An investigation of the mechanism of ZiyinDianji decoction in treating follicular dysplasia based on network pharmacology and molecular docking

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ABSTRACT

Follicular development disorder is a common gynaecological endocrine disease that can cause infertility, menstrual disorders, abortion, and other complications. ZiyinDianji decoction (ZYDJD) is a commonly used traditional Chinese medicine in clinical practice to promote follicular growth and development, but its pharmacological activity and mechanism of action are not clear. We combined network pharmacology with molecular docking and *in vivo* animal experiments to investigate the mechanism of ZYDJD in follicular development disorder. Cytoscape software was used for constructing ZYDJD-active component-target and PPI networks. GO biological process and KEGG pathway enrichment analyses were performed. The main components and key targets were selected for molecular docking. Finally, animal experiments were conducted for validation. The network pharmacology results showed that ZYDJD contained 83 active components and 159 core targets. The six most important active components were quercetin, luteolin, kaempferol, baicalein, isorhamnetin, and β-sitosterol, and the most important disease targets were AKT1, TNF, IL-6, and P53. GO analysis mainly involved 470 cell biological processes, including effect on hormones, vascular morphogenesis, development, and cell proliferation. KEGG analysis involved cancer pathways, lipid metabolism pathways, and PI3K/AKT signalling pathways. Molecular docking showed good results, and animal experiments further verified that ZYDJD prevented cyclophosphamide from causing excessive activation of primordial follicles. ZYDJD maintained ovarian reserve and reproductive function by inhibiting the hyperphosphorylation of key molecules of the PI3K/Akt pathway, reducing FOXO3a, thereby ensuring the development of normal follicles. In conclusion, based on network pharmacology, molecular docking, and animal experiments, ZYDJD may act through the PI3K/Akt/FOXO3a pathway.
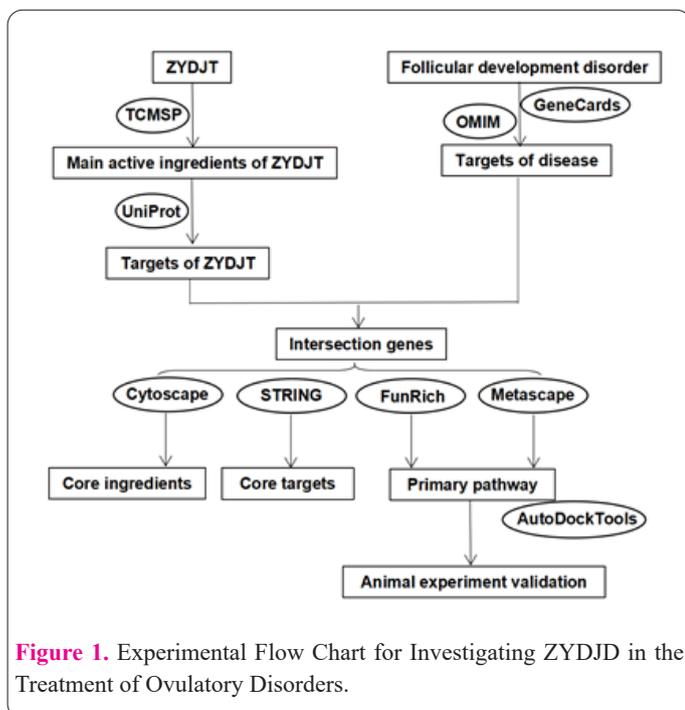
Introduction

Infertility is a medical and sociological problem that brings not only physical and mental harm to patients and their families but also affects family harmony and social stability. Surveys have shown that more than 60% of infertile families are infertile due to simple female factors, 10.5% of women worldwide suffer from secondary infertility, and 2% of women suffer from primary infertility (1). The causes of female infertility mainly include ovulatory dysfunction, tubal obstruction, and cervical factors (2). The most common cause is ovulatory dysfunction, which occurs in 30% to 40% of infertile women. Follicular dysplasia refers to diseases in which the size and function of follicles cannot reach the mature level due to hypothalamic-pituitary-ovarian reproductive axis disorders or paracrine or autocrine dysfunction of ovaries, mainly including abnormalities in the growth rate, shape, size, number and location of follicles, reflected as follicular failure, atresia and ovulation of immature follicles, which is more common in hypothalamic and hypopituitarism diseases, polycystic ovary syndrome, hyperprolactinemia, early-onset ovarian insufficiency, premature ovarian failure and other conditions (3). Symptoms often manifest as menstrual disorders (including abnormal menstruation, menstrual volume, and quality), luteal phase dysfunction, infertility, and abortion.

At present, treatments for follicular dysplasia include the application of Western medicine, such as ovulation induction, hormone support therapy, and assisted reproductive technology. However, improper use of expulsion-promoting drugs may lead to ovarian hyperstimulation syndrome, poor ovarian response, multiple pregnancies, decreased endometrial receptivity, and other adverse reactions. After drug withdrawal, the menstrual cycle and ovarian function of most patients cannot completely return to normal and may even be accompanied by serious complications (4-7). Although ART has been used to treat infertility, it cannot be widely used in middle-income and low-income areas and countries due to complex technology and expensive prices.

Clinical practice has demonstrated that TCM can not only promote follicular development but also regulate the overall function of the human body (8). Professor Zhou Huifang, guided by the theory of Professor Xia Guicheng, a famous doctor of traditional Chinese medicine, created ZYDJD based on many years of medical experience. ZYDJD is composed of 12 herbs, including Danggui, Fu-

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Figure 1. Experimental Flow Chart for Investigating ZYDJD in the Treatment of Ovulatory Disorders.

ling, Danpi, Shengdi and Nvzhenzi, and can nourish yin and blood, tonify the kidney, regulate menstruation, and tonify the liver and kidney. ZYDJD is mainly used to treat menstrual disorders, amenorrhea, and infertility in patients with liver-kidney yin deficiency. Because Chinese herbs have multiple components, targets, and mechanisms, it is inevitable to use modern medicine to analyse and study the mechanism of TCM. The overall systematic nature of network pharmacology and the characteristics of drug-drug interactions are in line with the understanding of the nature of Chinese herbal medicine for diseases (9). There-fore, network pharmacology has been increasingly applied for studying the mechanisms underlying TCM in treatment methods of various diseases (10, 11).

In this study, the main active components of ZYDJD and the main targets and pathways of action in the treat-ment of follicular development disorders were screened by network pharmacology and verified by in vivo expe-riments to preliminarily explore the molecular biological mechanism of action of ZYDJD in the treatment of folli-cular development disorders (Figure 1).

Materials and Methods

Screening of the main drug components of ZYDJD

According to the TCMSP database, we collected the main chemical components of ZYDJD with the keywords "Angelica sinensis", "Poria cocos", "Paeonia lactiflora", "Paeonia lactiflora", "Paeonia lactiflora", "Chishao", "Reh-mannia glutinosa", "Ligustrum lucidum", "Yam", "Cornus officinalis", "Cuscuta chinensis", and "Dipsacus". Com-pounds with oral bioavailability (OB) $\geq$ 30% and drug-like properties (DL) $\geq$ 0.18 were selected as active ingredients of each Chinese herb based on the UniProt database. The selected target protein names were annotated for genes. The main components of ZYDJD and the screened target genes were introduced into Cytoscape 3.9.1 to construct the "active component-target gene of ZYDJD compound" network.

Screening of disease targets and targets of ZYDJD for follicular dysplasia

Through the OMIM database (https://www.omim.org/) and the GeneCards database (https://www.genecards.org/), the gene targets of the disease were searched using the keywords "Follicular dysplasia", "Follicular maldeve-lopment", and "Ovulatory Disorder Infertility". The two databases were combined to obtain the ZYDJD targets in follicular development disorders.

Constructing a common target network of ZYDJD-ac-tive component-follicular dysplasia

The follicular dysplasia genes were mapped with the potential target genes of the ZYDJD compound to draw a Venn diagram and obtain the intersection genes. The active ingredients and intersection genes of ZYD-JD were imported into Cytoscape 3.9.1 to construct the "ZYDJD-active component-follicular dysplasia" network. Nodes in the network indicate targets or com-pounds, while edges indicate interactions between active ingredients of the drug and follicular dysplasia disease.

Construction of the Protein-Protein Interaction (PPI) Network

Entering drug-disease common targets into the protein interaction platform STRING, network construction was performed to set the protein species as "Homo sapiens" and obtain the interaction relationship between the targets of ZYDJD for the treatment of follicular dysplasia. TSV format files were downloaded and imported into Cytos-cape 3.9.1 software, and the "Network analyser" plugin was used to obtain target protein interaction network topo-logy parameters and network diagrams.

Functional analysis of gene ontology biological process (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were perfor-med

Using Metascape, 158 target genes were subjected to GO and KEGG analysis. GO analysis was used to obtain biological processes (BP), cellular components (CC), and molecular functions (MF). KEGG enrichment analy-sis can reveal important signalling pathways involved in biological processes. The top 25 GO and KEGG results with significant differences were obtained. Visual analysis was performed, and the enrichment results were output in a three-in-one histogram and bubble plot style using the Microbiology Information Software (http://www.bioinfor-matics.com.cn/).

Molecular docking

Molecular docking is the process of recognizing drugs from target proteins through the chemical environment and energy. The main active ingredients of ZYDJD were screened to determine the top three according to the de-gree value, and the mol2 files of the 3D structures of these active ingredients were downloaded from the TCMSP database and logged in the PDB database (http://www. rcsb.org/). PDB format files for the top 4 core target pro-tein structures were downloaded, and the core target pro-tein structures were dehydrated and hydrogenated using AutoDock tools 1.5.7 software, selected as receptors, and finally converted to PDBQT receptor format. Small mole-cules were hydrogenated and twist keys were detected and

converted to PDBQT ligand format for molecular docking. PyMOL software was used to visualize the molecular docking results. Absolute binding energy > 4.25 represents the preliminary binding ability of the molecule to the target, > 5.0 represents strong binding ability, and > 7.0 represents very strong binding ability, which is used to predict and evaluate the binding activity between the active ingredients of the drug and the target protein.

Experimental animals

Sixty 8-week-old SPF female rats weighing 230 g ± 20 g were housed in an SPF barrier environment after admission under licence No. SYXK (Su) 2018-0049. The animals were given food and water *ad libitum* and kept on a 12 h/12 h diurnal cycle, with room temperature (23 ± 3°C) and relative humidity 40% ± 10%.

Drug

Cyclophosphamide (Baxter Oncology GmbH, batch number: 1A443A) and complex packing oestradiol tablets/oestradiol and dydrogesterone tablets (trade name, Femoston: each tablet contains oestradiol 2 mg; oestradiol dydrogesterone tablets: each tablet contains oestradiol 2 mg + dydrogesterone 10 mg; Abbott Healthcare Products B. V, batch number: H20152345) were obtained.

Preparation of ZYDJD: The medicinal materials were verified by Associate Professor Liu Shengjin from the Department of Traditional Chinese Medicine Identification, Nanjing University of Traditional Chinese Medicine, which met the relevant requirements of the Pharmacopoeia of the People's Republic of China (2015 Edition). The total medicinal materials of ZYDJD (110 g) were purchased from the Outpatient Pharmacy of the First Affiliated Hospital of Nanjing University of Traditional Chinese Medicine. Finally, the aqueous extract of the three herbs was evenly mixed and filtered, followed by vacuum concentration to 2.8 g/mL using a rotary evaporator, sealed, and stored at 4°C until use.

Reagents

Rabbit PI3K antibody, rabbit AKT antibody and rabbit phospho-AKT (Ser473) antibody (Cell Signaling Technology, USA; batch numbers 4249T, 4691T, and 4060T, respectively), rabbit FOXO3a antibody and rabbit phospho-FOXO3a (S253) antibody (ABMAT, China, batch numbers T55898 and T55562, respectively), rabbit GAPDH antibody (ABMART, China, batch number: T40004); RIPA lysate, PMSF, phosphatase inhibitor, protease inhibitor (Biyuntian Biotechnology Co., Ltd., Shanghai), loading buffer, rapid blocking solution, and rabbit secondary antibody(ImmunoWay Biotechnology Company, batch number: RS0002) were purchased for this study.

Instruments

An MPRO multifunctional microplate reader (Tecan, Switzerland), a Mini-PROTEAN Tetra electrophoresis tank, a semidry and rapid transfer system, a tank transfer system, a Chemi DOC TMMP immunoblotting-multifunctional imaging analysis system (Bio-Rad, USA), an MB100-4P constant temperature mixer, a BioPhotometer D30 protein nucleic acid analyser, a 5427R high-speed refrigerated centrifuge (Eppendorf, Germany); a Sorvall ST8 high-speed table-top centrifuge, and a UXF40086v -80°C ultralow temperature freezer (Thermo, USA) were used in this study.

Animal treatment

After 7 days of adaptive feeding, vaginal smear was performed at 9 am every day to observe 2~3 oestrous cycles. Rats with regular cycles were selected and divided into 6 groups, the control group, the model group, the Femoston group and ZYDJD low-, medium-, and high-dose groups, by random number table, with 10 rats in each group. According to the results of the daily vaginal smear, after the rats were selected to enter metestrus, cyclophosphamide was prepared at 20 mg/ml with normal saline, intraperitoneally injected at a dose of 75 mg/kg, and the rats were observed for 14 days, during which a daily morning vaginal smear was performed, and irregular oestrous cycles were found in rats, showing that follicular dysplasia in rats was successfully modelled.

After 14 days of model establishment, the blank and model groups were given normal saline at 1 ml/100 g body weight once daily for 15 days. According to the Table of Ratios of Equivalent Doses Converted by Body Surface Area between Humans and Animals (12), the equivalent dose ratio converted according to body surface area between 60 kg adult females and 200 g rats was 6.25 in the treatment group, and one tablet was dissolved in 50 ml normal saline and administered at a dose of 0.5 ml/100 g in the Femoston group. Oestrogen was administered for 2 days, oestrogen + progesterone was administered for 3 days as a cycle, and the drug was discontinued 15 days later. The low-, medium-, and high-dose groups of ZYDJD were 0.5, 1, and 2 times the adult equivalent dose, respectively. The low, medium, and high doses were 5.7, 11.4 and 22.8 g/kg, respectively, and the equivalent dose was 11.4 g/kg, which was completed by continuous administration for 15 days.

Histopathological examination of rat ovaries

Rats in each group fasted after the last dose but had free access to water, and they were anaesthetized by intraperitoneal injection of 10% chloral hydrate solution at a dose of 3.5 mL/kg. After blood collection from the abdominal aorta, the rats were sacrificed by cervical dislocation, and the ovaries were rapidly removed. One part of the tissue was fixed in 4% paraformaldehyde solution for immersion and sent for pathology, and the other part was snap-frozen in liquid nitrogen and placed in a −80°C refrigerator for testing.

Rat ovarian tissues were immersed in 4% paraformaldehyde fixative for 24h, dehydrated with an ethanol gradient, cleared with xylene, embedded in paraffin, cut into 5 μm slices, deparaffinized with xylene, and eluted with an ethanol gradient for HE staining.

Western blotting validation

Total protein was extracted from rat ovarian tissue, the protein concentration was measured by the BCA method, and the concentration of each sample was adjusted to be consistent. For SDS–PAGE, a 10% layer gel and a separation gel were prepared, the loading amount of each well was not less than 20 μg, the layer gel was run at 80 V for 30 min, the separation gel was run at 110 V for 60 min, then 400 MA constant flow was used to transfer the protein onto a PVDF membrane for 30 min. Quick blocking solution was added for 30 min, and the primary antibody

was added for incubation overnight at 4°C on a horizontal shaker followed by the secondary antibody (diluted at 1:5000) for 1 h at room temperature. TBST was used to wash the membrane three times for 10 min each time. The membrane was exposed to ECL luminescent solution for 30 s to 5 min. The gel imaging system was used to collect the bands. Image Lab software was used to determine the grey value data.

Statistical analyses

Experimental data were analysed using GraphPad Prism 9.4.0 software. If the comparison between multiple groups conformed to a normal distribution and the variances were homogeneous, one-way ANOVA was used, and $P < 0.05$ indicated that the difference was statistically significant.

Results

Screening of the main active ingredients of Chinese herbal compounds

A total of 56 active ingredients were obtained from the TCMSP and BATMAN-TCM databases, as shown in Supplement Table 1. A total of 271 predicted drug targets were combined and reweighed. The main drug components and corresponding targets were imported into Cytoscape 3.9.1 to construct the network diagram of "active component-target gene of ZYDJD compound", which included 337 nodes and 793 edges (Figure 2).

Screening of disease targets and targets of ZYDJD in treating follicular dysplasia and the network construction

A total of 3125 targets for follicular dysplasia were obtained from the GeneCards database and the OMIM database. There are 158 common targets from both databases (Figure 3a). A total of 56 active ingredients in ZYDJD and 158 common targets were imported into Cytoscape 3.9.1 to construct the "ZYDJD-active component-follicular dysplasia" graph (Figure 3b), which contains a total of 135 nodes and 252 edges, and the top 16 TCM components in order of degree values are quercetin, luteolin, kaempferol, baicalein, isorhamnetin, β-sitosterol, diosgenin, stigmasterol, tetrahydroalstone, ellagic acid, grapsin, kadsurensin, campylamine, campylamine, AIDS180907, and campest-5-en-3beta-ol. These 16 active ingredients are believed to be the main drug components involved in treating follicular dysplasia. A total of 158 common targets were imported into the STRING database to construct the PPI

network. The following figure was obtained (Figure 3c). The plug-in CentiscaPe 2.2 was opened, and degree, closeness, and betweenness were analysed. The results obtained were Degree unDir: 41.745, Betweenness unDir: 126.11, and Closeness unDir: 0.00362242, and values greater than the above results were the screening conditions used to obtain the key targets (Figure 3d).

GO biological process and KEGG pathway enrichment analyses

Using FunRich mapping software 3.1.3, key targets were imported to generate a heatmap of gene expression in human tissues (Figure 4). A total of 158 common targets were introduced into Metascape, default settings were Min Overlap: 3, P Value Cut-off: 0.01, Min Enrichment: 1.5 for GO and KEGG functional enrichment analyses. GO enrichment analysis yielded 470 cell biological processes (Figure 5a), which mainly involved response to the hormone, organic cyclic compounds' cellular response to cyclic compound, cell stimulated response toxenobiotic, vascular morphogenesis, development, cell proliferation; molecular function was associated with cellular transcription factor, kinase binding, and protein homodimerization activity, etc. KEGG pathway analysis involved 103 signalling pathways. These pathways mainly involved the cancer pathway, the lipid metabolism pathway, the AGE-RAGE signalling pathway, and the TNF signalling pathway (Fi-
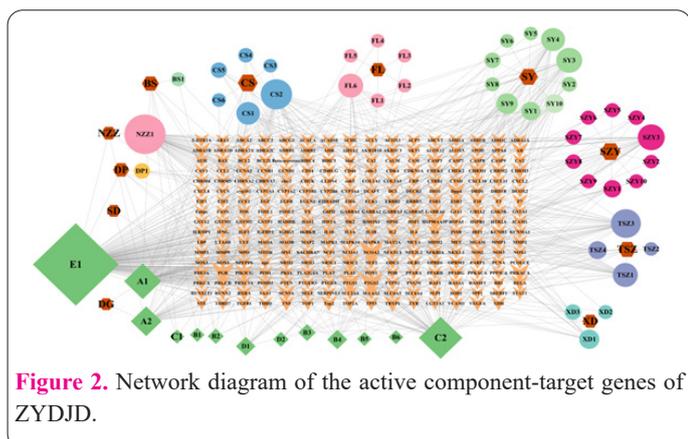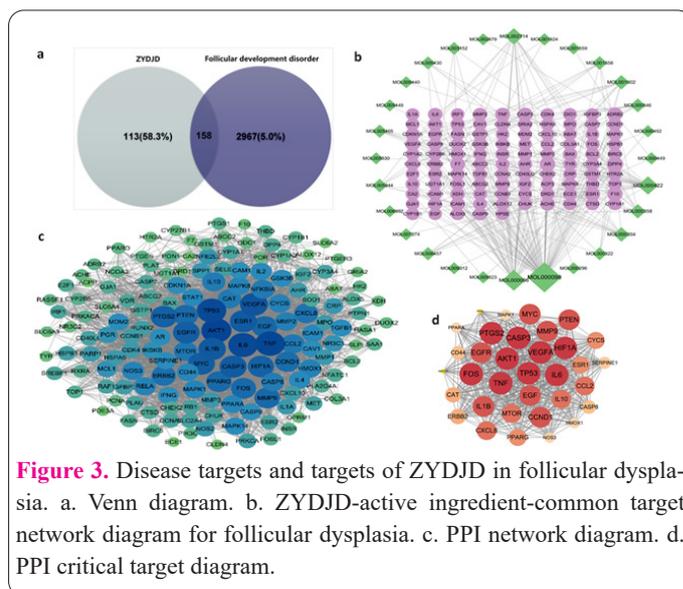

Figure 3. Disease targets and targets of ZYDJD in follicular dysplasia. a. Venn diagram. b. ZYDJD-active ingredient-common target network diagram for follicular dysplasia. c. PPI network diagram. d. PPI critical target diagram.
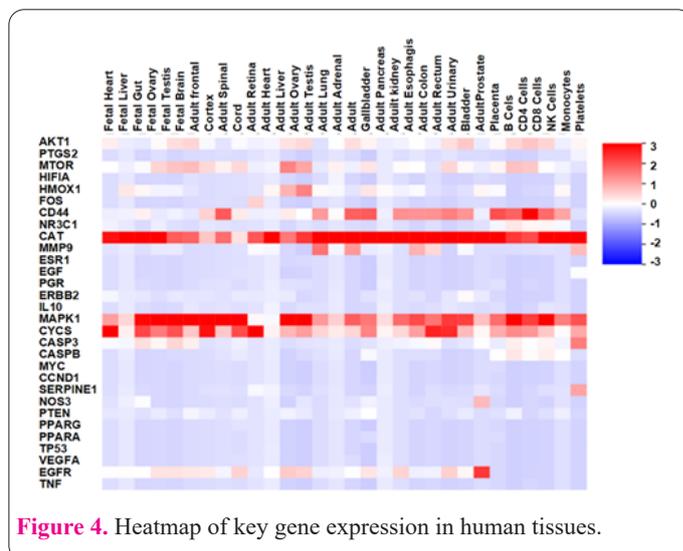

Figure 2. Network diagram of the active component-target genes of ZYDJD.

Figure 4. Heatmap of key gene expression in human tissues.

gure 5b). These results suggest that ZYDJD may act by regulating multiple signalling pathways.

Molecular docking

The 158 genes and enriched related pathways were imported into Cytoscape 3.9.1 to generate target gene-critical pathway network maps (Figure 6a). Quercetin, luteolin, and kaempferol were molecularly docked into the top 4 PPI network degree values. The binding energies of quercetin to AKT1, TNF, IL-6, and P53 were -6.3, -6.0, -7.5 and -6.9 kJ/mol^{-1}, respectively. The binding energies of luteolin to AKT1, TNF, IL-6, and P53 were -6.2, -6.2, -7.5 and -6.7 kJ/mol^{-1}, respectively. The binding energies of kaempferol to AKT1, TNF, IL-6 and P53 were -5.9, -6.7, -7.5, and -6.6 kJ/mol^{-1}, respectively. The molecular docking patterns of quercetin, luteolin, and kaempferol with Akt are shown in Figure 6b.

Effect of ZYDJD on ovarian morphology in rats with ovulatory dysfunction

The blank group showed growing follicles at all levels, with a large number and rare atretic follicles. Compared with the blank group, the overall number of follicles in the model group decreased, the follicles at all levels in the model group were atretic and reduced, the developing follicles were vacuolated, the granulosa cells were disorganized and detached, and the nucleus colour became lighter. The number of follicles in the traditional Chinese medicine group and the Western medicine group increased compared with that in the model group. In the ZYDJD medium- and high-dose groups, follicular development at all levels gradually recovered, the nuclear colour became darker, vacuolation and atretic follicles decreased, granulosa cells were densely arranged, and the normal structure of
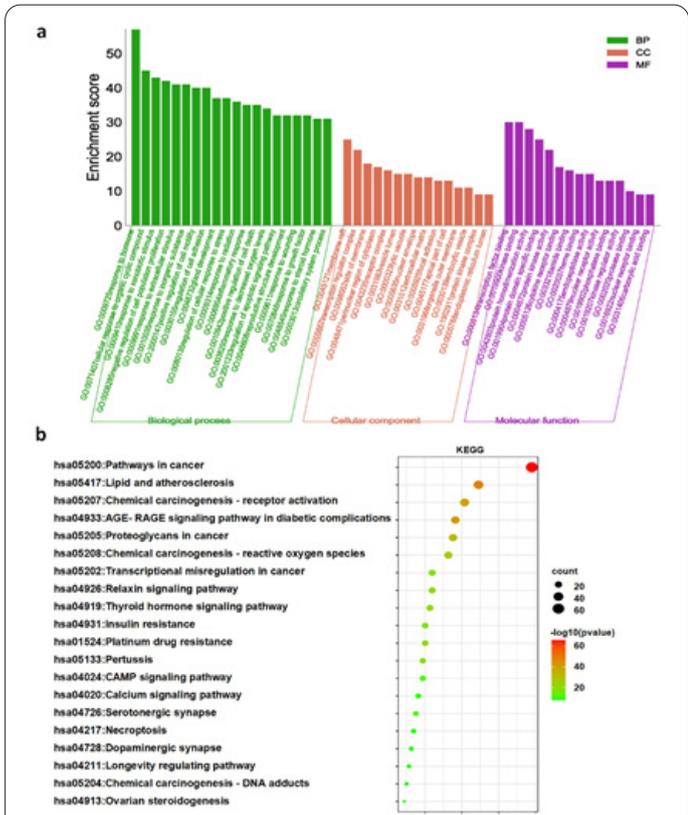

Figure 5. Functional enrichment analysis. a. GO Enrichment Analysis Plot. b. KEGG analysis bubble map.
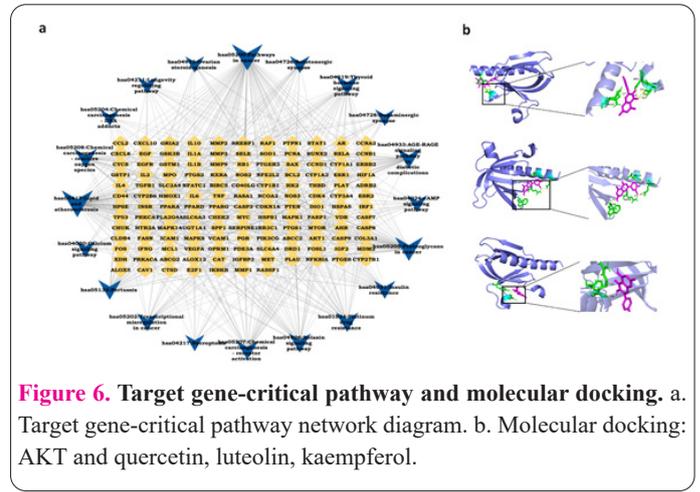

Figure 6. Target gene-critical pathway and molecular docking. a. Target gene-critical pathway network diagram. b. Molecular docking: AKT and quercetin, luteolin, kaempferol.
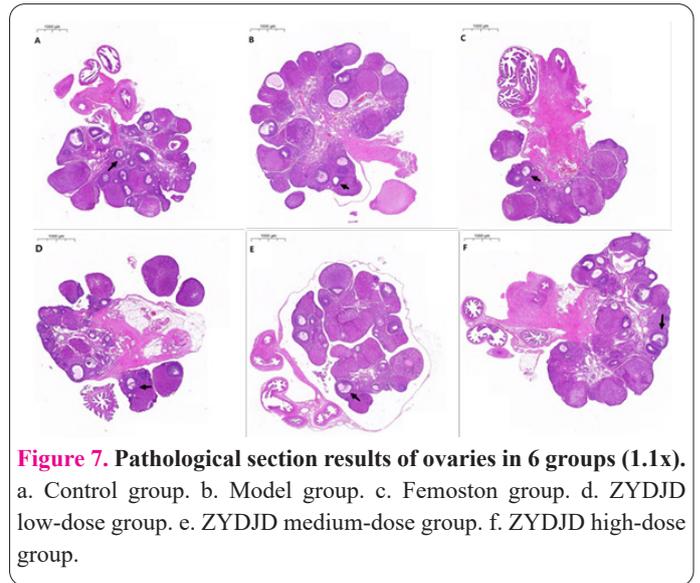

Figure 7. Pathological section results of ovaries in 6 groups (1.1x). a. Control group. b. Model group. c. Femoston group. d. ZYDJD low-dose group. e. ZYDJD medium-dose group. f. ZYDJD high-dose group.

the ovary gradually became clear (Figure 7 and Figure 8).

Effect of ZYDJD on PI3K, AKT, P-AKT, FOXO3a, P-FOXO3a and P53protein expression in ovulatory dysfunction rats

Compared with the blank group, the PI3K level, P-AKT/AKT ratio, P-FOXO3a/FOXO3a ratio, and P53 level were significantly increased in the model group (P < 0.01). Compared with the model group, these indexes were significantly decreased in the ZYDJD medium-dose group (P < 0.05) (Figure 9).

Discussion

According to TCM, "pregnancy must regulate menstruation first", the normal menstruation of women depends on the balance of the kidney-Tiangui-Chongren-uterus axis (13), the function of organs such as the heart, liver, spleen, and kidney, sufficient Chong Ren Xuehai with meridian constraints, and Du meridian Yang Qi filling. An imbalance of any aspect will cause follicular dysplasia. Adequate kidney essence and kidney Qi are important material bases for follicular development and maturation. The pathogenesis of this disease is based on kidney deficiency, as well as pathological factors such as liver-kidney yin deficiency, spleen-kidney yang deficiency, qi and blood deficiency, stagnation of liver-qi, stagnation of liver-fire, phlegm-dampness, or blood stasis. Professor Zhou Huifang believes that the late menstrual period is
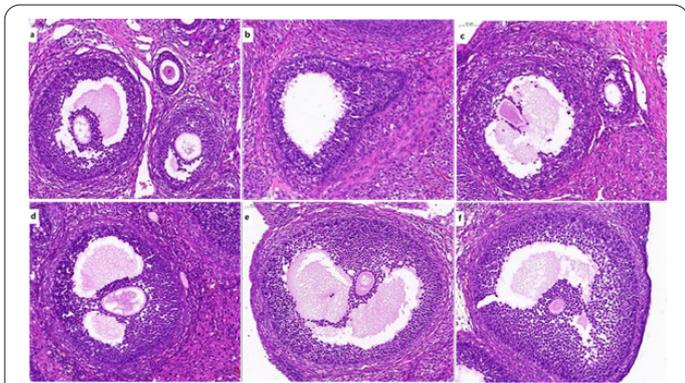
Figure 8. Follicular development in six groups shown by HE staining (20x). a: Control group. b: Model group. c: Femoston group. d: ZYDJD low-dose group. e: ZYDJD medium-dose group. f: ZYDJD high-dose group.
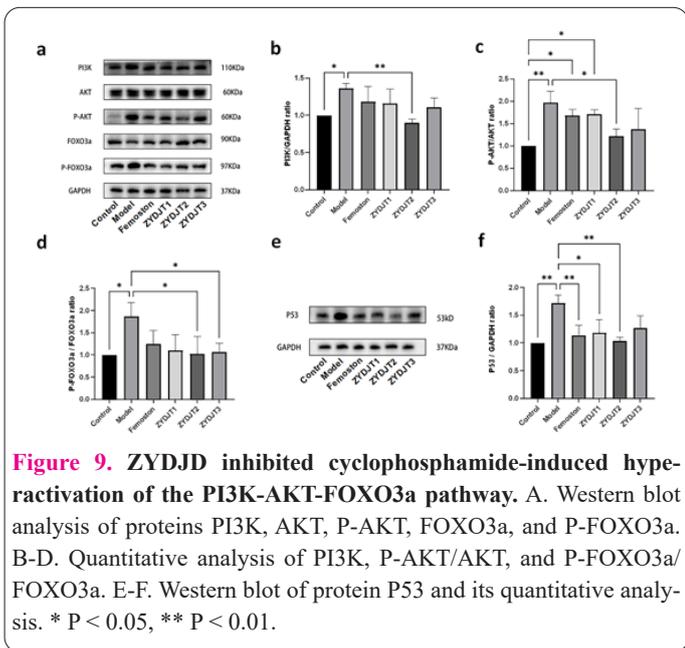

Figure 9. ZYDJD inhibited cyclophosphamide-induced hyperactivation of the PI3K-AKT-FOXO3a pathway. A. Western blot analysis of proteins PI3K, AKT, P-AKT, FOXO3a, and P-FOXO3a. B-D. Quantitative analysis of PI3K, P-AKT/AKT, and P-FOXO3a/FOXO3a. E-F. Western blot of protein P53 and its quantitative analysis. * $P < 0.05$, ** $P < 0.01$.

the length to weight of yin and is an important period for the development of sperm and eggs. If the liver and kidney are insufficient and the yin fluid is deficient, follicular development is impaired. ZYDJD is mainly composed of 12 herbs, including Danggui, Shengdi, Nvzhenzi, Fuling, Shanyao, and Yam, and the whole prescription has an important role in tonifying the kidney and nourishing yin. Modern pharmacological studies have demonstrated that kidney-invigorating herbs can significantly enhance the HPO axis and neuroendocrine regulation function, regulate serum E2, FSH, and LH levels, increase endometrial sensitivity to oestrogen, and promote the recovery of ovarian morphological structure and related functions.

In this paper, the mechanism of ZYDJD in improving follicular development disorders was investigated through constructing network pharmacology and molecular docking using bioinformatics tools as well as performing *in vivo* experiments for verification. In network pharmacology experiments, the six most effective active substances in this prescription were quercetin, luteolin, kaempferol, baicalein, isorhamnetin, and β-sitosterol. Experimental studies have found that quercetin reduces cyclophosphamide-induced loss of primordial follicles, rescues depletion of the primordial follicle pool, and protects reproductive function by inhibiting phosphoryla-

tion of key factors of the PI3K/AKT/FOXO3a pathway and nuclear exit of FOXO3a in mice (14). By observing the effect of adding quercetin to cryoprotectants on follicular activity after cryopreservation of sheep ovarian tissues, it was found that a low concentration of quercetin could reduce oxidative damage of ovarian tissue and preserve follicular activity (15). Kaempferol has various biological functions, such as antioxidation, anti-inflammation, and anticancer functions, and when the production of intracellular reactive oxygen species (ROS) is greater than the cellular consumption, ROS will accumulate in large amounts and then trigger oxidative stress, which is also one of the important main causes affecting follicular development. Kaempferol can reduce oxidative stress in tissues by inhibiting lipid peroxidation and scavenging superoxide anions in lipid microparticle systems induced by reduced coenzyme II (NADPH) or Fe^{2+} (16, 17). In addition, kaempferol has a very significant anti-inflammatory effect by regulating the activity of proinflammatory enzymes and inflammation-related gene expression and inhibiting transcription factors, adhesion molecules, and matrix metalloproteinases (18). Luteolin alleviates bisphenol A-induced ovariotoxicity by decreasing the expression of cleaved caspase-3 protein and decreasing the relative expression of phosphorylated p38 MAPK and ERK proteins (19). Baicalin has antioxidant, anti-apoptotic, and anti-inflammatory effects, and the addition of baicalin to porcine oocyte *in vitro* maturation medium and embryonic IVC medium can significantly inhibit oocyte and cumulus cell apoptosis, improve the ability of oocyte *in vitro* maturation, and improve oocyte quality (20). β-Sitosterol has biological activities such as antioxidation, anti-inflammation, anti-apoptosis, and neuroprotection and intervenes with upregulation of P-AKT and P-PI3KCA expression in granulosa cells, promoting granulosa cell proliferation. Expression of the apoptotic protein caspase-3 is downregulated, thereby inhibiting granulosa cell apoptosis (21).

Ovarian reserve in women is closely related to fertility. In clinical practice, cyclophosphamide can lead to massive loss of primordial follicles in patients and cause decreased ovarian reserve (22). When ovarian reserve decreases, the recruitment of follicles is promoted, the number and quality of recruited follicles are decreased, and the follicular phase and menstrual cycle are shortened (23). Therefore, cyclophosphamide can be used for constructing the rodent model of follicular dysplasia. In this experiment, compared with the normal group, the follicles at all levels in the model group were atretic and reduced, the developing follicles were vacuolated, the granulosa cells were disorganized and detached, and the nuclei became lighter, suggesting that the model was successfully established. After treatment with medium and high doses of ZYDJD, the development of follicles at all levels was gradually recovered, the colour of nuclei became darker, vacuolation and atretic follicles were decreased, granulosa cells were densely arranged, and the normal structure of ovaries gradually became clear. These results suggest that ZYDJD can effectively improve the histological structure of the ovary.

In this study, network pharmacology combined with molecular docking technology was used to explore the active substances, targets, and signalling pathways of ZYDJD in the treatment of follicular dysplasia or other follicular development disorders. The results showed

that AKT1, TNF, IL-6, P53, VEGFA, and IL1B were the molecular targets with higher PPI network degree values, indicating that the above targets may play a major role in treatment, with AKT1 degree having the highest value. In silico simulations confirmed that the six most potent active substances, quercetin, luteolin, kaempferol, baicalein, isorhamnetin, and β-sitosterol, were all able to dock well to AKT1 target. Therefore, the AKT1 signalling pathway is likely to play a key role in the treatment of follicular dysplasia with this prescription. AKT is a protein kinase that is involved in the regulation of cell proliferation, apoptosis, and metastasis after activation. AKT1 is present in oocytes and can promote the growth, development, and maturation of follicles and participate in the proliferation of follicular granulosa cells. As shown by the heatmap of AKT1 expression in the human body in Figure 4, AKT1 expression is low in the ovarian tissue of normal adults. If AKT1 is abnormal, follicular development is blocked, which in turn leads to low follicular quality (24). Follicular development is regulated by multiple signalling pathways, including Wnt, EGFR-ERK, and PI3K-AKT pathways (25). The PI3K/Akt pathway is an intracellular pathway that plays an important role in the proliferation and differentiation of gonadotropin-induced granulosa cells, early follicular activation, and follicular growth and maturation (26). PI3K is activated by growth factors, hormones, and cytokines binding to their cognate receptors to generate PIP3 in the cytoplasmic membrane, which binds to the substrate protein homology domain of Akt to translocate AKT from the cytoplasm to the cell membrane and is completely activated by phosphorylation at two important sites, serine 473 (Ser473) and threonine 308 (Thr308), thus causing interactions between its downstream phosphorylation cascade and target proteins.

FOXO3a is a member of the forkhead transcription factor family and a major downstream target of the PI3K pathway, regulating key factors in early ovarian follicle development. FOXO3a is a longevity gene, and it is mainly involved in biological processes such as cell cycle progression, DNA repair, autophagy, and apoptosis, which can regulate the stress response caused by oxidative stress and DNA damage (27). FOXO3a controls primordial follicle activation, and mice with FOXO3a gene knockout show premature primordial follicle activation in ovaries (28). After FOXO3a is phosphorylated by Akt, it is translocated from the nucleus to the cytoplasm and loses its transcriptional activity, thus relieving the inhibitory effect on primordial follicle activation, resulting in the premature development of primordial follicles and the reduction of reserve follicles. Under such conditions, it cannot provide a sufficient number of primordial follicles to be recruited into the growth follicle pool, follicular development is impaired, and mature eggs with fertilization ability are finally unable to form. According to the results of animal experiments, ZYDJD can inhibit the hyperphosphorylation of key molecules of the PI3K/Akt pathway, reduce the expression of PI3K and P-Akt, reduce the activation of the downstream protein FOXO3a, thereby preventing the cyclophosphamide-induced hyperactivation of primordial follicles and maintaining ovarian reserve and reproductive function, ensuring the development of normal follicles. These results suggest that ZYDJD can improve follicular development and ovarian reserve, and the *in vivo* pharmacological results are consistent with the network pharmacology and molecular docking results.

Granulosa cells are the basic functional units of ovaries, which provide nutrition and information for follicular development through gap junctions and participate in the whole process of follicular development, maturation, and ovulation. Granulosa cell apoptosis is the main cause of follicular atresia (29). Granulosa cells undergo apoptosis earlier than oocytes in atretic follicles and are the initiators of follicular atresia (30), and when granulosa cells are damaged by oxidative stress, they activate the tumour suppressor P53, inhibit cell cycle-related proteins, arrest in G1/S phase, and trigger granulosa cell apoptosis (31). Therefore, the P53 gene is closely related to the mechanism of follicular atresia (32). In addition, P53 is also a redox-active transcription factor, which can directly sense oxidative damage through DNA-mediated charge transport (33), regulate its target gene and initiate a stress response. The results showed that ZYDJD could decrease the expression of P53 protein after treatment, indicating that ZYDJD could improve P53-mediated ovarian oxidative stress and granulosa cell apoptosis probably.

In summary, this study used network pharmacology, molecular docking, and animal experiments to explore and verify the potential mechanism of action of ZYDJD in the treatment of ovulatory disorders. Our results showed that ZYDJD is beneficial in alleviating follicular development disorders by stereoscopic and reticular regulation, which is consistent with the concept that people are an organic whole in TCM theory. However, due to the incomplete database data involved in the current network pharmacology study and the possibility that TCM components will change during decoction, there are some limitations to the results of this study. Therefore, further basic experiments and clinical studies at a later stage are still needed for verification, so as to more scientifically and comprehensively elaborate the mechanism underlying ZYDJD.

Informed consent
The authors report no conflict of interest.

Availability of data and material
We declared that we embedded all data in the manuscript.

Authors' contributions
FX and ZH conducted the experiments and wrote the paper; LW, LX, WY and DY analyzed and organized the data; ZH conceived, designed the study and revised the manuscript.

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