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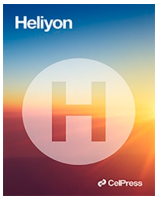

Research article

Exploring the effect of Gouqi Nuzhen Liuhe decoction on the PI3K/mTOR signaling pathway for premature ovarian insufficiency based on system pharmacology

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ABSTRACT

Objective: To explore the effect of Gouqi Nuzhen Liuhe Decoction (GNLHD) on the PI3K/mTOR Signaling Pathway for Premature Ovarian Insufficiency (POI) based on system pharmacology.
Methods: First, the system pharmacology approach was used to predict the mechanism of GNLHD. Then, mice were randomly divided into model group, positive group, GNLHD high-dose group, GNLHD medium-dose group, and GNLHD low-dose group. Hematoxylin-eosin (HE) staining was used to observe the pathological changes of ovarian tissue under light microscope. The expression levels of estradiol (E2), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were detected by enzyme-linked immunosorbent assay. The expressions of PI3K, AKT1 and mTOR proteins in ovarian tissue were detected by immunohistochemistry.
Results: The results of system pharmacology showed that GNLHD may regulate biological processes and signaling pathways such as: reproductive structure development, reproductive system development, Oocyte meiosis and so on. Compared with the model group, the levels of E2 in the GNLHD group were increased, and the levels of FSH and LH were decreased ($P < 0.05$). Compared with the model group, the number of mature follicles in the GNLHD group was significantly increased, the number of atretic follicles was relatively decreased, and the expressions of PI3K, AKT1, and MTOR proteins in the GNLHD group were significantly increased ($P < 0.05$).

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Conclusion: GNLHD may improve the ovarian function of POI mice by affecting the expression of PI3K, AKT1 and mTOR proteins, promote the growth and development of follicles, increase the E2 level, reduce FSH and LH level, and maintain the stability of the ovarian internal environment.

1. Introduction

Premature Ovarian Insufficiency (POI) is a reproductive endocrine disorder occurring in women before the age of 40, characterized by metabolic and reproductive dysfunction [1,2] such as menstrual irregularities, reduced fertility, irritability, insomnia, hot flashes, sweating, and decreased libido. Its onset is associated with ovarian aging [2]. The European Society of Human Reproduction and Embryology (ESHRE) diagnostic criteria define POI as oligomenorrhea or menopause for over 4 months with at least 2 serum basal follicle-stimulating hormone (FSH) levels >25IU/L (interval >4 weeks) or a single FSH level >40IU/L [3]. The incidence of POI ranges from 1 % to 5 % [4,5], with causes not fully elucidated but potentially linked to genetic, immune, psychological, iatrogenic, and environmental factors [6,7]. Hormone replacement therapy (HRT) is the primary treatment for POI, supplemented by immunotherapy, stem cell therapy, fertility therapy, and gene therapy [8,9]. While these therapies offer short-term clinical benefits, they face technical and ethical challenges. HRT, despite being the mainstay for POI management, has been associated with increased risks of breast cancer, stroke, and other conditions [10,11].

POI is classified under traditional Chinese medicine (TCM) as a condition falling into the realm of "menstrual disorders" and "infertility," encompassing "menorrhagia, delayed menstruation, early menstruation, and pre- and post-menstrual symptoms" [12]. TCM attributes the disease primarily to kidney-related imbalances closely linked to liver and spleen functions. TCM therapy, known for its comprehensive regulatory effects, offers a distinct advantage of minimizing adverse reactions compared to hormone replacement therapy [13]. Clinical investigations have demonstrated the effectiveness of Gouqi Nuzhen Liuhe Decoction (GNLHD), an herbal mixture comprising Siwu Decoction as the foundational formula supplemented with *Lycii fructus*, *Ligustri lucidi* fructus, *Cuscutae semen*, and *Cistanches herba*, in ameliorating POI symptoms, reducing follicle-stimulating hormone levels, and enhancing pregnancy rates. To investigate the therapeutic mechanism of GNLHD in POI treatment further, this study employs network pharmacology and multidirectional pharmacology to delineate the targets and associated pathways of GNLHD, aiming to provide insights and rationale for the clinical application of GNLHD in POI management [14,15].

Current research indicates that network pharmacology serves as a novel approach to elucidating the intricate mechanisms of traditional Chinese medicine. By adopting a network perspective, it elucidates the interactions among biological systems, drugs, and complex diseases, aligning closely with the overarching principles of Chinese medicine [16]. Network pharmacology, rooted in systems biology theory [17,18], integrates computer science, metabolomics, multi-directional pharmacology, bioinformatics, and other disciplines to systematically unravel the drug mechanisms via construction of networks linking active ingredients-gene targets-disease, gene targets-function/pathways, and other elements [19,20]. With its holistic and systematic nature, network pharmacology transforms the conventional single drug research paradigm into a novel approach that interconnects multiple targets and pathways with complex diseases. It serves as a crucial methodology for TCM research and a vital strategy for novel drug development [21,22]. The experimental group employed network pharmacology to elucidate the mechanisms of action of GNLHD in treating POI, with the aim of identifying targets and signal pathways associated with GNLHD efficacy in POI treatment. This effort seeks to provide a theoretical foundation for the clinical application of GNLHD in POI management and pave the way for the further development and utilization of GNLHD.

2. Materials and methods

2.1. GNLHD active components and target proteins screening

TCMSP (http://tcmspw.com/tcmsp.php) was used to search for the main chemical components of *Lycii Fructus*, *Ligustri Lucidi Fructus*, *Cuscutae Semen*, *Cistanches Herba*, *Angelicae Sinensis Radix*, *Chuanxiong Rhizoma*, *Paeoniae Radix Alba*, *Rehmanniae Radix Praeparata*, *Ziziphi Spinosae Semen*, *Cyperi Rhizoma*, *Cyathulae Radix*; and select the potential pharmacologically active compounds of GNLHD through pharmacokinetic parameters (ADME) [23]. The screening criteria were defined as oral bioavailability (OB) ≥30 %, Caco-2 parameter > -0.4, and drug-likeness (DL) ≥0.18 [23]. Then Pharmmapper (http://lilab-ecust.cn/pharmmapper/) was used to predict the target of GNLHD [24]. The UniProt database (https://www.uniprot.org/) was used to standardize the names of target proteins and obtained their official gene symbol (Table S1).

2.2. POI related gene collection

The POI related genes were collected from DisGeNet (https://www.disgenet.org/) [25], OMIM (OMIM http://omim.org/) database [26] and Genecards (http://www.genecards.org) [27]. The genes of the three databases are merged and deduplicated, and the final gene list was considered to be POI-related genes (Table S2).

2.3. Network construction and analysis

GNLHD targets and POI genes were imported into the STRING database (https://string-db.org), the biological type was set to "Homo Sapiens", the minimum interaction threshold was set to "medium confidence >0.4″, free nodes were hidden, and the rest of the settings were default, so as to construct a protein-protein interaction (PPI) network [28]. Cytoscape 3.7.0 software was used to visualize the network [29]. Metascape (http://metascape.org/gp/index.html#/main/step1) was used for enrichment analysis of GNLHD targets and POI genes [30].

2.4. Experimental materials

The timeline of the experiment is shown in Fig. 1.

2.4.1. Experimental animal

Healthy female Balb/c mice, 60, SPF grade, 8 weeks old, body weight 20±2 g, were purchased from and raised in the Experimental Animal Center of Hunan University of Traditional Chinese Medicine. The environment is maintained at 12 h bright and 12 h dark every day, allowing the animals to drink and eat by themselves. [Animal license number: SCXK (Xiang) 2016–0002]. Animal experiments were performed in accordance with the guidelines of the Animal Ethics Committee of Hunan University of Chinese Medicine and the guidelines for the care and use of experimental animals.

2.4.2. Experimental drugs

GNLHD is composed of *Lycii Fructus* 15 g, *Ligustri Lucidi Fructus* 15 g, *Cuscutae Semen* 30 g, *Cistanches Herba* 10 g, *Angelicae Sinensis Radix* 15 g, *Chuanxiong Rhizoma* 10 g, *Paeoniae Radix Alba* 10 g, *Rehmanniae Radix Praeparata* 30 g, *Ziziphi Spinosae Semen* 10 g, *Cyperi Rhizoma* 10 g, *Cyathulae Radix* 10 g. These Chinese medicinal materials were identified, screened, washed, processed, sliced, dried, and crushed by the Pharmacy Department of the First Affiliated Hospital of Hunan University of Chinese Medicine (Batch number: 20180908).

2.4.3. Instruments and reagents

METTLER TOLEDO PL203 precision balance (METTLER TOLEDO), LEICA RM223 5 slicer, LEICA HI1210 spreader, LEICA DMLB2 binocular microscope (German LEICA company). Motic BA410 biological microscope (Mike Audi Company). MIAS Medical Image Analysis System (Beihang Technology Co., Ltd.)

Mouse zona pellucida polypeptide solution: the 330342th amino acid sequence of mouse zona pellucida 3 (ZP3) (NSSSSQFQIHGPR), analytical purity>90 % (Hangzhou Zhongfu Biochemical Co., Ltd.). Freund's complete adjuvant and Freund's incomplete adjuvant (American Sigma company). Rabbit anti-mouse PI3K antibody (bs-0128R), AKT 1 antibody (bs-0115R), MTOR antibody (bs-1992R) (Beijing Boaosen Biotechnology Co., Ltd.). PV-9000 universal two-step detection kit (batch number: K183316B; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.). DAB color reagent kit (batch number: 107058A07); E2, FSH, LH enzyme-linked immunoassay kit (batch numbers: E20181010001, E20181010002, E20181010003) were purchased from Shanghai Jingtian Biotechnology Co., Ltd.

2.4.4. Quality control of GNLHD

The Inertsil ODS-SP (250 mm × 4.6 mm, 5 μm) column was used. The mobile phase was acetonitrile-0.1 % phosphoric acid solution, eluted with the following gradient, the flow rate was 1 mL/min, the column temperature was 30 °C, the detection wavelength was 225 nm, and the injection volume was 10 μL. It was finally found that the mass fractions of paeoniflorin and ferulic acid in GNLHD were 2122.3–2782.9 and 59.2–121.3 μg/g, respectively (Fig. S1).

2.5. Experimental methods

2.5.1. Animal modeling

6 mg ZP3 zona pellucida polypeptide powder was diluted with 6 mL of double-distilled water, and formulated into an immunological reagent in a ratio of 1:1 with Freund's complete adjuvant. In the same way, it is formulated with Freund's incomplete adjuvant

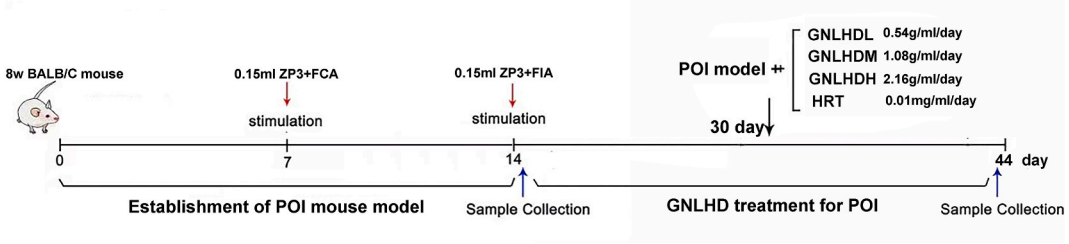

Fig. 1. The timeline of the experiment.

into an immune boosting reagent. In the model group, mice were injected with 0.15 mL of immune reagent into the soles of the feet and subcutaneously in the abdominal cavity; 14 days later, 0.15 mL of immune booster reagent was injected into the same site again to establish a mouse immune POI model. Mice in the blank group were injected with 0.15 mL of saline at the same location. The general conditions of the mice, such as drinking water, eating, fur color and mental state, were regularly observed, and the mice's body mass was recorded. One week after the injection of the immune reagents, vaginal exfoliated cell smears and HE staining were performed at 9:30 a.m. every day, and observation was continued for 7 days to determine the changes in the estrus cycle of the mice. The presence of estrus cycle disorder indicates the successful preparation of the immune POI model.

2.5.2. Animal grouping and intervention

10 mice were randomly selected as the blank group, and the remaining 50 mice were divided into modeling group. After modeling, the mice were randomly divided into model group, positive group, GNLHD high, medium, and low dose groups, with 10 mice in each group. The concentration of GNLHD is converted to 1:2:4 times the clinical adult dose according to the "Equivalent Metering Table for Conversion of Human and Animal Body Surface Areas". The drug concentrations of the low, medium and high dose groups were 0.54

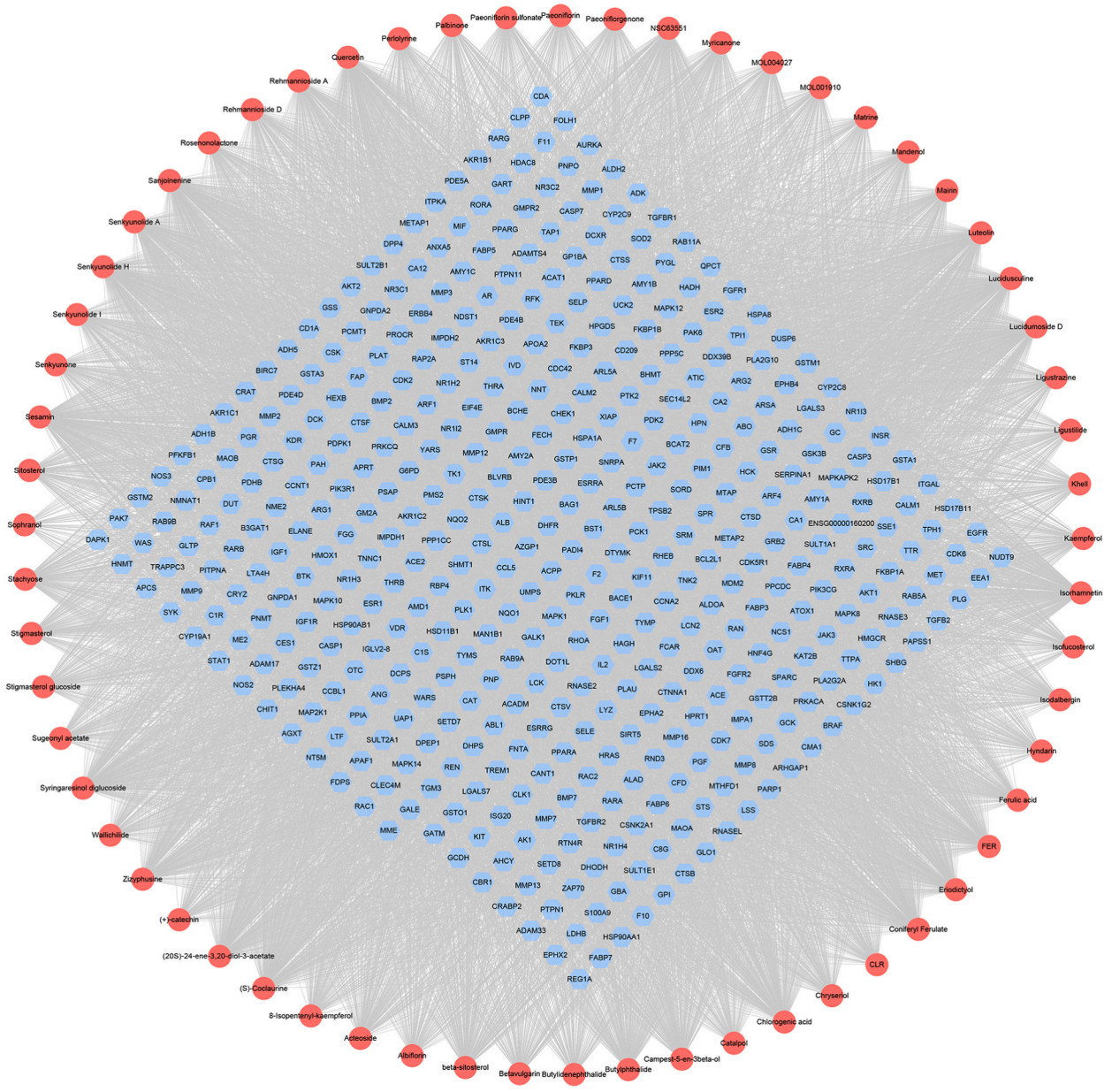

Fig. 2. Component-target network of GNLHD (Red circle stands for GNLHD component. Blue hexagon stands for GNLHD targets). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

g/mL, 1.08 g/mL, 2.16 g/mL of crude drug, respectively. The positive group of estradiol valerate tablets is 1 mg/tablet, and adults take 1 mg per day. After conversion, it is configured to be 0.01 mg/mL.

After the preparation of the model is completed, they will be reared routinely for 1 week. The administration was started from the second mouth, and gastric administration was adopted. The blank group and the model group were given 0.3 mL of normal saline. The positive group was given 0.3 mL of estradiol valerate aqueous solution. GNLHD high, medium and low dose groups were given 0.3 mL of the same concentration of concentrate. The administration lasted 30 days.

2.5.3. Sample collection

Blood was taken from the mice under anesthesia, and then they were sacrificed by cervical dislocation. The ovarian tissues on both sides of the mouse were taken out, weighed and recorded. One side of the ovary was fixed in an EP tube with 4 % paraformaldehyde solution, paraffin sections were made in the later stage, and HE staining and immunohistochemical detection were performed. The ovarian tissue on the other side was quick-frozen and stored in a −80 °C refrigerator, waiting for subsequent testing. Similarly, the spleen, thymus, etc. are taken out, weighed, recorded, and the organ index is calculated (organ index = organ mass/body mass).

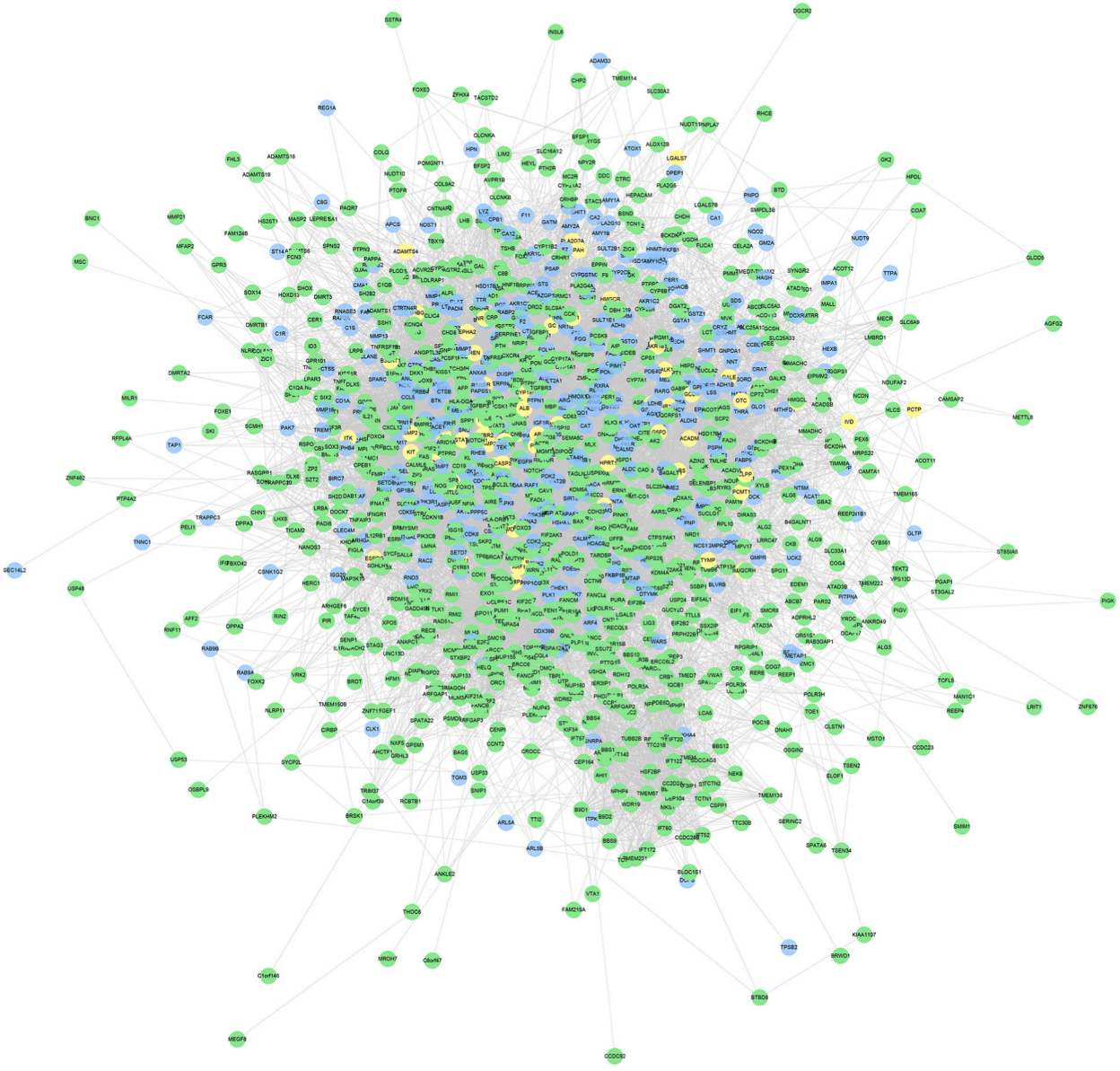

Fig. 3. GNLHD-POI PPI Network (Green, blue, yellow nodes stand for GNLHD targets, POI genes and GNLHD-POI targets, res.). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.5.4. Serum E2, FSH, LH level detection

Serum E2, FSH, and LH levels were tested strictly in accordance with the instructions of the ELISA kit. The linear regression equation of the standard curve was calculated based on the concentration of the standard in the corresponding kit and the corresponding absorbance (OD) value, and then based on the calculated sample OD value, the corresponding sample concentration was calculated on the regression equation.

2.5.5. Detection of PI3K, AKT1, MTOR protein expression in ovarian tissue

Immunohistochemistry was used to detect the protein expression of PI3K, AKT1, and MTOR in ovarian tissue. After dewaxing, hydration, inactivation, and antigen retrieval of ovarian tissue, the primary antibody was added to the humidified chamber at 4 °C and incubated overnight, and then the secondary antibody was added. Afterwards, DAB color development was carried out. After mounting the slide, under an optical microscope, the granular cells in the tissue can be observed as brown or brown, which is the positive expression area. The MIAS medical image analysis software is used to analyze the pictures. Five fields of view are selected for each slide to measure the average gray density value and the average optical density value.

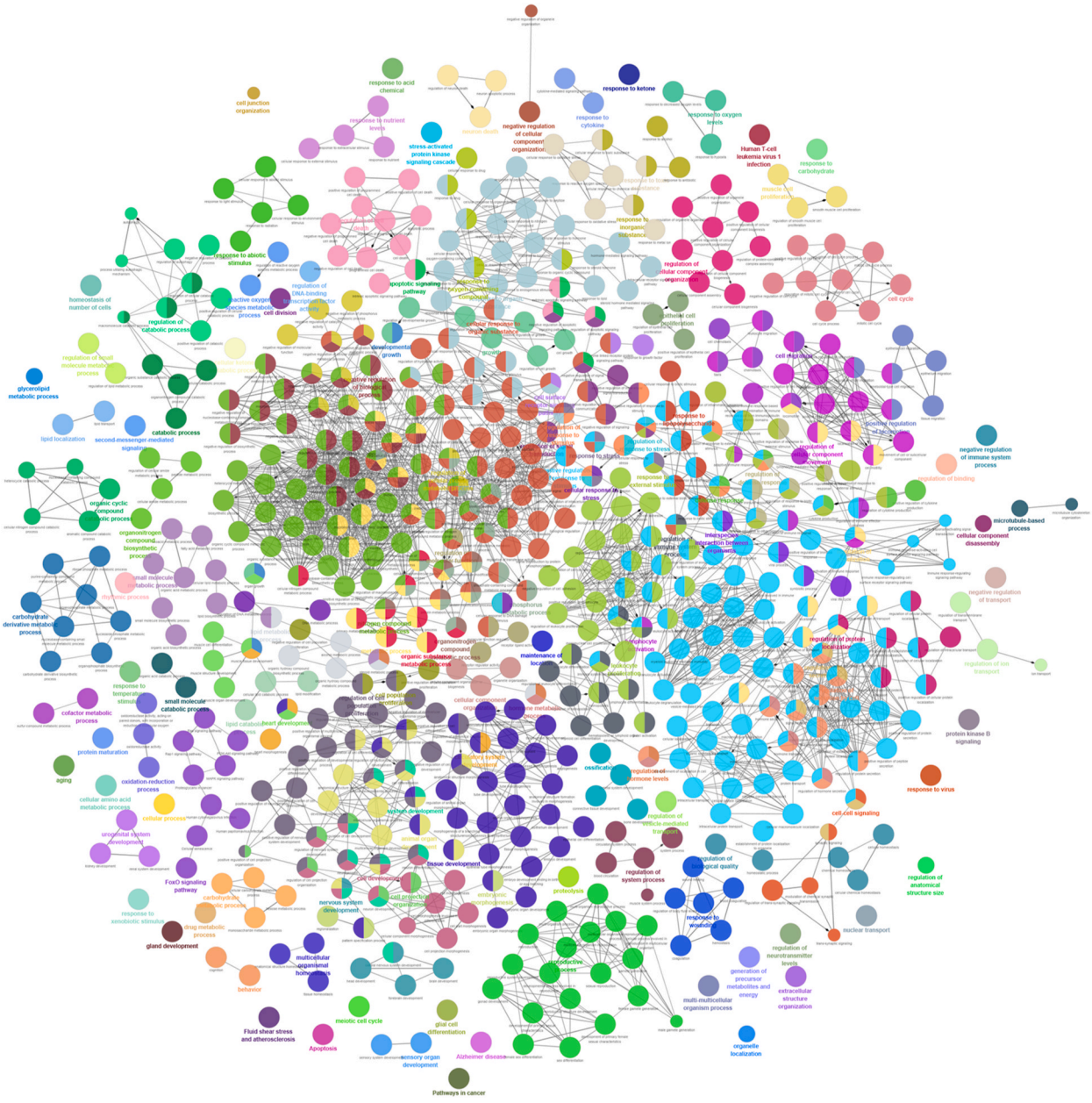

Fig. 4. The preliminary enrichment results.

2.6. Statistical analysis

The data of this study are selected SPSS22.0 version software for statistics and analysis, and expressed by mean ± SD. If the measurement data conforms to the normal distribution and the homogeneity of variance, a one-way analysis of variance was used; Otherwise, Tamhane's T2 method was used. When $P < 0.05$, the difference was considered to be statistically significant.

3. Results

3.1. GNLHD-POI PPI network analysis

A total of 61 components and 433 targets of GNLHD, and 1372 POI genes were obtained. The Component-target network of GNLHD were shown in Fig. 2. The relationship among GNLHD targets and POI genes were showed in Fig. 3. There were 59 interconnectable genes (GNLHD-POI targets) in GNLHD targets and POI genes. The top 20 GNLHD-POI targets were: AKT1 (334 edges), ALB (313 edges), ESR1 (231 edges), CASP3 (215 edges), HRAS (205 edges), IGF1 (199 edges), MDM2 (142 edges), AR (142 edges), IL2 (138 edges), STAT1 (134 edges), KIT (129 edges), PARP1 (121 edges), JAK2 (121 edges), KDR (119 edges), NOS3 (117 edges), MMP2 (116 edges), PGR (105 edges), NR3C1 (97 edges), CYP19A1 (96 edges), ESR2 (92 edges). The top 10 GNLHD targets were: EGFR (258 edges), HSP90AA1 (234 edges), SRC (229 edges), PPARG (173 edges), MMP9 (171 edges), CAT (163 edges), MAPK1 (162 edges), RHOA (159 edges), ANXA5 (148 edges), BCL2L1 (146 edges). The top 10 POI genes were: TP53 (360 edges), ACTB (343 edges), INS (313 edges), MYC (276 edges), TNF (276 edges), IL6 (264 edges), VEGFA (227 edges), STAT3 (220 edges), PTEN (211 edges), CD4 (194 edges). The preliminary enrichment results are shown in Fig. 4.

3.2. Enrichment analysis of GNLHD-POI PPI network

The targets and genes in GNLHD-POI PPI network were input into Metascape for enrichment analysis (Table S3). The biological processes include: response to hormone, cellular response to hormone stimulus, reproductive structure development, reproductive system development, response to peptide hormone, response to steroid hormone, gland development, regulation of hormone levels, regulation of cell adhesion, development of primary female sexual characteristics, negative regulation of cell population proliferation, response to growth factor, cellular response to growth factor stimulus, female gamete generation, gamete generation, hormone-

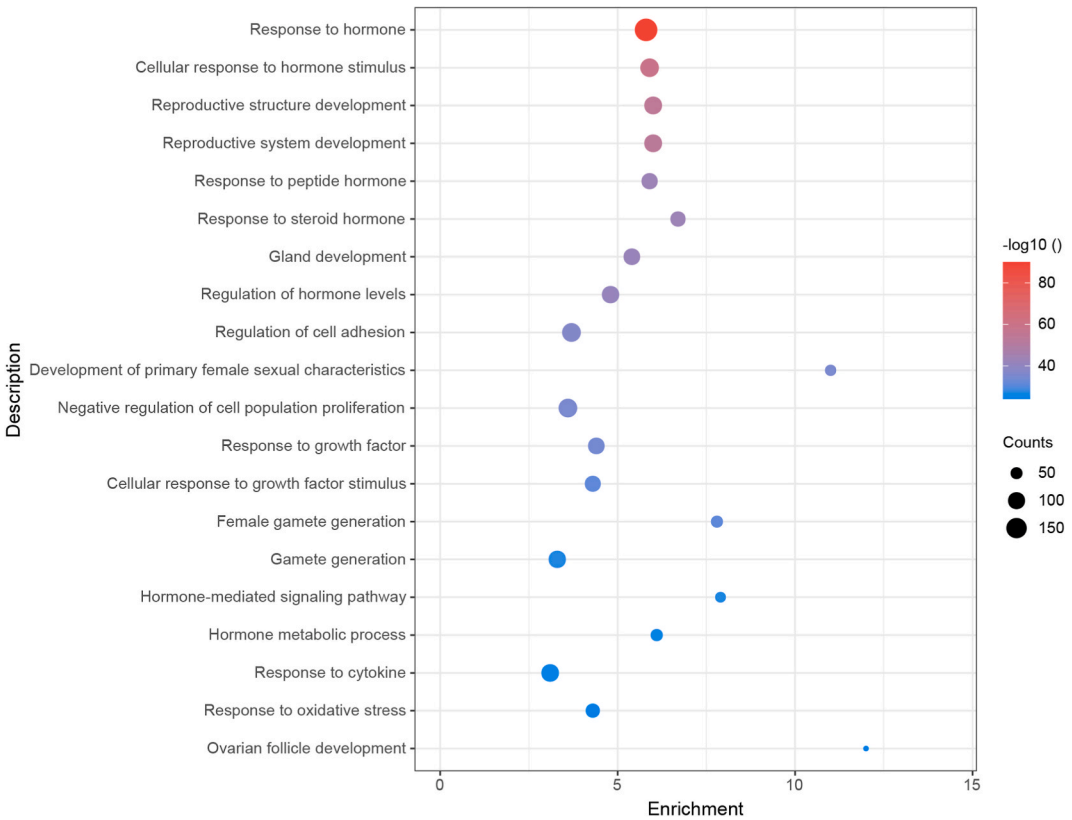

Fig. 5. Bubble chart of biological processes (X-axis stands for fold enrichment).

mediated signaling pathway, hormone metabolic process, response to cytokine, response to oxidative stress, ovarian follicle development (Fig. 5). The cell components include: vesicle lumen, cytoplasmic vesicle lumen, centrosome, secretory granule lumen, mitochondrial matrix, ciliary transition zone, extracellular matrix, external encapsulating structure, cilium, membrane raft, membrane microdomain, chromosomal region, collagen-containing extracellular matrix, ficolin-1-rich granule, ficolin-1-rich granule lumen, mitochondrial envelope, side of membrane, chromosome, telomeric region, mitochondrial membrane, perinuclear region of cytoplasm (Fig. 6). The molecular function include: kinase activity, phosphotransferase activity, alcohol group as acceptor, protein kinase activity, oxidoreductase activity, protein homodimerization activity, protein serine/threonine/tyrosine kinase activity, ligand-activated transcription factor activity, nuclear receptor activity, carboxylic acid binding, kinase binding, endopeptidase activity, protein kinase binding, peptidase activity, amide binding, lipid binding, protein domain specific binding, monocarboxylic acid binding, protein serine/threonine kinase activity, organic acid binding, transcription factor binding (Fig. 7). The signaling pathway include mTOR signaling pathway, PI3K-Akt signaling pathway, Ovarian steroidogenesis, Oocyte meiosis, Apoptosis, Prolactin signaling pathway, T cell receptor signaling pathway, Th17 cell differentiation, Estrogen signaling pathway, Progesterone-mediated oocyte maturation, JAK-STAT signaling pathway, Fc epsilon RI signaling pathway, Focal adhesion, HIF-1 signaling pathway, Autophagy - animal, PPAR signaling pathway, Cytokine-cytokine receptor interaction, Chemokine signaling pathway, Th1 and Th2 cell differentiation, Toll-like receptor signaling pathway (Fig. 8). The relationship among core signaling pathways and core targets were shown in Fig. 9. The role of GNLHD targets and POI genes in mTOR signaling pathways was shown in Fig. 10.

3.3. *Pathological changes of ovarian tissue*

In the blank group, the ovaries were normal, the internal structure of the tissues was clear, and normal follicle morphology could be observed under the microscope (Figs. 11A and 12). In the model group, the ovarian tissue structure is relatively disordered, the number of mature follicles in the ovary is greatly reduced, a small number of primary follicles can be seen, and the number of atretic follicles increases significantly (Figs. 11B and 12). In the GNLHD high-dose group, the ovarian tissue structure was relatively normal, a large number of primary follicles could be observed, and the number of atretic follicles was significantly reduced (Figs. 11C and 12). In the GNLHD medium-dose group, the number of primary and mature follicles in the ovarian tissue was relatively increased, while the number of atresia follicles was relatively reduced (Figs. 11D and 12). In the GNLHD low-dose group, the ovarian tissue morphology has a certain change, which is better than the model group, and multiple atretic follicles could be observed (Figs. 11E and 12). In the positive group, the morphology of the ovarian tissue was better improved, the number of primary follicles increased, and a small

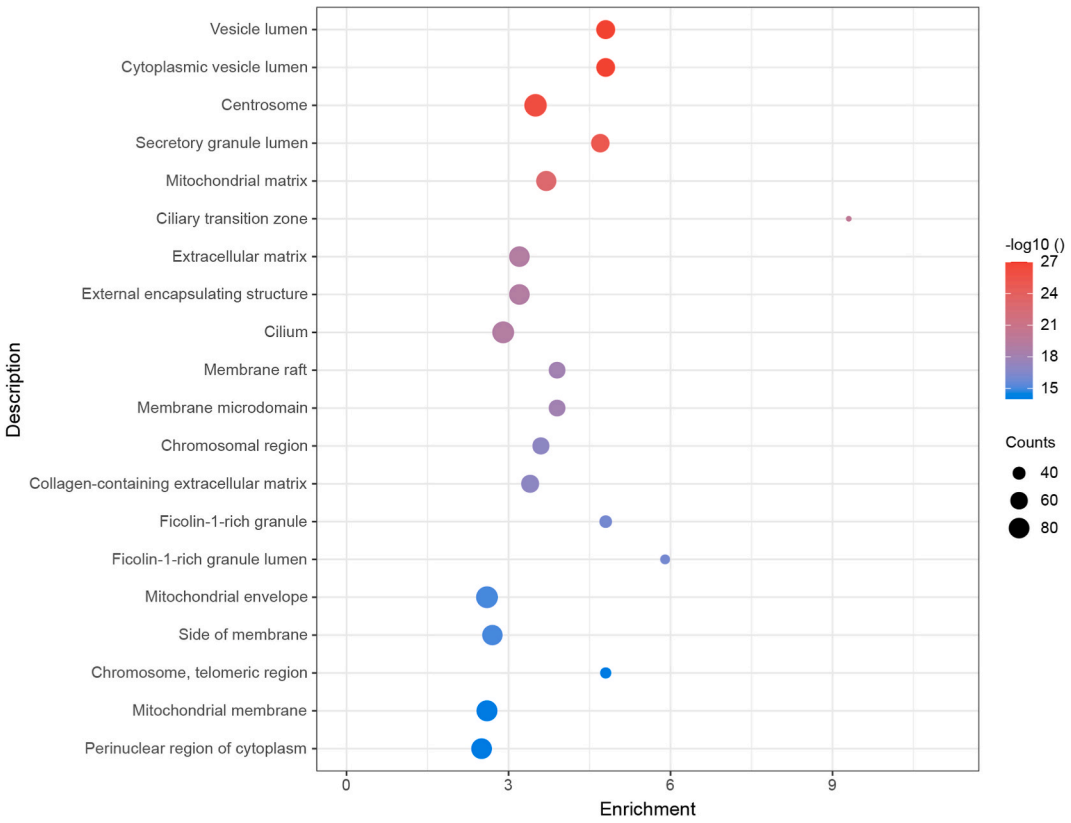

Fig. 6. Bubble chart of cell components (X-axis stands for fold enrichment).

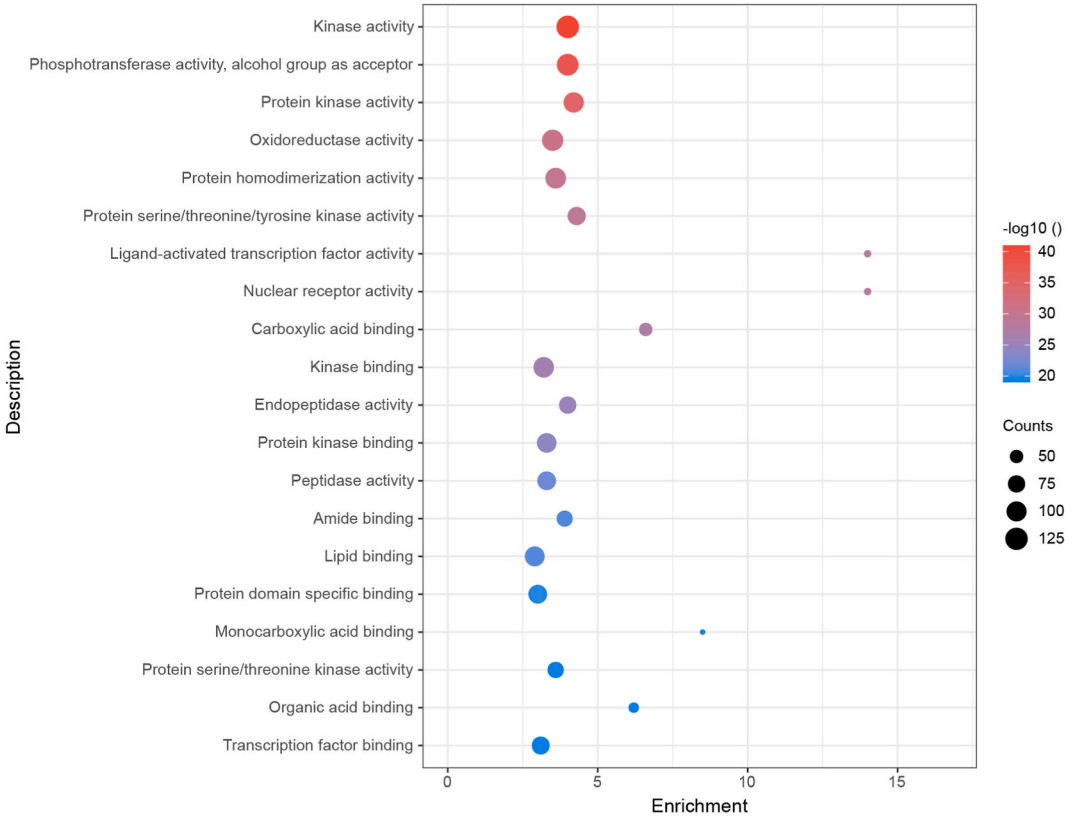

Fig. 7. Bubble chart of molecular functions (X-axis stands for fold enrichment).

amount of atretic follicles could be observed (Figs. 11F and 12).

3.4. Effect of GNLHD on serum E2, FSH, LH level

Compared with the blank group, the E2 level of the model group decreased, and the FSH and LH levels increased ($P < 0.05$). Compared with the model group, the E2 level of GNLHD groups and positive group increased, while the FSH and LH levels decreased, and the GNLHD high-dose group had the best effect ($P < 0.05$) (Fig. 13).

3.5. Effect of GNLHD on expression of PI3K, AKT1, MTOR protein in ovarian tissue

Compared with the blank group, the expression of PI3K, AKT1 and MTOR protein in the ovarian tissue of the model group was significantly different ($P < 0.05$). Compared with the model group, the PI3K and AKT1 proteins of the positive group, GNLHD high and medium dose groups were all increased ($P < 0.05$). Compared with the model group, the PI3K and AKT1 proteins of the GNLHD low-dose group were not significantly different ($P > 0.05$). Compared with the model group, the MTOR of the positive group, GNLHD high, medium, and low dose groups were significantly increased ($P < 0.05$) (Figs.14-17).

4. Discussion

In recent years, the incidence of premature ovarian insufficiency has been steadily rising, increasing the risk of conditions like osteoporosis, diabetes, hypertension, cardiovascular diseases, and cerebrovascular diseases, significantly impacting the physical and mental health as well as the quality of life of women of childbearing age [31]. Currently, hormone replacement therapy (HRT) is the primary treatment method for POI both domestically and internationally. HRT aids in regulating the hypothalamic-pituitary-ovarian (HPO) axis function by replenishing the deficient sex hormones in the body. However, discontinuing HRT often leads to recurrence and elevates the risk of breast cancer, endometrial cancer, stroke, and other associated diseases. Long-term management and treatment of POI have certain limitations and contraindications [32]. Traditional Chinese medicine (TCM) has shown effectiveness in regulating women's endocrine function. Several studies have demonstrated that TCM can enhance ovarian function recovery through its estrogen-mimicking effects, regulate endocrine function, and maintain the balance of the kidney, "tiangui," "chongren," and uterine axis functions. This approach aligns somewhat with modern medical HRT treatment for POI, offering fewer adverse reactions and

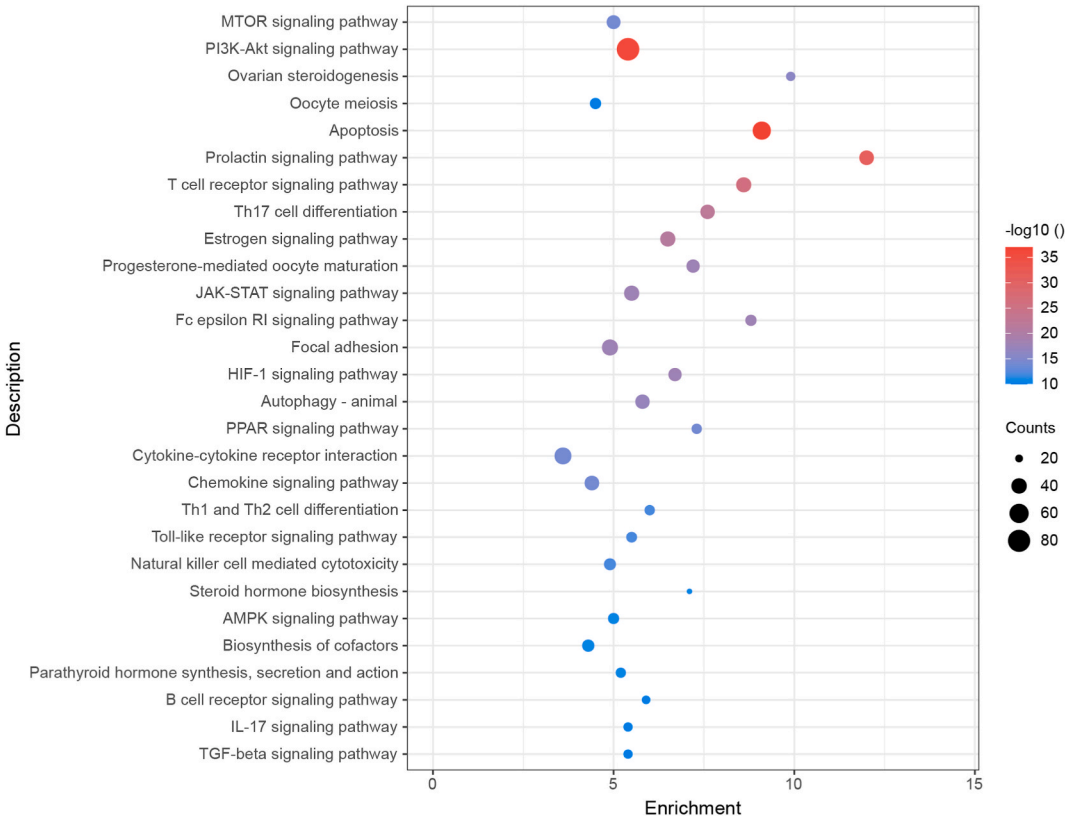

Fig. 8. Bubble chart of signaling pathways (X-axis stands for fold enrichment).

lower costs, making it more readily accepted by patients in clinical settings [33–35]. GNLHD is one of the commonly used basic prescriptions for the treatment of gynecological diseases, which is composed of *Lycii Fructus* 15 g, *Ligustri Lucidi Fructus* 15 g, *Cuscutae Semen* 30 g, *Cistanches Herba* 10 g, *Angelicae Sinensis Radix* 15 g, *Chuanxiong Rhizoma* 10 g, *Paeoniae Radix Alba* 10 g, *Rehmanniae Radix Praeparata* 30 g, *Ziziphi Spinosae Semen* 10 g, *Cyperi Rhizoma* 10 g, *Cyathulae Radix* 10 g.

Modern pharmacological studies have demonstrated the ovarian protective effects of Lycium barbarum polysaccharide in a cisplatin chemotherapy-induced premature ovarian insufficiency (POI) model [36]. Sun et al. identified that Lycium barbarum polysaccharide may ameliorate lipid metabolism disorders by upregulating the expression of *p*-GSK-3β and PPARγ in the liver [37]. Huang et al. observed that Lycium barbarum polysaccharide could enhance sexual cycle regulation in mice, decrease serum anti-zona pellucida antibody and FSH concentrations, and elevate serum E2 concentration, indicating its protective effects against autoimmune premature ovarian failure [38]. Wei et al. reported that Lycium barbarum polysaccharide significantly increased estrogen, progesterone, and IGF-I levels while decreasing IGFBP-1 levels in naturally aging female rats, suggesting a potential role in resisting ovarian function decline [39].

The primary pharmacological action of Ligustri Lucidi Fructus lies in its constituents having estrogen-like effects [40]. Ongoing pharmacological investigations have revealed that Ligustri Lucidi Fructus can stimulate bone, muscle, and body mass growth while enhancing the body's defense function [41]. Its mechanisms of growth promotion and physique enhancement may involve insulin-like effects, estrogen-like effects, and the promotion of cell differentiation, showing promise for addressing slow growth and wasting diseases in children [42–44]. Regarding ovarian follicle regulation, Ligustri Lucidi Fructus decoction significantly boosts the proliferation activity of ovarian cells under heat stress, reduces cell apoptosis rates, increases SOD activity in cell culture supernatants, and enhances the antioxidant function of ovarian cells [45].

Cuscutae Semen is predominantly utilized for treating female conditions such as infertility, premature ovarian failure, and recurrent miscarriage [46]. Current research indicates that the main active substances of Cuscutae Semen, total flavonoids, and quercetin can partially enhance ovarian function in a Tripterygium wilfordii-induced premature ovarian failure model [47]. Wang et al. highlighted the significant restorative effects of Cuscuta chinensis total flavonoids on rats with chemotherapy-induced premature ovarian failure, leading to increased ovarian weight and follicle numbers, elevated estrogen levels, and notable therapeutic effects on premature ovarian failure [48]. Li et al. demonstrated that quercetin can prevent primordial follicle depletion induced by cyclophosphamide by inhibiting the PI3K/AKT/FOXO3a pathway, offering novel therapeutic approaches to mitigate chemotherapy side effects in cancer patients with premature ovarian failure and infertility [49]. Adam et al. explored the potential of quercetin in preventing or counteracting xylene effects on ovarian cells, indicating a possible role in safeguarding female reproduction from

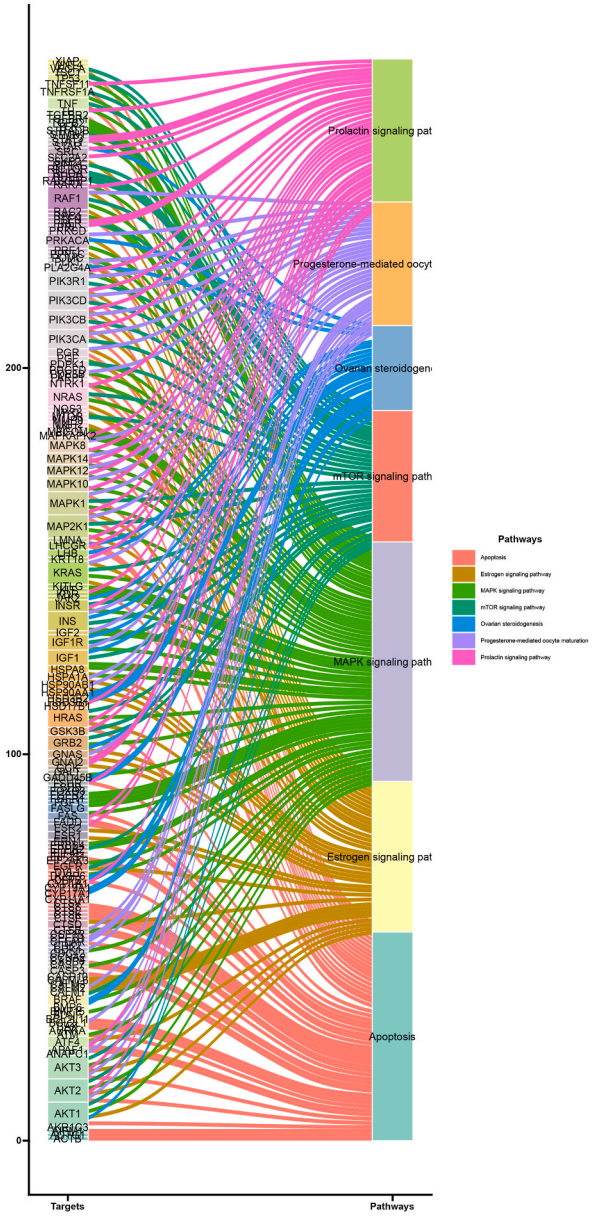

Fig. 9. The relationship among core signaling pathways and core targets.

xylene-related risks [50]. Alexandre et al. noted quercetin's direct impact on inhibiting basal ovarian cell function and responses to FSH stimulation [51]. Yi et al. discovered that chlorogenic acid could mitigate zearalenone-induced injury-mediated apoptosis by decreasing the protein expression of cleaved caspase-3, altering the Bax/Bcl-2 ratio, and modifying cleaved-PARP levels [52]. Li et al. revealed that isorhamnetin enhances estrogen secretion while inhibiting progesterone and testosterone secretion by regulating steroidogenic proteins and mRNAs like CYP19A1, StAR, and 3β-HSD [53]. Tang et al. demonstrated luteolin's effective promotion of estradiol secretion from ovarian granulosa cells without influencing normal cell differentiation but influencing their proliferation to some extent [54]. Zhang et al. found that luteolin could alleviate bisphenol A-induced ovarian toxicity in mice potentially through modulation of the p38 MAPK and ERK signaling pathways [55].

PPI network analysis found that GNLHD may play a therapeutic role through AKT1, ESR1, CASP3, HRAS, IGF1, MDM2, AR, IL2, STAT1, KIT, PARP1, JAK2, KDR, NOS3, MMP2, PGR, NR3C1, CYP19A1, ESR2 and so on. Current studies have found that AKT1 is involved in mediating the downstream mTOR signaling pathway and its FOXP3 signaling pathway, which plays an important role in the development of ovarian follicles [56,57]. For example, conditional knockout (cKO) of Mtor results in differential effects on oocyte quality, granulosa cell fate, and follicular development [58]. In non-growing primordial oocytes, cKO of Mtor causes defects in follicular development, resulting in progressive oocyte degeneration and loss of granulosa cell properties [58]. Increased expression of

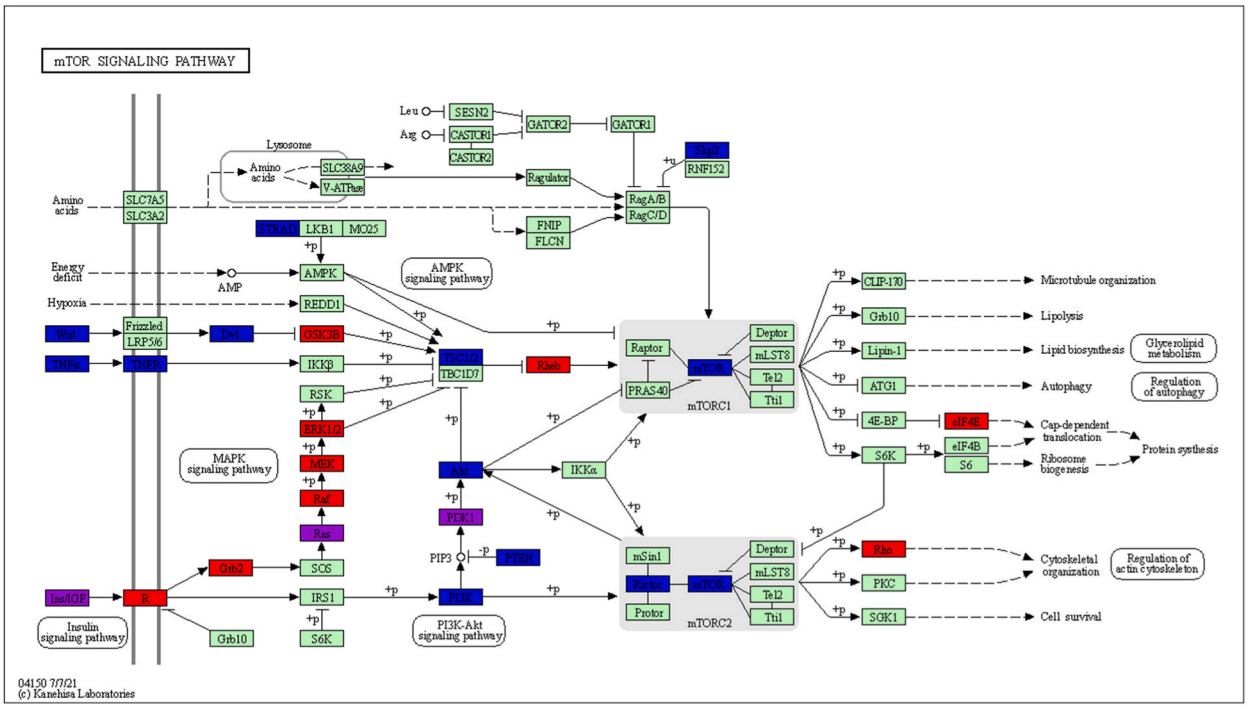

Fig. 10. KEGG mapper modified from hsa 04150 (POI genes marked in blue; GNLHD targets marked in red; GNLHD-POI targets marked in purple). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

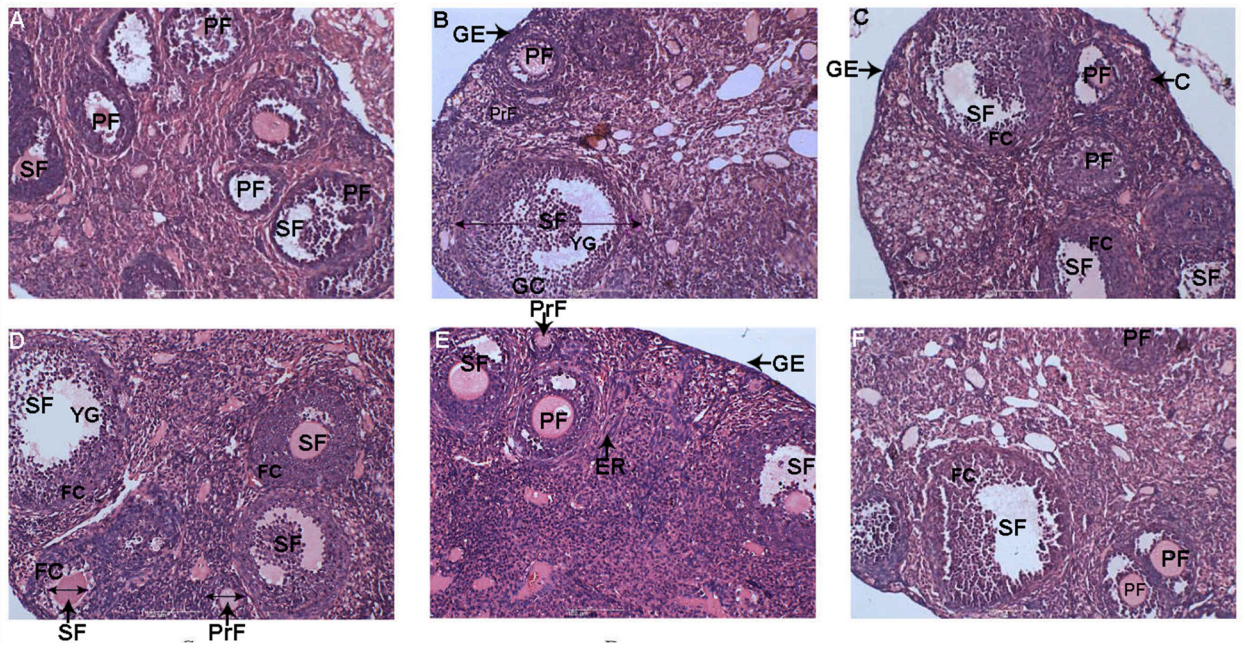

Fig. 11. Pathological changes of ovarian tissue (A: blank group; B: Model group; C: GNLHD high-dose group; D: GNLHD medium-dose group; E: GNLHD low-dose group; F: positive group. PrF: primordial follicle; GE: germinal epithelium; PF: primary follicle; YG: yolk granules; SF: secondary follicle; ER: extraovarian rete; C: cortex; FC: follicular cell layer).

steroid synthesis-regulating genes such as ESR1, IGF1, AR, PGR, CYP19A1, and ESR2 also plays an important role in follicular development at all levels [59,60]. These steroid hormones can activate extracellular signal-regulated protein kinase (ERK1/2), PI3K and other downstream signaling pathways, and cooperate with FSH to effectively reduce the apoptosis of ovarian granulosa cells and

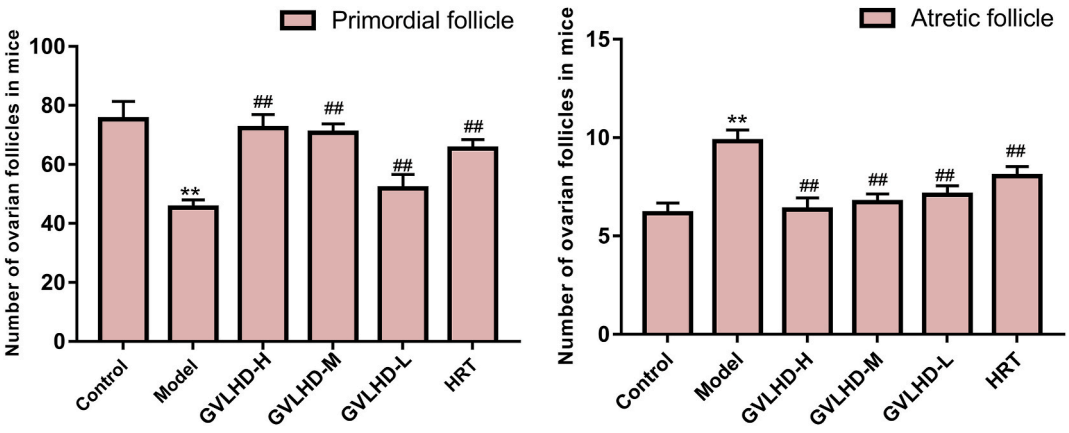

Fig. 12. The number of follicles (**Compared with blank group, $P < 0.05$; ##compared with model group, $P < 0.05$).

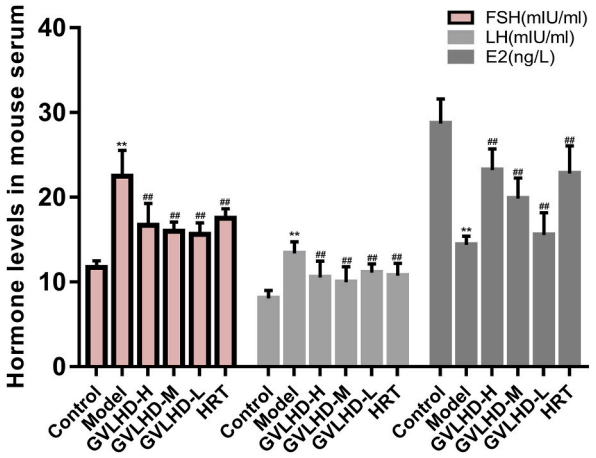

Fig. 13. Effect of GNLHD on serum E2, FSH, LH level (**Compared with blank group, $P < 0.05$; ##compared with model group, $P < 0.05$).

reduce the damage of ovarian granulosa cells, and enhance the body's antioxidant capacity, provide an appropriate environment for the growth, maturation and development of oocytes, and protect ovarian granulosa cells and oocytes [61–63]. The normal expression of NOS3 can improve the endometrial receptivity to a certain extent, increase the blood supply of the local tissue of the endometrium, thereby improving the reproductive function of patients with premature ovarian insufficiency [64]. It can be seen that GNLHD may play a role in the treatment of POI by enhancing the antioxidant capacity of cells, reducing apoptosis, regulating immunity, and promoting fertility through the above core targets.

The enrichment analysis results showed that GNLHD may regulate response to hormone, response to oxidative stress, ovarian follicle development, cellular response to hormone stimulus, reproductive structure development, reproductive system development, mTOR signaling pathway, PI3K-Akt signaling pathway, Ovarian steroidogenesis, Oocyte meiosis and so on. Current studies have shown that FSH has the ability to regulate anti-oxidative stress in antral follicles, and its content is positively correlated with total oxidant status and Oxidative Stress index. When the antioxidant defense mechanism upregulated by FSH is unbalanced and withdrawn, or when exposed to reactive oxygen species (ROS), ROS will further activate downstream information molecules through the OS process, causing follicular atresia and reducing follicle quality and quantity. At the same time, too much hydrogen peroxide in the body will seriously inhibit the sensitivity of LH and the production of steroids, resulting in the reduction of E2 secretion by granulosa cells, which further leads to the destruction of ovarian function from the upstream [65–67]. When the body is in the state of Oxidative Stress, adding antioxidant substances can significantly increase the activities of CAT and superoxide dismutase (SOD), and reduce the level of ovarian ROS, thereby protecting the normal function of the ovary and reducing the occurrence of POI [68,69].

Mechanistic analysis of key signaling pathways revealed that mTOR signaling pathway is one of the core pathways for GNLHD treatment of POI. Studies have found that there is a large amount of mTOR expression in oocytes, and the mTOR signaling pathway is involved in the regulation of oocyte growth, primordial follicle development, and granulosa cell proliferation and differentiation [70]. When mTOR is knocked out, primordial follicles can develop prematurely and undergo rapid apoptosis, leading to the occurrence of POI [71]. mTOR is a serine-threonine protein kinase downstream of PI3K/AKT. Once AKT is regulated by upstream PI3K, it can regulate mTOR and other downstream molecules and sites that regulate follicle development [72]. If the mTOR pathway is disturbed, it

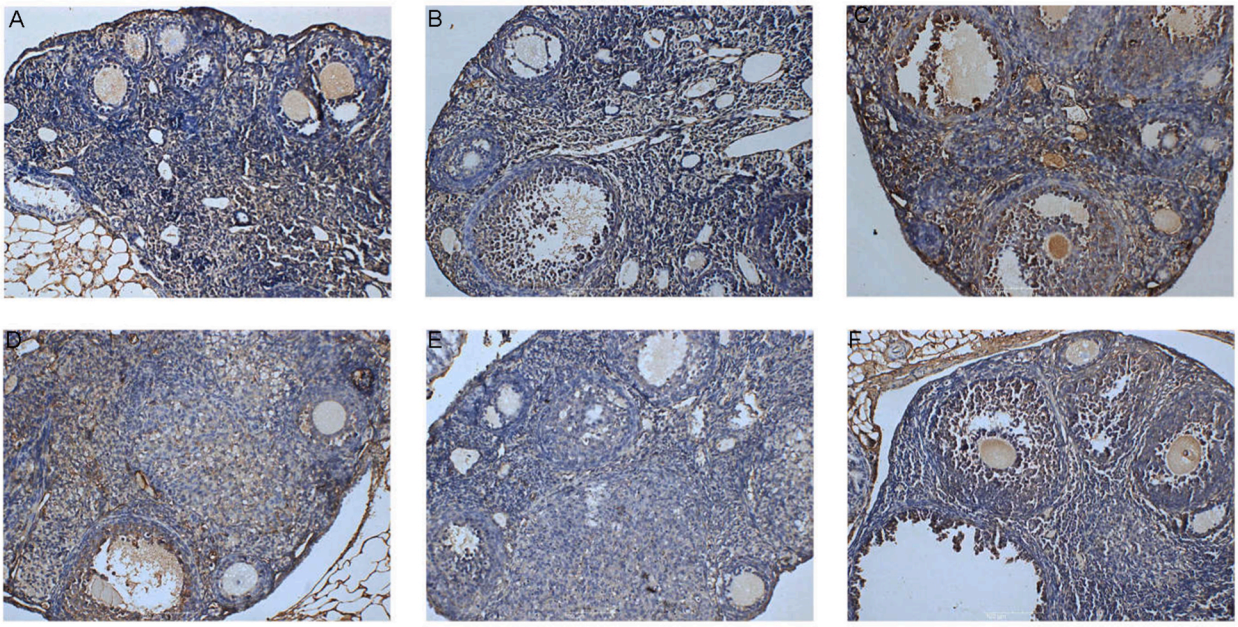

Fig. 14. Effect of GNLHD on Expression of PI3K protein in ovarian tissue (A: blank group; B: Model group; C: GNLHD high-dose group; D: GNLHD medium-dose group; E: GNLHD low-dose group; F: positive group).

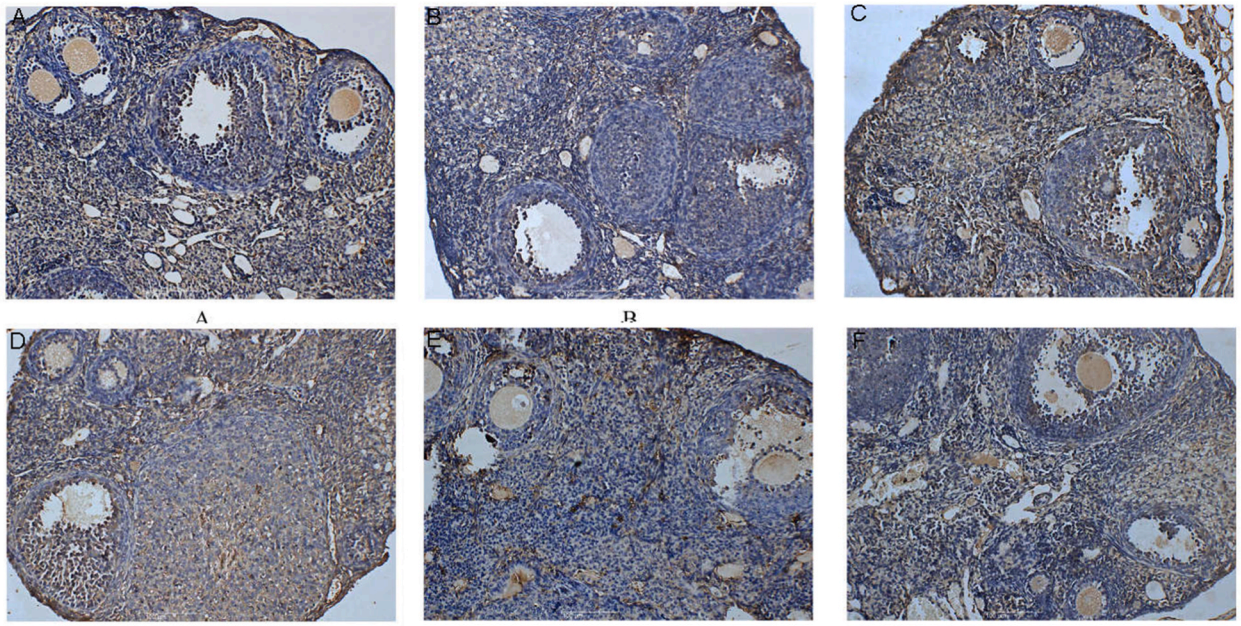

Fig. 15. Effect of GNLHD on Expression of AKT1 protein in ovarian tissue (A: blank group; B: Model group; C: GNLHD high-dose group; D: GNLHD medium-dose group; E: GNLHD low-dose group; F: positive group).

may be associated with abnormal follicles [73].

This study observed a relatively disordered structure of mouse ovarian tissue, with a significant decrease in the number of mature follicles and a notable increase in atretic follicles. Additionally, serum E2 levels were reduced while FSH and LH levels increased, confirming the success of the modeling. The decreased E2 level in the model group impacted the expression of PI3K, AKT1, and MTOR proteins. Following treatment with GNLHD, the ovarian tissue function in mice gradually recovered, with an increase in mature follicles and the presence of numerous primary follicles. The expression of PI3K, AKT1, and MTOR proteins gradually elevated, serum E2 levels began to rise, and FSH and LH levels decreased. These findings indicate that GNLHD can enhance the internal environment of

ovarian tissue and gradually restore ovarian tissue function, effectively regulating ovarian endocrine function and exhibiting potential in the treatment of POI.

5. Conclusion

Based on the related methods of network pharmacology, this study combined modern biological information technology and TCM theory to analyze the compounds, active ingredients, targets and signaling pathways of GNLHD, and preliminarily discussed the mechanism of GNLHD in the treatment of POI. Through animal experiments, it was found that it may improve the ovarian function by increasing the expression level of E2, reducing the expression level of FSH and LH, regulating the expression of mTOR protein, promoting the growth and development of follicles, and maintaining the stability of the ovarian internal environment.

Ethics approval and consent to participate

Animal experiments were performed in accordance with the guidelines of the Animal Ethics Committee of Hunan University of Chinese Medicine and the guidelines for the care and use of experimental animals (Approve No. LL20170702).

Consent for publication

Not applicable.

Data availability statement

Data included in article/supp. material/referenced in article.

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CRediT authorship contribution statement

Kailin Yang: Writing – original draft, Software, Formal analysis, Data curation, Conceptualization. **Lingyu Wu:** Methodology, Formal analysis, Data curation. **Liuting Zeng:** Writing – original draft, Methodology, Formal analysis, Data curation,

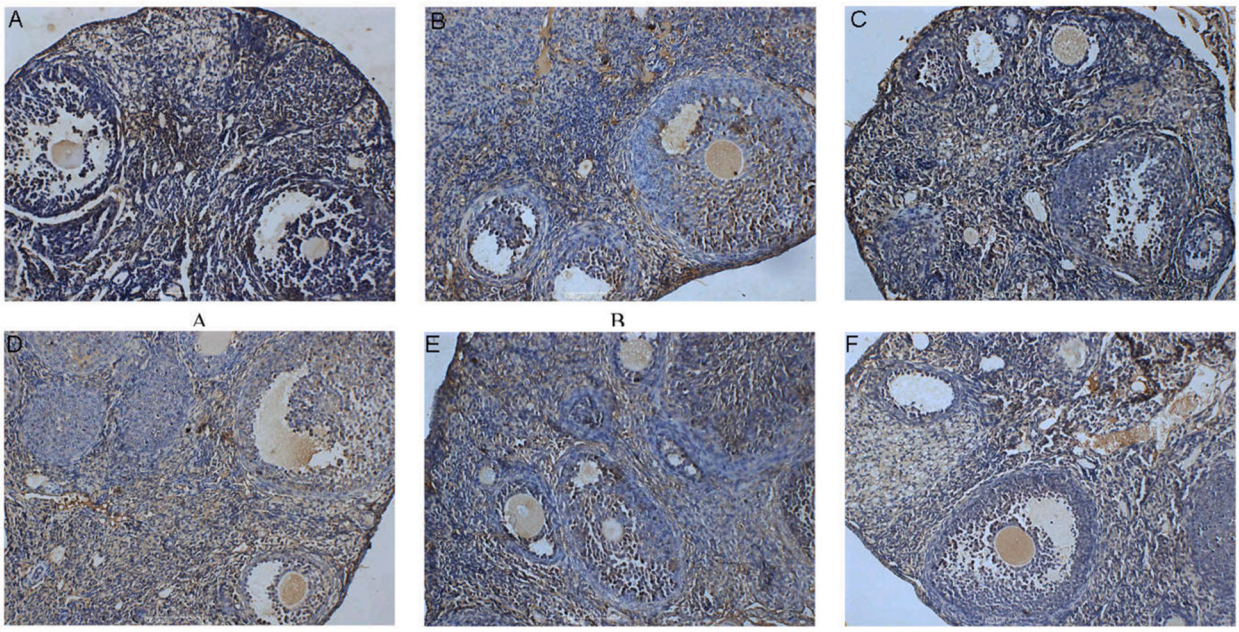

Fig. 16. Effect of GNLHD on Expression of mTOR protein in ovarian tissue (A: blank group; B: Model group; C: GNLHD high-dose group; D: GNLHD medium-dose group; E: GNLHD low-dose group; F: positive group).

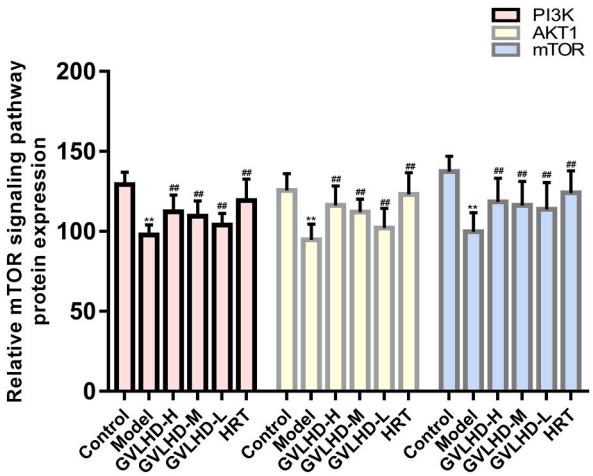

Fig. 17. Expression of PI3K, AKT1, MTOR protein in ovarian tissue (**Compared with blank group, $P < 0.05$; ##compared with model group, $P < 0.05$).

Conceptualization. **Wang Xiang:** Validation, Formal analysis, Data curation. **Junpeng Chen:** Data curation, Methodology, Resources. **Yexing Yan:** Data curation, Methodology, Software. **Moujia Hao:** Data curation, Methodology, Software. **Tian Song:** Data curation, Methodology, Software. **Enjian Zhai:** Validation, Formal analysis, Data curation. **Guomin Zhang:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Huiping Liu:** Writing – review & editing, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33105.

References

[1] J.S. Laven, Primary ovarian insufficiency, Semin. Reprod. Med. 34 (4) (2016) 230–234, https://doi.org/10.1055/s-0036-1585402.
[2] S.D. Sullivan, P.M. Sarrel, L.M. Nelson, Hormone replacement therapy in young women with primary ovarian insufficiency and early menopause, Fertil. Steril. 106 (7) (2016) 1588–1599, https://doi.org/10.1016/j.fertnstert.2016.09.046.
[3] S. Tsiligiannis, N. Panay, J.C. Stevenson, Premature ovarian insufficiency and long-term health consequences, Curr. Vasc. Pharmacol. 17 (6) (2019) 604–609, https://doi.org/10.2174/1570161117666190122101611.
[4] S. Jaillard, K. Bell, L. Akloul, K. Walton, K. McElreavy, W.A. Stocker, M. Beaumont, C. Harrisson, T. Jääskeläinen, J.J. Palvimo, G. Robevska, E. Launay, A. P. Satié, N. Listyasari, C. Bendavid, R. Sreenivasan, S. Duros, J. van den Bergen, C. Henry, M. Domin-Bernhard, L. Cornevin, N. Dejucq-Rainsford, M.A. Belaud-Rotureau, S. Odent, K.L. Ayers, C. Ravel, E.J. Tucker, A.H. Sinclair, New insights into the genetic basis of premature ovarian insufficiency: novel causative variants and candidate genes revealed by genomic sequencing, Maturitas 141 (2020) 9–19, https://doi.org/10.1016/j.maturitas.2020.06.004.
[5] P. Touraine, Premature ovarian insufficiency: step-by-step genetics bring new insights, Fertil. Steril. 113 (4) (2020) 767–768, https://doi.org/10.1016/j.fertnstert.2019.12.032.
[6] L. Yeganeh, J.A. Boyle, A. Wood, H. Teede, A.J. Vincent, Menopause guideline appraisal and algorithm development for premature ovarian insufficiency, Maturitas 130 (2019) 21–31, https://doi.org/10.1016/j.maturitas.2019.09.009.
[7] R. Giri, A.J. Vincent, Prevalence and risk factors of premature ovarian insufficiency/early menopause, Semin. Reprod. Med. 38 (4–05) (2020) 237–246, https://doi.org/10.1055/s-0040-1722317.
[8] C.L. Benetti-Pinto, H.P. Giraldo, A.E. Giraldo, T.A. Mira, D.A. Yela, Interferential current: a new option for the treatment of sexual complaints in women with premature ovarian insufficiency using systemic hormone therapy: a randomized clinical trial, Menopause 27 (5) (2020) 519–525, https://doi.org/10.1097/GME.0000000000001501.

[9] J. Paciuc, Hormone therapy in menopause, Adv. Exp. Med. Biol. 1242 (2020) 89–120, https://doi.org/10.1007/978-3-030-38474-6_6.
[10] K. Maclaran, N. Panay, Current concepts in premature ovarian insufficiency, Womens Health (Lond). 11 (2) (2015) 169–182, https://doi.org/10.2217/whe.14.82.
[11] P. Anagnostis, S.A. Paschou, N. Katsiki, D. Krikidis, I. Lambrinoudaki, D.G. Goulis, Menopausal hormone therapy and cardiovascular risk: where are we now? Curr. Vasc. Pharmacol. 17 (6) (2019) 564–572, https://doi.org/10.2174/1570161116666180709095348.
[12] Jie Shen, Research on the Medication Rule of Premature Ovarian Failure Based on Ancient and Modern Literature [D], Nanjing University of Traditional Chinese Medicine, 2013 (in chinese).
[13] Huici Ye, Research and Clinical Practice of Prescription and Treatment of Premature Ovarian Failure Based on Data Analysis of Modern Chinese Medicine Literature [D], Guangzhou University of Traditional Chinese Medicine, 2017 (in chinese).
[14] Qihu Dong, The Clinical Evaluation and Mechanism of Xiangfu Siwu Decoction in the Treatment of Primary Dysmenorrhea Due to Qi Stagnation and Blood stasis [D], Nanjing University of Traditional Chinese Medicine, 2012 (in chinese).
[15] Shen Jun, Research Progress on Treatment of Irregular Menstruation with Siwu Decoction [D], Beijing University of Traditional Chinese Medicine, 2008 (in chinese).
[16] K. Yang, L. Zeng, T. Bao, Z. Long, B. Jin, Exploring the pharmacological mechanism of quercetin-resveratrol combination for polycystic ovary syndrome: a systematic pharmacological strategy-based research, Sci. Rep. 9 (1) (2019 Dec 5) 18420, https://doi.org/10.1038/s41598-019-54408-3. Erratum in: Sci Rep. 2020,10(1):3002.
[17] S.Y. Zhang, K.L. Yang, Z.Y. Long, W.Q. Li, H.Y. Huang, Use of a systematic pharmacological methodology to explore the mechanism of shengmai powder in treating diabetic cardiomyopathy, Med Sci Monit 26 (2020) e919029, https://doi.org/10.12659/MSM.919029.
[18] K. Yang, L. Zeng, A. Ge, X. Pan, T. Bao, Z. Long, Q. Tong, M. Yuan, X. Zhu, J. Ge, Z. Huang, Integrating systematic biological and proteomics strategies to explore the pharmacological mechanism of danshen yin modified on atherosclerosis, J. Cell Mol. Med. 24 (23) (2020) 13876–13898, https://doi.org/10.1111/jcmm.15979.
[19] K. Yang, L. Zeng, A. Ge, Y. Shi, X. Zhu, W. Liu, J. Ge, The effect of HedysarummultijugumMaxim.-chuanxiong rhizoma compound on ischemic stroke: a research based on network and experimental pharmacology, Oxid. Med. Cell. Longev. 2020 (2020) 6072380, https://doi.org/10.1155/2020/6072380.
[20] X. Yuan, W. Xiang, K. Yang, H. Liu, G. Zhang, Q. He, J. Fan, Systematic pharmacology-based strategy to explore the molecular network mechanism of modified taohong Siwu decoction in the treatment of premature ovarian failure, Evid Based Complement Alternat Med 2022 (2022 Jan 21) 3044463, https://doi.org/10.1155/2022/3044463. PMID: 35096106; PMCID: PMC8799328.
[21] L. Zeng, K. Yang, L. Liu, T. Zhang, H. Liu, Z. Tan, L. Lei, Systematic biological and proteomics strategies to explore the regulation mechanism of Shoutai Wan on recurrent spontaneous Abortion's biological network, J. Ethnopharmacol. 263 (2020) 113156, https://doi.org/10.1016/j.jep.2020.113156.
[22] H. Liu, L. Zeng, K. Yang, G. Zhang, A network pharmacology approach to explore the pharmacological mechanism of xiaoyao powder on anovulatory infertility, Evid Based Complement Alternat Med 2016 (2016) 2960372, https://doi.org/10.1155/2016/2960372.
[23] J. Ru, P. Li, J. Wang, W. Zhou, B. Li, C. Huang, P. Li, Z. Guo, W. Tao, Y. Yang, X. Xu, Y. Li, Y. Wang, L. Yang, TCMSP: a database of systems pharmacology for drug discovery from herbal medicines, J. Cheminf. 6 (2014) 13, https://doi.org/10.1186/1758-2946-6-13.
[24] X. Liu, S. Ouyang, B. Yu, Y. Liu, K. Huang, J. Gong, S. Zheng, Z. Li, H. Li, H. Jiang, PharmMapper server: a web server for potential drug target identification using pharmacophore mapping approach, Nucleic Acids Res. 38 (Web Server issue) (2010) W609–W614, https://doi.org/10.1093/nar/gkq300.
[25] J. Piñero, J.M. Ramírez-Anguita, J. Saüch-Pitarch, F. Ronzano, E. Centeno, F. Sanz, L.I. Furlong, The DisGeNET knowledge platform for disease genomics: 2019 update, Nucleic Acids Res. 48 (D1) (2020) D845–D855, https://doi.org/10.1093/nar/gkz1021.
[26] A. Hamosh, A.F. Scott, J.S. Amberger, C.A. Bocchini, V.A. McKusick, Online Mendelian Inheritance in Man (OMIM), a knowledgebase of human genes and genetic disorders, Nucleic Acids Res. 33 (Database issue) (2005) D514–D517, https://doi.org/10.1093/nar/gki033.
[27] G. Stelzer, R. Rosen, I. Plaschkes, S. Zimmerman, M. Twik, S. Fishilevich, T. Iny Stein, R. Nudel, I. Lieder, Y. Mazor, S. Kaplan, D. Dahary, D. Warshawsky, Y. Guan - Golan, A. Kohn, N. Rappaport, M. Safran, D. Lancet, The GeneCards suite: from gene data mining to disease genome sequence analysis, Current Protocols in Bioinformatics 54 (3) (2016) 1, 30.1 - 1.30.33.
[28] D. Szklarczyk, FranceschiniA, S. Wyder, K. Forslund, D. Heller, J. Huerta-Cepas, STRING v10:protein-protein interaction networks, integrated over the tree of life, Nucleic Acids Res. 43 (1) (2015) D447–D452.
[29] G.D. Bader, C.W. Hogue, An automated method for finding molecular complexes in large protein interaction networks, BMC Bioinf. 4 (2003) 2, https://doi.org/10.1186/1471-2105-4-2.
[30] Y. Zhou, B. Zhou, L. Pache, M. Chang, A.H. Khodabakhshi, O. Tanaseichuk, C. Benner, S.K. Chanda, Metascape provides a biologist-oriented resource for the analysis of systems-level datasets, Nat. Commun. 10 (1) (2019) 1523, https://doi.org/10.1038/s41467-019-09234-6.
[31] S. Tsiligiannis, N. Panay, J.C. Stevenson, Premature ovarian insufficiency and long-term health consequences, Curr. Vasc. Pharmacol. 17 (6) (2019) 604–609, https://doi.org/10.2174/1570161117666190122101611.
[32] J.S. Laven, Primary ovarian insufficiency, Semin. Reprod. Med. 34 (4) (2016) 230–234, https://doi.org/10.1055/s-0036-1585402.
[33] Wenwen Yang, Clinical Study on the Treatment of Ovarian Function Decline by Invigorating the Kidney and Filling the Essence, Chinese Academy of Chinese Medical Sciences, 2021 (in chinese).
[34] Yunhang Zhou, Systematic Review and Meta-Analysis of Traditional Chinese Medicine in the Treatment of Premature Ovarian Insufficiency [D], Guangzhou University of Traditional Chinese Medicine, 2018, https://doi.org/10.27044/d.cnki.ggzzu.2018.000180 (in chinese).
[35] S. Chen, Clinical Study on the Improvement of Early-Onset Ovarian Insufficiency with Bushen Huoxue Decoction and the Mechanism of Regulating Keap 1/Nrf 2/ARE Signaling Pathway in Model Mice [D], Nanjing University of Traditional Chinese Medicine, 2021, https://doi.org/10.27253/d.cnki.gnjzu.2021.000159 (in chinese).
[36] Huixia Sun, Zhe Guo, Xu Jing, Ovarian protective effect of Lycium barbarum polysaccharide on cisplatin chemotherapy-induced premature ovarian failure model in rats, J. Clin. Pathol. 40 (3) (2020) 578–584 (in chinese).
[37] Yixin Ma, Song Nan, Lianqun Jia, Aijie Hou, Fang Yang, Hongwei Zhao, Study on the effect of Lycium barbarum polysaccharide in improving lipid metabolism disorder in ovariectomized rats, Shizhen Traditional Chinese Medicine 28 (4) (2017) 774–777 (in chinese).
[38] Huang Tian, Zheng Xiaoxia, Xiaohua Qiu, Wang Yan, Protective effect of Lycium barbarum polysaccharide on autoimmune premature ovarian failure model mice, Pharmaceut. Res. 33 (8) (2014) 437–440, https://doi.org/10.13506/j.cnki.jpr.2014.08.002 (in chinese).
[39] Wei Min, Zheng Shengzhi, Ma Hong, Lv Ye, Study on the protective mechanism of Lycium barbarum polysaccharide on the ovary of naturally aging female rats, Chinese Materia Medica 34 (12) (2011) 1915–1918, https://doi.org/10.13863/j.issn1001-4454.2011.12.038 (in chinese).
[40] B. Chen, L. Wang, L. Li, R. Zhu, H. Liu, C. Liu, R. Ma, Q. Jia, D. Zhao, J. Niu, M. Fu, S. Gao, D. Zhang, Fructus LigustriLucidi in osteoporosis: a review of its pharmacology, phytochemistry, pharmacokinetics and safety, Molecules 22 (9) (2017) 1469, https://doi.org/10.3390/molecules22091469.
[41] L. Gao, C. Li, Z. Wang, X. Liu, Y. You, H. Wei, T. Guo, Ligustrilucidi fructus as a traditional Chinese medicine: a review of its phytochemistry and pharmacology, Nat. Prod. Res. 29 (6) (2015) 493–510, https://doi.org/10.1080/14786419.2014.954114.
[42] Mingfa Zhang, Yaqin Shen, Research progress of Ligustrum lucidum and its active components in promoting growth and constitution, Drug Evaluation Research 39 (3) (2016) 474–481 (in chinese).
[43] Yubo Guo, The Mechanism of Ligustrum Lucidum on Osteoporosis in Ovariectomized Rats Based on the Regulation of Wnt/β-Catenin Pathway [D], Beijing University of Traditional Chinese Medicine, 2016, https://doi.org/10.26973/d.cnki.gbjzu.2016.000061 (in chinese).
[44] Mingfa Zhang, Yaqin Shen, Research progress of Ligustrum lucidum and its active components in anti-osteoporosis, Drug Evaluation Research 37 (6) (2014) 566–57113 (in chinese).
[45] Hui Zou, Li Dandan, Jing Chen, Lao Xuefen, Zheng Cao, Qiuchen Shang, Xiaoqin Xu, Effects of Se-enriched Ligustrum lucidum on heat-stressed ovarian cells, J. Yangzhou Univ. (Agric. Life Sci. Ed.) 37 (4) (2016) 27–31+48, https://doi.org/10.16872/j.cnki.1671-4652.2016.04.006 (in chinese).
[46] Wang Qi, Song Fang, Ying Kang, Zhaojin Luan, Meixia Yang, Research progress on the protective effect of Dodder on the female reproductive system, Journal of Baotou Medical College 37 (9) (2021) 117–119, https://doi.org/10.16833/j.cnki.jbmc.2021.09.026 (in chinese).

[47] Changsheng Huang, Shoudi He, Yancheng Guan, Ruyun Zhou, Tan Ning, Shicun Luo, Mingli Luo, Effects of dodder flavonoids and quercetin on ovarian function in rats with premature ovarian failure induced by Tripterygium wilfordii polyglycosides, Chinese Clinical Pharmacology Journal of Science 36 (6) (2020) 667–670, https://doi.org/10.13699/j.cnki.1001-6821.2020.06.021 (in chinese).
[48] Yongxia Wang, Na Ma, Xingming Zhong, Cui Rong, Zhulin Miao, The effect of total flavonoids of Cuscuta dodder on ovarian function in rats with premature ovarian failure, Medical Review 25 (13) (2019) 2695–2699 (in chinese).
[49] Li Jianghui, Quercetin Inhibits Cyclophosphamide-Induced Primordial Follicle Depletion by Regulating PI3K/AKT/FOXO3a pathway[D], Shanghai Jiaotong University, 2020, https://doi.org/10.27307/d.cnki.gsjtu.2020.000349 (in chinese).
[50] A. Tarko, A. Štochmalova, S. Hrabovszka, A. Vachanova, A.H. Harrath, S. Alwasel, R. Grossman, A.V. Sirotkin, Can xylene and quercetin directly affect basic ovarian cell functions? Res. Vet. Sci. 119 (2018) 308–312, https://doi.org/10.1016/j.rvsc.2018.07.010.
[51] AV Sirotkin, A Štochmaľová, R Alexa, A Kádasi, M Bauer, R Grossmann, A Alrezaki, S Alwasel, AH Harrath, Quercetin directly inhibits basal ovarian cell functions and their response to the stimulatory action of FSH, Eur J Pharmacol 860 (2019) 172560, https://doi.org/10.1016/j.ejphar.2019.172560.
[52] Y. Yi, S. Wan, Y. Hou, J. Cheng, J. Guo, S. Wang, A. Khan, N. Sun, H. Li, Chlorogenic acid rescues zearalenone induced injury to mouse ovarian granulosa cells, Ecotoxicol. Environ. Saf. 194 (2020) 110401, https://doi.org/10.1016/j.ecoenv.2020.110401.
[53] X. Li, H. Chen, Z. Zhang, D. Xu, J. Duan, X. Li, L. Yang, R. Hua, J. Cheng, Q. Li, Isorhamnetin promotes estrogen biosynthesis and proliferation in porcine granulosa cells via the PI3K/akt signaling pathway, J. Agric. Food Chem. 69 (23) (2021) 6535–6542, https://doi.org/10.1021/acs.jafc.1c01543.
[54] Xiaojing Tang, Effects of Codonopside and Luteolin on Ovarian Granulosa Cell Hormone Secretion and Their Mechanism of action[D], Minzu University of China, 2015 (in chinese).
[55] Xiqian Zhang, Yao Li, Luo Yanqun, Yi Yanhong, Mei Dong, Fenghua Liu, Study on the effect of luteolin on bisphenol A-induced ovarian toxicity in mice, New Med. 51 (7) (2020) 539–543 (in chinese).
[56] Y. Lou, W. Yu, L. Han, S. Yang, Y. Wang, T. Ren, J. Yu, A. Zhao, ROS activates autophagy in follicular granulosa cells via mTOR pathway to regulate broodiness in goose, Anim. Reprod. Sci. 185 (2017) 97–103, https://doi.org/10.1016/j.anireprosci.2017.08.008.
[57] M. Shen, Y. Jiang, Z. Guan, Y. Cao, L. Li, H. Liu, S.C. Sun, Protective mechanism of FSH against oxidative damage in mouse ovarian granulosa cells by repressing autophagy, Autophagy 13 (8) (2017) 1364–1385, https://doi.org/10.1080/15548627.2017.1327941.
[58] J Guo, T Zhang, Y Guo, T Sun, H Li, X Zhang, H Yin, G Cao, Y Yin, H Wang, L Shi, X Guo, J Sha, JJ Eppig, YQ Su, Oocyte stage-specific effects of MTOR determine granulosa cell fate and oocyte quality in mice, Proc Natl Acad Sci U S A 115 (23) (2018) E5326–E5333, https://doi.org/10.1073/pnas.1800352115.
[59] A. Uyar, S. Torrealday, E. Seli, Cumulus and granulosa cell markers of oocyte and embryo quality, Fertil. Steril. 99 (4) (2013) 979–997, https://doi.org/10.1016/j.fertnstert.2013.01.129.
[60] Y.X. Guo, C.H. Duan, Q.H. Hao, Y.Q. Liu, T. Li, Y.J. Zhang, Effect of short-term nutritional supplementation on hormone concentrations in ovarian follicular fluid and steroid regulating gene mRNA abundances in granulosa cells of ewes, Anim. Reprod. Sci. 211 (2019) 106208, https://doi.org/10.1016/j.anireprosci.2019.106208.
[61] R. Wen, S. Hu, Q. Xiao, C. Han, C. Gan, H. Gou, H. Liu, L. Li, H. Xu, H. He, J. Wang, Leptin exerts proliferative and anti-apoptotic effects on goose granulosa cells through the PI3K/Akt/mTOR signaling pathway, J. Steroid Biochem. Mol. Biol. 149 (2015) 70–79, https://doi.org/10.1016/j.jsbmb.2015.01.001.
[62] Y.J. Chen, P.W. Hsiao, M.T. Lee, J.I. Mason, F.C. Ke, J.J. Hwang, Interplay of PI3K and cAMP/PKA signaling, and rapamycin-hypersensitivity in TGFbeta1 enhancement of FSH-stimulated steroidogenesis in rat ovarian granulosa cells, J. Endocrinol. 192 (2) (2007) 405–419, https://doi.org/10.1677/JOE-06-0076.
[63] L. Li, X. Shi, Y. Shi, Z. Wang, The signaling pathways involved in ovarian follicle development, Front. Physiol. 12 (2021) 730196, https://doi.org/10.3389/fphys.2021.730196.
[64] M. Taguchi, J. Alfer, K. Chwalisz, Endothelial nitric oxide synthase is differently expressed in human endometrial vessels during themenstrual cycle, Mol Hum Reprod 6 (2) (2000) 185–190.
[65] J. Lu, Z. Wang, J. Cao, Y. Chen, Y. Dong, A novel and compact review on the role of oxidative stress in female reproduction, Reprod. Biol. Endocrinol. 16 (1) (2018) 80, https://doi.org/10.1186/s12958-018-0391-5.
[66] Rizzo A,Roscino MT,Binetti F. Roles of reactive oxygen species in female reproduction. ReprodDomestAniml,20147 (2) : 344-352.
[67] E. Agaçayak, N. YamanGörük, H. Küsen, et al., Role of inflammation and oxidative stress in the etiology of primary ovarian insufficiency, Turk J ObstetGynecol 13 (3) (2016) 109–115.
[68] Z. Yan, Y. Dai, H. Fu, Y. Zheng, D. Bao, Y. Yin, Q. Chen, X. Nie, Q. Hao, D. Hou, Y. Cui, Curcumin exerts a protective effect against premature ovarian failure in mice, J. Mol. Endocrinol. 60 (3) (2018) 261–271, https://doi.org/10.1530/JME-17-0214.
[69] M. Thakur, G. Feldman, E.E. Puscheck, Primary ovarian insufficiency in classic galactosemia: current understanding and future research opportunities, J. Assist. Reprod. Genet. 35 (1) (2018) 3–16, https://doi.org/10.1007/s10815-017-1039-7.
[70] K. Papageorgiou, E. Mastora, A. Zikopoulos, M.E. Grigoriou, I. Georgiou, T.M. Michaelidis, Interplay between mTOR and hippo signaling in the ovary: clinical choice guidance between different gonadotropin preparations for better IVF, Front. Endocrinol. 12 (2021) 702446, https://doi.org/10.3389/fendo.2021.702446.
[71] E.H. Ernst, S. Franks, K. Hardy, P. Villesen, K. Lykke-Hartmann, Granulosa cells from human primordial and primary follicles show differential global gene expression profiles, Hum. Reprod. 33 (4) (2018) 666–679, https://doi.org/10.1093/humrep/dey011.
[72] A. Kaur, S. Sharma, Mammalian target of rapamycin (mTOR) as a potential therapeutic target in various diseases, Inflammopharmacology 25 (3) (2017) 293–312, https://doi.org/10.1007/s10787-017-0336-1.
[73] T.T. Jesus, P.F. Oliveira, M. Sousa, C.Y. Cheng, M.G. Alves, Mammalian target of rapamycin (mTOR): a central regulator of male fertility? Crit. Rev. Biochem. Mol. Biol. 52 (3) (2017) 235–253, https://doi.org/10.1080/10409238.2017.1279120.