) at a dose of 40 IU. After 48 hours, the mice were euthanized using CO_2 inhalation anesthesia. The ovaries were collected under sterile conditions in pre-cooled sterile phosphate balanced solution (PBS). The follicles were punctured under an anatomical microscope, and the granulosa cells were released into pre-cooled DMEM culture medium. After digestion with 0.25% trypsin (containing 0.02% EDTA at 37°C and 5% CO_2 for 40 minutes to 1 hour, the digestion was terminated by adding a culture medium containing 15% FBS. The cells were filtered using a 200-mesh cell sieve and centrifuged for 5 minutes. The supernatant was discarded, and the MOGC were collected. The cells were cultured overnight at 37°C with 5% CO2. On the second day, the non-attached parietal cells were discarded after changing the solution, and the purity of the MOGC was confirmed for subsequent experiments.

Cell Viability Assay

For the cell viability assay, primary MOGC cells (5×10^4 cells/well) and HUVEC (3×10^3 cells/well) was seeded in a 96-well plate and incubated for 24 hours. Then, the cells were treated with different concentrations of YJKL (ranging from 0.16 mg/mL to 5.12 mg/mL) for 48 hours.

After washing away the residual culture medium with PBS, a 3-[4,5-Dimethyl-2-thiazolyl]-diphenyltetrazolium bromide (MTT) solution containing 5mg/mL was added to each well (50μL/well). The plate was incubated in a cell culture incubator at 37°C in the dark for 2–4 hours. After incubation, Dimethyl sulfoxide (DMSO) was added (150μL per well) and the plate was placed on a shaker for 5 to 10 minutes until the crystals were completely dissolved. The optical

density 570 (OD_{570}) value was measured using a full-wavelength enzyme-linked instrument. The data was then analyzed using GraphPad Prism 8.0 software.

Construction of Mouse Model of PCOS Induced by Dehydroepiandrosterone

C57BL/6J mice raised in an SPF level barrier system animal room, were randomly divided into a normal control group and a model group. The mice in the model group were subcutaneously injected with 0.2 mL of dehydroepiandrosterone (70 mg/kg), while the normal control group was given 0.2 mL glycerol once a day for 20 days. The model mice were randomly divided into the model group, YJD group and YJKL group. The YJD group and YJKL group were administered raw medicine at a dosage of 25g/kg through gavage, while the normal control group and model group were given physiological saline through gavage. Each group received continuous gavage for 12 days to observe the clinical manifestations of the animals. On the second day after the previous administration, the mice were weighed. Finally, the ovaries and uterus of mice were dissected, collected, and weighed. They were then fixed in a 10% formalin solution for preservation.

Construction of Mifepristone-Induced Implantation Disorder in Mouse Embryos

C57BL/6 mice (aged 6–8 weeks) were raised in the SPF animal laboratory. After the quarantine period, female mice were randomly divided into a normal control group and a model group. Female and male mice were bred in cages at a ratio of 2:1, with the first day of pregnancy recorded as the presence of vaginal emboli. The model group received a single subcutaneous injection of 0.1 mL of mifepristone (0.08 mg of mifepristone dissolved in 0.1 mL of propylene glycol) on the fourth day of pregnancy, while the normal control group received 0.1 mL of propylene glycol. On the 12th day, the experimental animals were euthanized, and the uterus and ovary tissues were collected to observe the number of embryo implantations in each group. Randomly divide the female mice after modeling into a model control group, a YJD group YJKL group, with a normal control group. Apply physiological saline (normal control group and model control group), basic formula, and the present invention formula continuously to the corresponding experimental group for 12 days, and observe the clinical symptoms of the experimental animals once a day. After the experiment, the experimental mice were euthanized and the uterus and ovaries of each group of experimental animals were collected for observation of embryo implantation, as well as morphological and histological analysis.

Construction of Ethanol-Induced Model of Endometrial Injury in Mice

SPF C57BL/6 mice, aged 6–8 weeks, were housed in a constant temperature facility. They were fed a standard diet and had unrestricted access to food and water. After the quarantine period, female mice were randomly divided into a normal control group and a model group. In the model group, 0.5 mL of alcohol was infused into the uterine horn of female mice during the estrus phase of their physiological cycle for 5 minutes. Afterward, the uterine cavity was rinsed with N.S. to remove the remaining ethanol. The normal control group was treated with intrauterine perfusion and N.S. flushing with an equal volume of PBS. After successfully establishing the model of ethanol-induced endometrial injury, the mice were divided into four groups: the model group, the YJD group, the YJKL group, and the normal control group. The treatment lasted for twelve days. The clinical symptoms of the experimental animals were observed every day. After the experiment, the mice were euthanized, and the uteri of mice in each group were collected for histomorphological observation.

Hematoxylin and Eosin (H&E) Staining

The tissues obtained from the mice were fixed in a 4% PBS-paraformaldehyde solution (pH 7.4) at room temperature for 48 hours and embedded in paraffin. After dehydration and clearing, the tissues were cut into slices that were 5 μm thick. The slices were then dewaxed and stained with H&E.[23]

Immunohistochemistry (IHC) Staining

Sections were prepared and treated with citrate buffer for repair. After blocking with sheep embryo serum, the sections were incubated with the primary antibody overnight at 4°C. Afterwards, the sections were incubated with a fluorescently labeled secondary antibody and stained with DAB. The sections were then photographed using a microscope.[24]

Detection of Estrus Cycle

All mice underwent estrus cycle phase determination between 08:00 and 10:00 each day. (1) Vaginal lavage using a pipette to circulate 100 μL of normal saline throughout the vaginal canal to collect a sample of vaginal epithelial and blood cells, (2) crystal violet staining of the mounted vaginal sample followed by two washes with ddH_2O and cover-slipping with 15 μL of glycerol; (3) microscopic cytological determination of which estrus cycle phase the vaginal sample represented.

Western Blot Analysis

Ovarian tissue was fully ground with a mortar and pestle, followed by treatment with lysate. Next, grinding machine was used to turn the tissue into a homogenate, which was centrifuged at 12,000 rpm for 10 min at 4 °C, and the supernatants were collected. The protein concentration was measured by bicinchoninic acid (BCA) assay. Protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (soaked in methanol approximately 10s before use) in transfer buffer. The membranes were incubated for 2 h in blocking solution (5% skim milk). Then, the membranes were washed with TBST and incubated with primary antibodies at 4°C overnight. The membranes were washed three times in TBST for 10 min each time and incubated with secondary antibody for 1 h at room temperature. β-actin was used as a loading control.

The immune complexes were detected using enhanced chemiluminescence. The density of specific bands was analyzed using Image J software.

qRT-PCR Analysis

RNA extraction and qRT-PCR The total RNA of the ovarian tissue was extracted using an RNA extraction solution according to the manufacturer's instructions. According to the instructions of the NovoScript Plus All-in-one 1st Strand cDNA Synthesis SuperMis (gDNA Purge) kit, 1μg of RNA was utilized in reverse transcriptase reaction to gain cDNA.

The expression of AKT1 and HIF-1α was investigated using RT-PCR. 2×NovoStart SYBR qPCR SuperMix Plus kit and LightCycler 480 System were employed to perform qRT-PCR. The threshold cycle (CT) is defined as the PCR cycle at which the fluorescent signal of the reporter dye crosses the automatically placed threshold. Genic transcript levels were calculated using the formula $2^{-\Delta\Delta CT}$. The mRNA levels of tested genes were normalized glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference. The qRT-PCR experiment was conducted in biological triplicate for each group.

Statistical Analysis

All statistical calculations for the one-way analysis of variance were performed using GraphPad Prism 8.0 software. The experimental results were presented as means ± SEM, and a p-value of less than 0.05 indicated statistical significance.

Results

Active Ingredients and Targets of YJKL Decoction

A total of 159 unique compounds were collected from the TCMSP database, and after removing duplicate values of active ingredients, we obtained 130 drugs. Additionally, from the TCMSP databases, we collected the targets of the active compounds in YJKL Decoction and obtained 250 drug targets (Supplemental Tables 1 and 2).

Therapeutic Targets of PCOS

To search for candidate targets related to PCOS, "Polycystic ovary syndrome" was used as an index keyword to identify 998 potential targets from the DisGeNET database and 1092 potential targets from the GeneCards database (with a score ≥15) (Supplemental Table 3). After removing duplicates, there were still 1785 targets remaining. The Venn diagram results (Figure 2) revealed that there were 105 overlapping targets between the YJKL targets and PCOS disease targets (Supplemental Table 4).

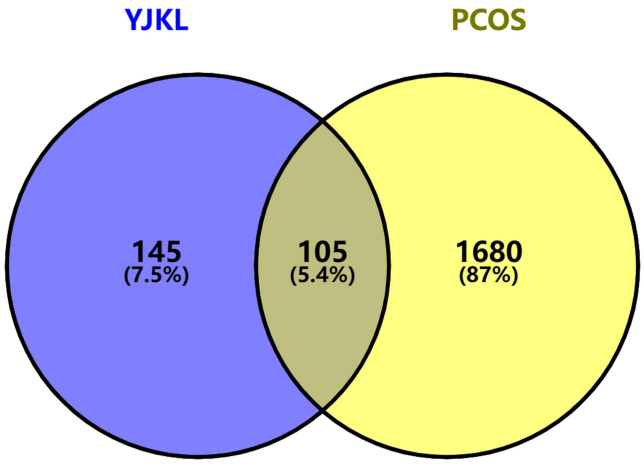

Figure 2 Venn diagram of potential targets of YJKL for the treatment of PCOS.

YJKL-Active Compound-Target Network

We constructed the YJKL-component-target network by considering the characteristics of multiple components and multiple targets in TCM compound prescriptions(Figure 3). In this network, the orange hexagon represents YJKL, the yellow triangle represents the compound contained in YJKL, the green circle represents the active ingredient, and the blue diamond represents the target. More information about the active ingredients at the core can be found in Table 1. (Supplemental Table 5)

PPI Network and Core Genes of Disease-Drug Targets

We utilized the String database to establish a PPI network consisting of 104 nodes and 1732 edges (The free target is hidden). The PPI network was visualized using Cytoscape 3.8.2 software (Figure 4). The targets with the highest degree value were selected as the core targets, which include AKT1, TP53, TNF, ALB, and VEGFA (Supplemental Table 6). We then utilized these core targets to construct the PPI network and visualize it. More information about the core targets is shown in Table 2.

GO Enrichment and KEGG Pathway Analysis Results

The intersection targets were inputted into the MetaScape database for GO and KEGG pathway enrichment analyses (Supplemental Tables 7 and 8). Based on a P value of < 0.01, the top ten GO terms were selected in BP, CC, and MF. It was suggested that biological processes such as hormone response, cellular response to organic cyclic compounds, and response to xenobiotic stimuli, cellular components such as membrane rafts, transcription regulator complexes, and protein kinase complexes, and molecular functions such as cytokine receptor binding, protein domain-specific binding, and nuclear receptor activity are important for YJKL treatment against PCOS (Figure 5A). The top 20 pathways were selected in KEGG, indicating a close relationship between the AGE-RAGE signaling pathway in diabetic complications, the TNF signaling pathway, and the HIF-1 signaling pathway (Figure 5B).

Molecular Docking

Study Based on the network pharmacology results, we screened the top five compounds: quercetin, kaempferol, luteolin, (-)-Tabernemontanine, and 7-Methoxy-2-methyl isoflavone. We then performed molecular docking with the core proteins AKT1, TP53, TNF, ALB, and VEGFA, respectively (Supplemental Table 9).

The compounds formed hydrogen bonds with the core targets. The 3D structure information of important proteins is displayed in Table 3. Molecular docking results showed the following: the free binding energy between AKT1 and quercetin was −6.7 kcal/mol, the free binding energy between TP53 and luteolin was −7.2 kcal/mol, the free binding energy between TNF and kaempferol was −8.9 kcal/mol, the free binding energy between ALB and 7-Methoxy-2-methyl

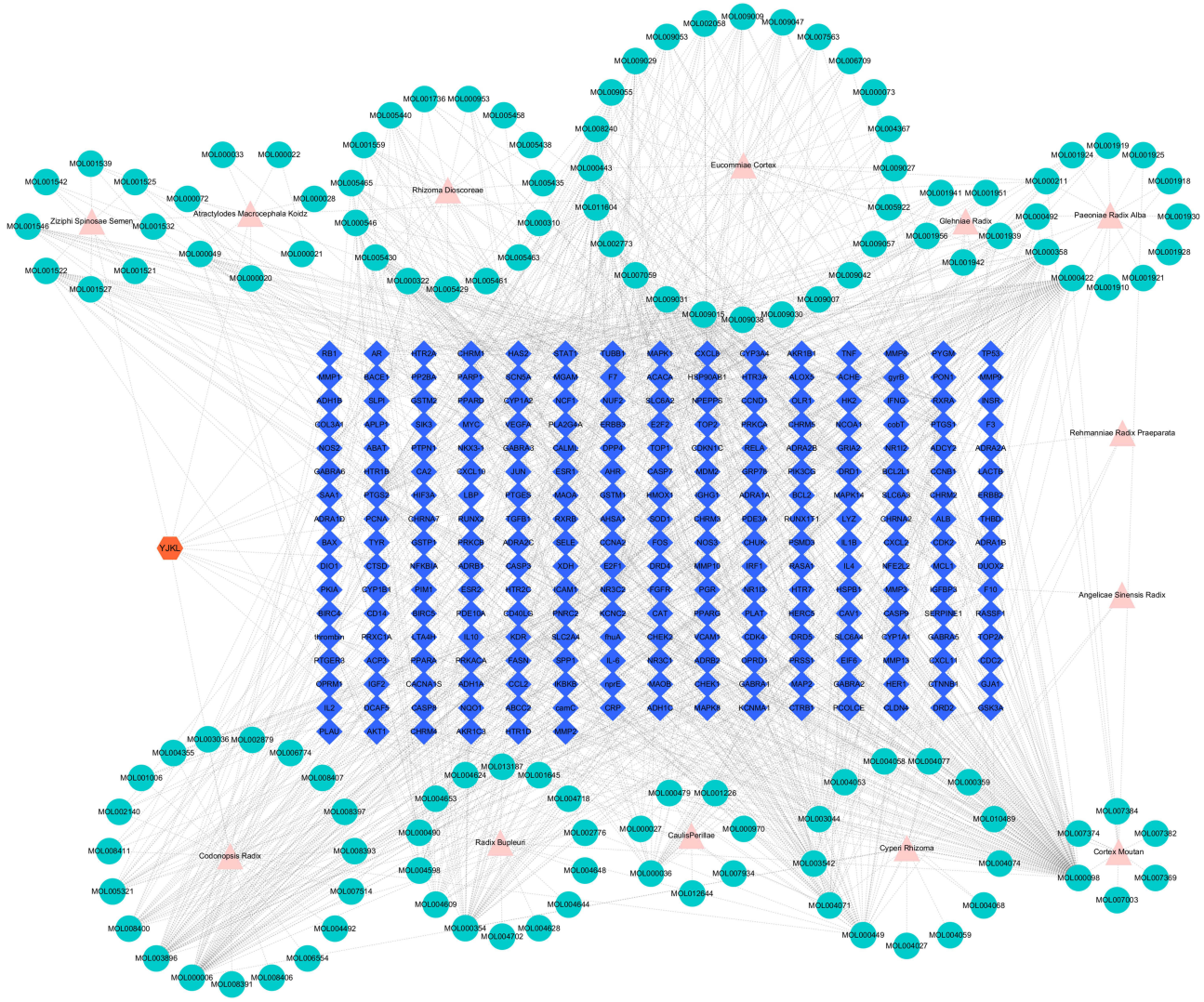

Figure 3 YJKL-component-target network.(The Orange hexagon represents YJKL Decoction. The pink triangle represents the compound contained in YJKL Decoction. The green circle represents the active ingredient. The blue diamond represents the target).

isoflavone was −8.4 kcal/mol, the free binding energy between VEGFA and (-)-Tabernemontanine was −7.9 kcal/mol, and the free binding energy between 8-isopentenyl kaempferol was not determined (Figure 6). All molecular docking data were used to generate a heat map, as shown in Figure 7.

Table 1 Information About the Core Active Ingredients

Mol IDs	Molecule Names
MOL000098	Quercetin
MOL000422	Kaempferol
MOL000006	Luteolin
MOL009015	(-)-Tabernemontanine
MOL003896	7-Methoxy-2-methyl isoflavone

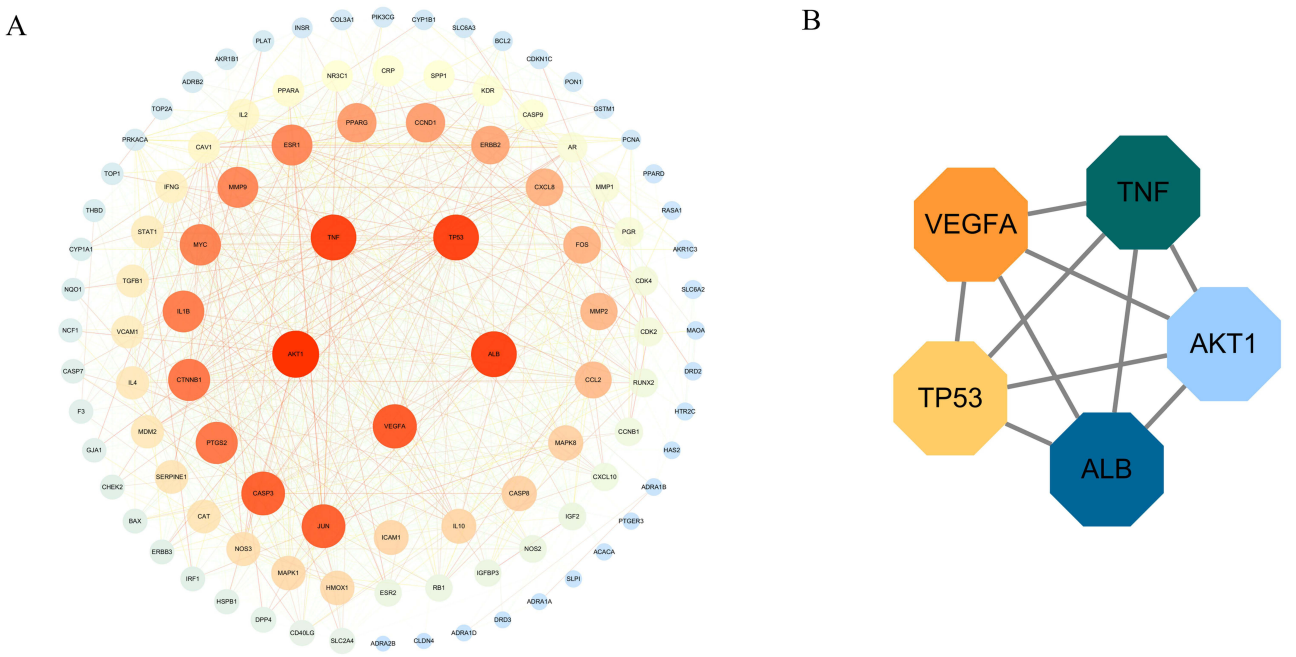

Figure 4 (**A**) Network diagram of common targets of YJKL and PCOS. (**B**) Network diagram of the core target.

A lower binding energy indicates a stronger binding activity between the ligand and receptor, with a binding energy of less than −5 kcal/mol generally indicating strong binding activity.[25] The results indicated that the binding between the active compounds and the core targets is tight.

MD

Simulation MD simulation is a key theoretical method used to determine the stability of a protein-ligand complex. To determine the binding mass of the small molecular ligands with tyrosinase after docking, the complex was further analyzed through MD simulation. The root-mean-square deviation (RMSD) of TNF with luteolin and quercetin remained stable throughout the entire simulation, indicating the high stability of these two complexes. Violent fluctuations in RMSD indicate a violent motion of the complex, whereas stable motion suggests stability (Figure 8).

Successful Construction of PCOS Mouse Model

As shown in Figure 9A, the estrus cycle of the model group mice is disordered, while the estrus cycle of other mice is more regular.

Figure 9B and C shows that compared with the normal control group, the weight of the uterus decreased significantly ($p<0.001$), while the weight of the ovary increased significantly ($p<0.001$). These findings are consistent with obesity or

Table 2 Information About the Core Targets

Gene Names	Protein Complete Names	UniProt Number
AKT1	Threonine kinase 1	P31749
TP53	Tumor protein p53	P04637
TNF	Tumor necrosis factor	P01375
ALB	Albumin	P02768
VEGFA	Vascular endothelial growthfactor A	P15692

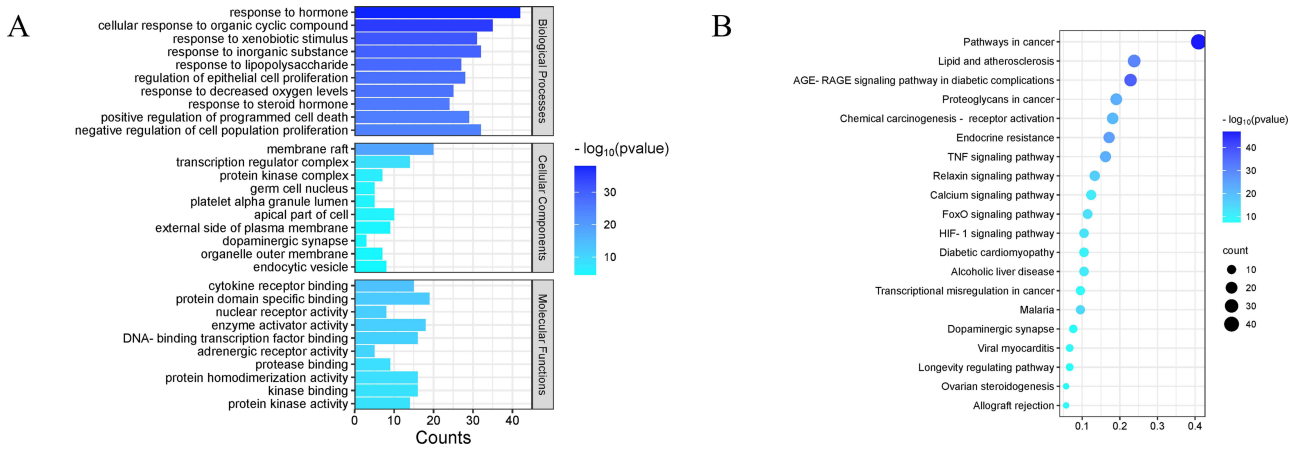

Figure 5 GO and KEGG enrichment analysis for identification of the mechanism of YJKL in treating PCOS. (**A**) The top 10 significantly enriched (P<0.01) terms in BP, CC and MF of GO analysis were selected. (**B**) The top 20 pathways with significantly enriched (P<0.01) were selected.

overweight and ovarian enlargement observed in most PCOS patients. Compared to the model group, the uterine weight of mice in the YJD group and YJKL group returned to normal levels. Additionally, the recovery effect of the YJKL group was better than that of the YJD group. Compared to the model group, the ovarian indexes of mice in the YJD group and YJKL group were lower. The YJKL group had the most significant effect (P<0.001). Therefore, the results showed that the PCOS mouse model was successfully constructed. (Figure 9).

YJKL Can Regulate Core Targets and Key Pathways

The mRNA levels and protein levels of AKT and HIF-1α were reduced in the model group compared to the control group. Protein and mRNA levels recovered to varying degrees after YJD and YJKL treatment (Figure 10).

YJKL Can Improve Symptoms of PCOS

After treating MOGC and HUVEC with various concentrations of YJKL Decoction for 48 hours, an MTT assay was conducted to measure cell proliferation. The results are shown in Figure 11A. The MTT experimental results showed that the proliferation activity of MOGC was induced in a concentration-dependent manner after 48 hours of treatment with YJKL. Additionally, YJKL Decoction effectively induced the proliferation of HUVEC. This finding demonstrates that YJKL Decoction plays a role in promoting angiogenesis.

In the normal control group, the uterus exhibited a healthy red color with a large number of embryo implantation sites. These sites were swollen like beads and evenly distributed on both sides. However, in the mifepristone model group, the uterus appeared thinner and smaller, and had a pale color. Very few embryos were implanted in this group. The basic prescription group showed some improvement compared to the misoprostol group, with the uterus remaining small and fine, although the number of embryo implantations was still higher than the misoprostol model group. However, the

Table 3 The 3D Structure Information of Key Proteins

Target Names	PDB Names
AKT1	7MXY
TP53	7LIN
TNF	2E7A
ALB	6R7S
VEGFA	1MKK

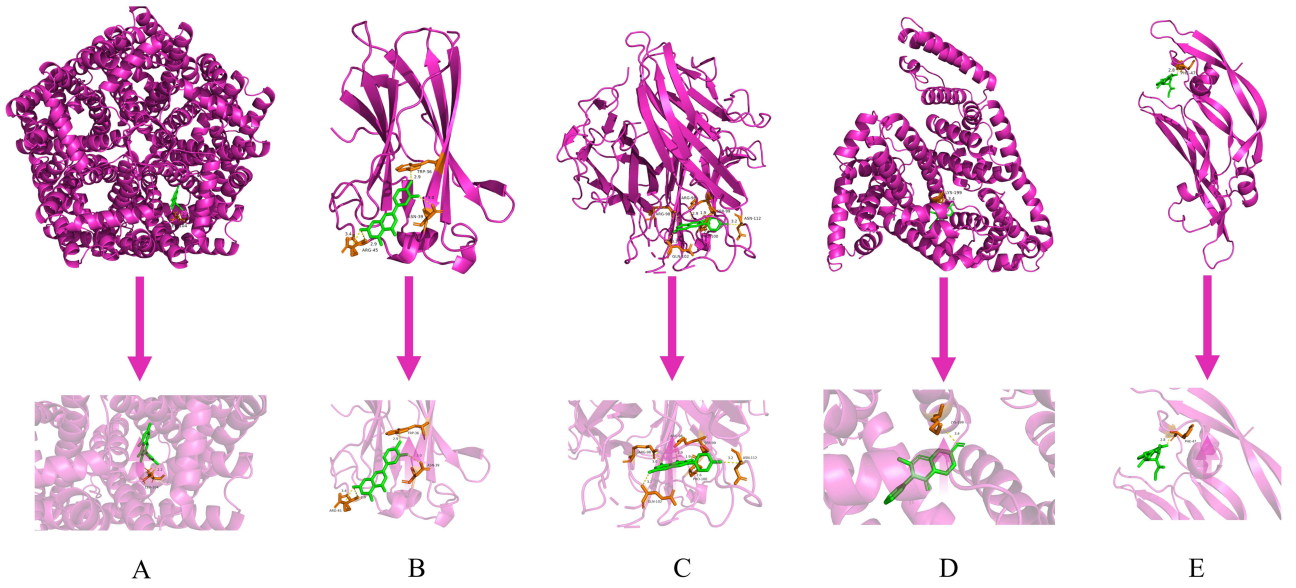

Figure 6 Partial molecular docking results. (**A**) AKT1 with quercetin molecular docking result. (**B**)TP53 with luteolin molecular docking result. (**C**)TNF with kaempferol molecular docking result. (**D**)ALB with 7-Methoxy-2-methyl isoflavone molecular docking result. (**E**) VEGFA with (-)-Tabernemontanine molecular docking result.

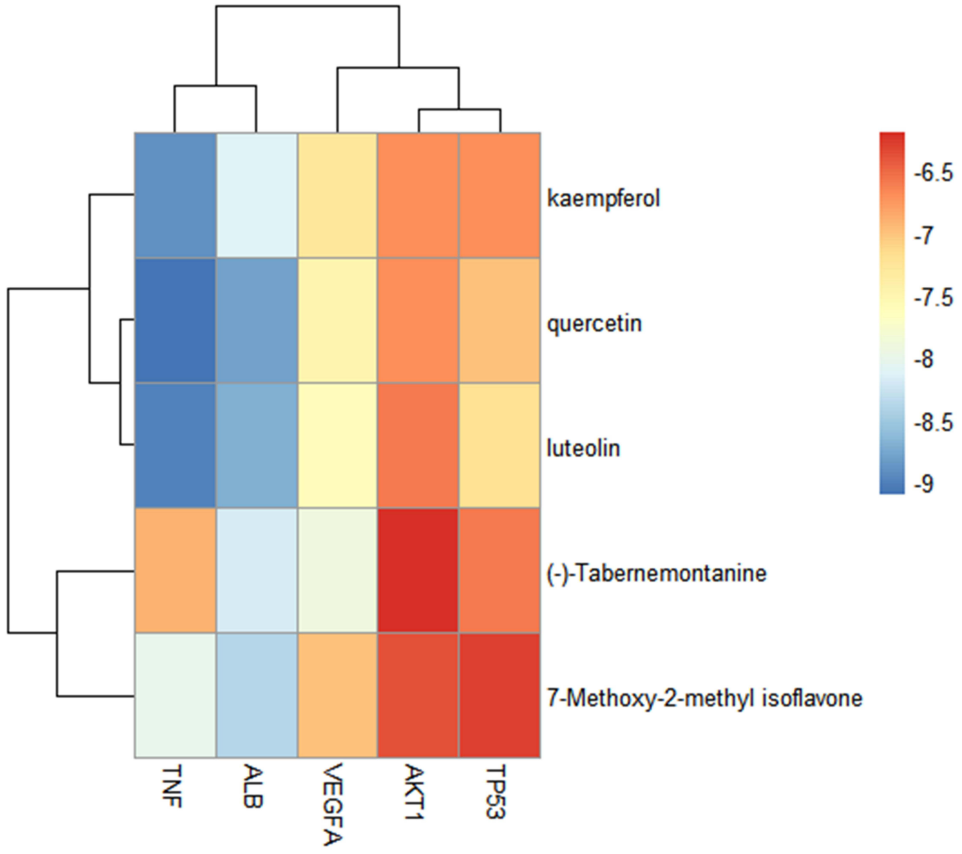

Figure 7 Molecular docking heatmap. The closer the color in the figure is to blue, the smaller the numerical value, indicating a closer molecular docking.

distribution of implantation sites in this group was uneven. In the YJKL Decoction group, the uterus gradually returned to its original size and the number of embryo implantations increased. The implantation sites were symmetrical and there was a slight recovery in the color of the uterus. No obvious abnormalities were found in the mice treated with YJKL

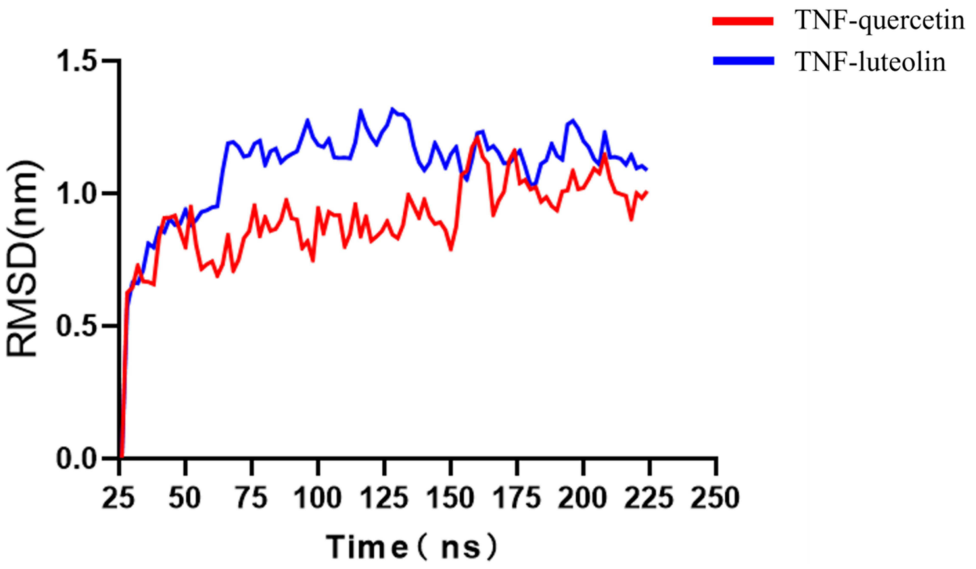

Figure 8 Molecular dynamics simulation results. The red line represents the molecular dynamics simulation results of TNF and quercetin; The blue line represents the molecular dynamics simulation results of TNF and luteolin.

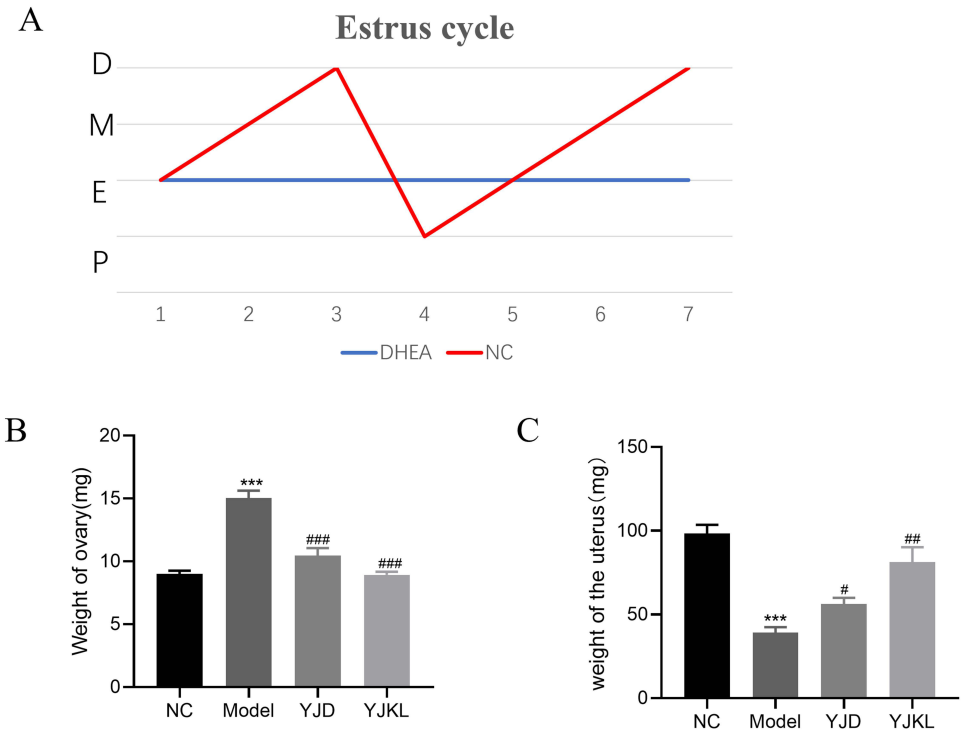

Figure 9 Prompt for successful model making. (**A**) The model group showed obvious disorder in the estrus cycle, while the other groups had relatively normal estrus cycles. (**B**) The ovarian weight of the model group mice was higher than that of the control group, while the YJD and YJKL groups were lower compared to the model group, with YJKL also lower than YJD. (**C**) Comparison chart of mouse uterine weight. It can be seen that the uterine weight of the model group was lower than that of the control group, and the uterine weight recovered after treatment, and the treatment effect of YJKL was better than that of the YJD group. (*Refers to the model group relative to the control group, #refers to the relative to the model group. #Refers to P<0.05, ##Refers to P<0.01,####Refers to P<0.001, ***Refers to P<0.001).

Decoction after 12 days of treatment or in the control group. This indicates that YJKL Decoction can effectively improve mifepristone-induced embryo implantation dysfunction in mice (Figure 11B).

Histological observation using HE staining showed that the endometrial structure of the normal control group was intact. The mouse uterus was composed of a serous layer, muscular layer, and endometrial layer, with the endometrial

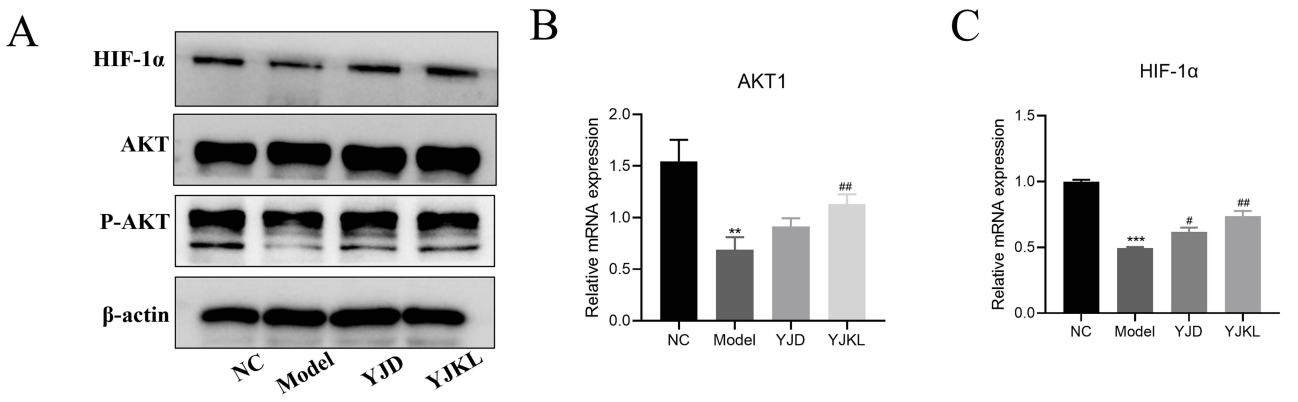

Figure 10 (**A**) HIF-1α and P-AKT protein expression was different in different groups. (**B**) The mRNA expression of AKT1 was different among different groups. (**C**) The mRNA expression of HIF-1α was different among different groups. (*Refers to the model group relative to the control group, # refers to the relative to the model group. #Refers to P<0.05, ##Refers to P<0.01, **Refers to P<0.01, ***Refers to P<0.001).

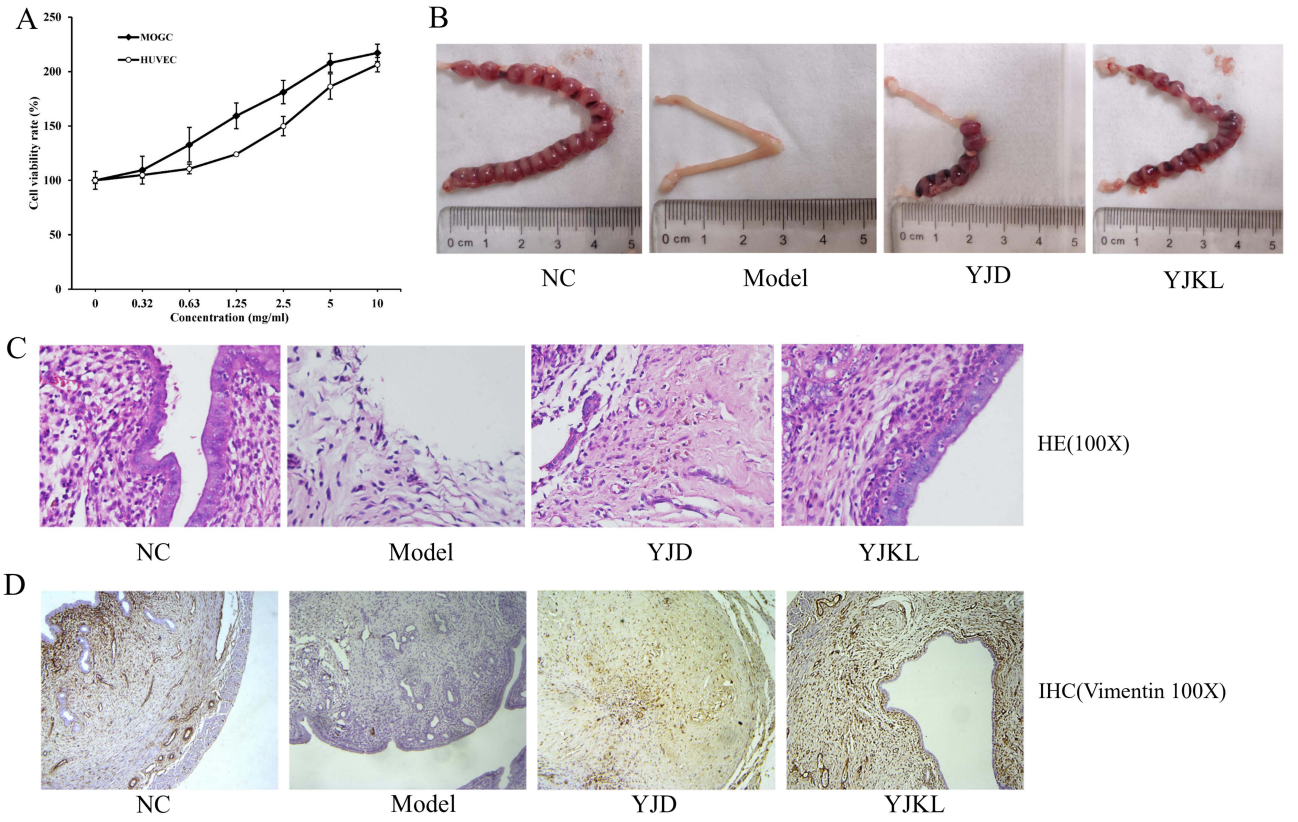

Figure 11 Pharmacodynamic experiments. (**A**) MTT experimental results. The addition of YJKL Decoction promotes the proliferation of MOGC and HUVEC. (**B**) Comparison of implantation maps of mouse embryos in each group. (**C**) Comparison of HE staining patterns of mouse endometrium in each group. (**D**) Comparison of Immunohistochemical staining patterns of vimentin protein in different groups of mice.

layer being the thickest and having a wavy surface. The endometrial and glandular epithelial cells were intact and arranged tightly in a single-layer cubic shape. The blood vessels and glands were also normal. In the ethanol model group, the endometrium became significantly thinner and the glandular arrangement was disorderly, with a decrease in their number. The endometrial epithelium and glandular epithelial cells became flattened and disordered, and local blood vessels appeared sparse. However, in the basic prescription treatment group, there was an improvement in the endometrium with an increase in the number of glands. The morphology of endometrial epithelium and glandular

epithelial cells also improved compared to the ethanol model group. Notably, there was almost no difference between the YJKL Decoction group and the normal control group, indicating that YJKL Decoction has the ability to repair endometrial lesions induced by alcohol in model mice(Figure 11C).

To further verify the effect of YJKL Decoction in promoting endometrial cell proliferation and angiogenesis in vivo, uterine tissues of the animals were collected at the end of experiment, fixed in a 4% PFA solution, and sliced. Immunohistochemical staining using Vimentin, a marker related to cell proliferation and angiogenesis, was performed. The immunohistochemical results indicated that, compared with the normal control group, the content of Vimentin in the ethanol model group was significantly reduced ($P<0.05$). However, both the basic prescription treatment group and the YJKL Decoction group showed an increase in the content of Vimentin compared to the ethanol model group ($P<0.05$). Therefore, it can be concluded that YJKL Decoction can effectively repair damaged endometrial proliferation and angiogenesis markers in model mice(Figure 11D).

Discussion

Perimenopausal dysfunction, which includes conditions like PCOS infertility, follicular hypotonia, and delayed follicular development, is a common gynecological disease. Previous research has shown that the overall effective rate of YJT treatment for patients with perimenopausal dysfunction is 90%.[26] However, there are few reports on the treatment of PCOS infertility specifically related to insufficient follicular tension and delayed follicular development.

In order to address this gap, we developed a modified prescription called YJKL Decoction, which is derived from the YJT Decoction formula. Traditional Chinese medicine compounds, YJKL Decoction, have the advantage of containing multiple components and targeting multiple pathways. They also have minimal toxic side effects, making them potentially effective remedies for the treatment of infertility caused by PCOS.

Through our study, we aim to provide a better understanding of the therapeutic effect of YJKL Decoction and offer an effective solution for the treatment of PCOS infertility in clinical practice. By exploring the mechanism of action of YJKL Decoction, we hope to contribute to the development of novel treatments for infertility and improve the overall reproductive health of women affected by PCOS.

In the process of obtaining visual images of the relationship between herbal medicine and disease targets, five targets with the highest degree values were selected as the core targets of YJKL Decoction for treating PCOS infertility: AKT1, TP53, TNF, ALB, and VEGFA. The AKT family, also known as the protein kinase B family, plays a crucial role in cell survival and apoptosis. Among the AKT family members, AKT1 is considered key. AKT1 is an important factor in placental development and fetal growth in mice. Its absence leads to defective placental development.[27] Insulin is secreted by pancreatic beta cells, and extensive studies have demonstrated that AKT1 plays a critical role in the proliferation of these cells.[28] Additionally, insulin resistance is a significant clinical feature in PCOS patients. Furthermore, reducing AKT1 levels has been found to decrease fertility in mice, which is attributed to the altered insulin and insulin-like growth factor signaling caused by the absence of AKT1.[29]

The TP53 gene encodes TP53, which is an important tumor suppressor gene. Previous studies have shown that high levels of TP53 protein are expressed in the nuclei of apoptotic granulosa cells during follicle development.[30] Additionally, TP53 levels are significantly lower in patients with PCOS.[31] TNF is a cytokine with multiple biological activities and can be mainly classified into TNF-α and TNF-β. While TNF-α is secreted by macrophages, TNF-β is secreted by T lymphocytes. TNF-α is an important adipokine involved in systemic inflammation and can contribute to obesity. Obesity is one of the major clinical manifestations in PCOS patients, and high expression of TNF-α has been found in these patients.[32] Moreover, TNF-α is involved in glucolipid metabolism and has been associated with insulin resistance.[33]

During acute ischemic conditions, the metal-binding capacity of ALB to transition metals such as copper, nickel, and cobalt is reduced. This reduction leads to the generation of a metabolic variant of the protein known as ischemia-modified albumin (IMA).[34] IMA, as a reflection of oxidative stress in the follicular endocrine microenvironment, may be linked to impaired oocyte developmental competence and embryo quality, along with increased testosterone levels.[35] VEGFA is secreted by endothelial cells and multiple tumor cells and acts as a regulator of endothelial growth factor and vascular

permeability.[36,37] VEGFA is closely associated with endometrial receptivity, and it has been shown that its expression in the endometrium significantly increases during the mid-luteal phase of the menstrual cycle.[38,39]

When constructing the YJKL-active compound-target network, the top three active ingredients with the highest degree values were identified as quercetin, kaempferol, and luteolin. All three ingredients contain significant amounts of flavonoids, which are well-known for their anti-tumor biological activity. Quercetin exhibits various biological activities, including antioxidant, antiviral, and anti-inflammatory properties.[40] Studies have shown that PCOS is associated with insulin resistance, primarily due to decreased luteinizing hormone levels and increased lipocalin activity.[41] Furthermore, recent research has demonstrated that quercetin activates HIF-1α similarly to hypoxia and stabilizes HIF-1α.[42] Kaempferol, as a natural and highly significant active flavonoid molecule, inhibits tumors by activating the AMPK/Nrf2/HO-1 signaling pathway, thereby reducing apoptosis, decreasing inflammatory responses, and improving oxidative stress.[43]

KEGG enrichment analysis results reveal that YJKL Decoction involves multiple signaling pathways in the treatment of PCOS, primarily the TNF signaling pathway and the HIF-1 signaling pathway. The TNF-α signaling pathway is associated with glucose uptake by tissues, potentially contributing to reduced fertility in women.[44] The HIF-1a signaling pathway plays a vital role in regulating the development of the ovarian luteum and offers a potential therapeutic approach for ovarian dysfunction diseases, such as PCOS.[45] Multiple animal experimental studies suggest that HIF-1 promotes follicular development and interacts with FSH receptors on granulosa cells to regulate E2 secretion.[46] Therefore, it can be inferred that the YJKL Decoction primarily exerts its therapeutic effect on PCOS by modulating the TNF signaling pathway and the HIF-1 signaling pathway. In vitro experimental results suggest that YJKL Decoction can improve various clinical symptoms in patients with infertility caused by polycystic ovary syndrome.

However, this study still has some limitations. First, we may have overlooked the targets of some drugs due to the large number and diversity of targets in each database, as well as the various algorithms used. Second, we have screened the core active ingredients for molecular docking and subsequent experiments. However, it is important to note that these components do not fully represent YJKL Decoction. Third, there have been few experiments conducted to validate the conclusions of network pharmacology. For example, we can conduct more cell experiments to knock down the level of P-AKT gene in MOGC cells and detect the expression levels of HIF-1a proteins. Due to HIF-1 α The signaling pathway is closely related to oxidative stress and ferroptosis. Test the levels of reactive oxygen species and GSH again, and repeat the above molecular mechanism experiments using cells. Finally, the core active ingredient monomers were added to detect the proliferation activity of cells. It is precisely because the effects and mechanisms of these potential active ingredients on sepsis have not been fully explained and verified that we believe there is significant potential for further development and research. In the future, we will conduct more experiments to explore the mechanisms of the key compounds in YJKL Decoction.

Conclusion

YJKL can be accessed through AKT-HIF-1α signaling pathway is used to treat polycystic ovary syndrome type infertility.

Ethical Approval

The animal study was approved by the Ethics Committee of Anhui Medical University (protocol code 82230030) for studies involving humans and (protocol code LLSC20231914) for studies involving animals. The animal protocols were reviewed and approved by the Experimental Animal Ethics Committee of Anhui Medical University (Hefei, China). The studies involving animals were reported in accordance with the ARRIVE guidelines for reporting experiments involving animals(Kilkenny et al, 2010; McGrath et al, 2010).

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Disclosure

The authors declare that they have no conflicts of interest in this work.

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