ORIGINAL RESEARCH

The Network Pharmacology Analysis of Tonifying Yang Formula for the Treatment of Diminished Ovarian Reserve

Qianwen Ma, PhD; Xuanzhu Wu, BS; Dandan Chen, BS; Wei Li, MSc; Jianfei Wu, BS; Mingxia Shen, BS; Chenshu Ye, MSc; Yong Tan, PhD; Qi Guo, PhD

ABSTRACT

Background • Diminished ovarian reserve (DOR) can lead to amenorrhea, infertility, and even the development of premature ovarian insufficiency, severely affecting the quality of life for women. Therefore, it is important to determine the main components of Tonifying Yang Formula, analyze the active substances and effective targets for treating DOR using Tonifying Yang Formula, and explore its potential mechanisms of action.

Objective • The study is aim to determine the main components of Tonifying Yang Formula, analyze the active substances and effective targets for treating DOR using Tonifying Yang Formula, and explore its potential molecular mechanisms of action, providing important theoretical basis for clinical application.

Methods • The main active components of Tonifying Yang Formula and their potential therapeutic targets for DOR were searched using the Chinese Medicine Systems Pharmacology Database and Analysis Platform, BATMAN-TCM, GeneCards, OMIM, and Uniprot databases. The protein-protein interaction network of shared targets between drugs and diseases was constructed using the STRING database. The shared targets of drugs and diseases were subjected to GO analysis and KEGG pathway enrichment analysis using the DAVID database. AutoDock Vina was used to perform molecular docking between the active substances and key targets of the drug to validate their interaction activities.

Results • The key chemical components in the Tonifying Yang Formula for DOR treatment include quercetin, luteolin, beta-sitosterol, stigmasterol, and kaempferol. The 164 key targets for treating DOR with Tonifying Yang Formula included AKT1, TNF, JUN, TP53, IL6, IL1B, EGFR, VEGFA, INS, and CASP3, among others. GO enrichment analysis revealed that the Tonifying Yang Formula mainly regulates gene expression positively, negatively regulates the apoptotic process, and affects signal transduction. KEGG pathway enrichment analysis showed that Tonifying Yang Formula is mainly involved in cancer-related pathways, the AGE-RAGE signaling pathway in diabetic complications, prostate cancer, lipid and atherosclerosis, fluid shear stress and atherosclerosis, and the IL-17 signaling pathway. Molecular docking results indicated that the core components of the Tonifying Yang Formula had higher docking energies and stable binding with targets such as AKT1, IL6, JUN, TNF, and TP53. This study selected the PI3K/AKT signaling pathway for validation. Through experimental research, we found that Tonifying Yang Formula could improve ovarian reserve function by activating the PI3K/AKT signaling pathway.

Conclusions • The potential mechanism of Tonifying Yang Formula therapy for DOR may be related to the influence of Chinese herbal compounds on pathways such as AKT1, IL6, JUN, TNF, and TP53, regulating the proliferation and apoptosis of ovarian granulosa cells, maintaining the function of the ovarian corpus luteum, regulating the secretion of related hormones, and alleviating ovarian tissue inflammation. (*Altern Ther Health Med.* [E-pub ahead of print.])

Qianwen Ma, PhD; Xuanzhu Wu, BS; Dandan Chen, BS; Jianfei Wu, BS; Mingxia Shen, BS; Chenshu Ye, MSc, Traditional Chinese Medicine Department, Hangzhou Ninth People's Hospital, Hangzhou, China. **Wei Li, MSc,** Traditional Chinese Medicine Department, Afffliated Hospital of Jiangsu University, Zhenjiang, China. **Yong Tan, PhD,** Reproductive Medicine Department, Afffliated Hospital of Nanjing University of Traditional Chinese Medicine, Nanjing, China. **Qi Guo, PhD,** Medical college, Jiangsu University, Zhenjiang, China.

Corresponding author: Qi Guo, PhD
E-mail: guoqiujs@163.com

INTRODUCTION

Background

Diminished ovarian reserve (DOR) refers to the decrease in the number and quality of ovarian follicles in women before age 40. In clinical practice, it is often manifested as menstrual irregularities or a decline in fertility and may even be accompanied by various symptoms of perimenopause. Serum follicle-stimulating hormone level, luteinizing hormone level, anti-Mullerian hormone level, number of sinus follicles under ultrasound, and number of growing follicles and oocytes after ovarian stimulation are all markers reflecting DOR. Prolonged DOR can lead to amenorrhea, infertility, and even the development of premature ovarian insufficiency, severely affecting the quality of life for women.[1-2] In recent years, with increasing life stress and irregular lifestyle, the incidence of DOR has been on the rise, significantly affecting patients' fertility, increasing their physiological and psychological burden, and lowering their quality of life.[3-4] Therefore, how to improve ovarian reserve function and slow down its progression to premature ovarian insufficiency has become an increasingly hot topic in women's

health and reproductive health, as well as a challenging issue in medical research.

Current Medical Treatments and Limitations

Modern medical treatment for DOR generally involves hormone replacement therapy. Still, the long-term therapeutic effect is not ideal, and there are many side effects, such as an increased risk of stroke, breast cancer, and thrombosis.

Traditional Chinese Medicine in DOR Treatment

Traditional Chinese medicine (TCM) treatment of DOR has shown good therapeutic effects, including improving ovarian reserve function, with fewer adverse reactions.[5] Given this, we have accumulated abundant research experience in TCM treatment of DOR. Through our research, we have found that the sequential use of Nourishing Yin and Tonifying Yang formulas can promote follicular development, improve egg quality, establish a natural menstrual cycle, and significantly improve ovarian growth and development.[6] The sequential use of Nourishing Yin and Tonifying Yang Formulas involves dividing the menstrual cycle into the post-menstrual phase and pre-menstrual phase, using Nourishing Yin Formula during the post-menstrual phase and Tonifying Yang Formula during the pre-menstrual phase for the treatment of DOR. This therapy has achieved satisfactory clinical outcomes.[7] The Nourishing Yin Formula sequentially uses Nourishing Yin and Tonifying Yang Formulas include Chinese angelica, Dogwood fruit, White peony root, Dodder seed, Rrehmannia dried root, and Placenta hominis. Tonifying Yang Formula includes Morinda officinalis, Dipsacus asper, Psoralea corylifolia, Epimedium, Codonopsis pilosula, and Chinese yam rhizome. The pre-menstrual phase is a period of Yang activity, during which Tonifying Yang Formula can maintain luteal function and prepare for pregnancy. Therefore, the treatment during the pre-menstrual phase mainly consists of tonifying the kidney and assisting Yang herbs such as Morinda officinalis, Dipsacus asper, Psoralea corylifolia, and Epimedium herb. However, so far, the underlying mechanisms of Tonifying Yang Formula in treating DOR are still unclear and require further investigation.

Research Objectives

In finding disease targets and drug design on DOR, network pharmacology and bioinformatics of TCM are important. In finding disease targets and drug design on DOR, network pharmacology and bioinformatics of traditional Chinese medicine are essential. This can provide a better understanding of drug targets and molecular mechanisms, playing an important role in guiding clinical practice. By studying the "Disease gene target drug" interaction network of Tonifying Yang Formula in treating ovarian reserve dysfunction, we aim to understand the molecular mechanisms and targets of Tonifying Yang Formula in treating DOR, providing an important theoretical basis for clinical application.

DATA SOURCE AND RESEARCH METHODS[8-9]

Collection of Chemical Composition and Target Prediction of Tonifying Yang Formula

The Traditional Chinese Medicine Database and Analysis Platform (TCMSP, https://tcmsp-e.com/) was used to screen for active ingredients of Tonifying Yang Formula, including Morinda officinalis, Dipsacus asper, Psoralea corylifolia, Epimedium, Codonopsis pilosula, and Dioscorea opposita, with oral bioavailability (OB) ≥30% and drug-likeness (DL) ≥ 0.18 as selection criteria. The obtained targets were then screened, non-human genes were removed based on data calibration using Uniprot (https://www.uniprot.org/), and invalid repeated targets were deleted. For drugs that could not be retrieved from TCMSP, high-credibility proteins were obtained as candidate genes by logging into the BATMAN-TCM database (http://bionet.ncpsb.org.cn/batman-tcm/). The obtained targets were then screened, non-human genes were removed based on data calibration using Uniprot (https://www.uniprot.org/), and invalid repeated targets were deleted to obtain standardized gene names. (Note: TCMSP is a unique pharmacology platform for Chinese herbal medicine systems to capture the relationship between drugs, targets, and diseases. The UniProt database is the most informative and extensive protein database.

The BATMAN-TCM database is a TCM target database that analyzes the relationship between compounds and targets through the components of TCM compounds, and makes relevant connections between the action pathways of targets and diseases.)

Acquisition of target genes related to decreased ovarian reserve

Searching for the keywords "decreased ovarian reserve, diminished ovarian reserve" in both GeneCards (https://www.genecards.org/) and OMIM (https://www.omim.org/) databases allowed us to obtain disease-related targets. All targets were integrated into an Excel sheet, and duplicate genes were removed. The obtained target genes were then calibrated using the Uniprot database to obtain disease target gene information. (Note: Genecards is a comprehensive database of the human genome, synthesizing 125 web-sourced genome, transcriptome, and proteomics databases. OMIM is a comprehensive database of human genes and genetic diseases, which includes information on Mendelian genetic diseases and human genes.)

Drug-disease target prediction results

The obtained drug component targets are mapped to disease targets and a Venn diagram is used to obtain intersection genes. Next, the "Drug-Component-Target" network is built using Cytoscape 3.7.2 software. (Cytoscape is a software that graphically displays the network for analysis and editing.)

Construction of target protein interaction network

To further study the interactions between proteins involved in the treatment of diminished ovarian reserve using Tonifying Yang Formula, the drug-intersection genes were uploaded to the String protein interaction network database (https://string-db.

org/) to construct a protein-protein interaction (PPI) network. The species was set to "Homo sapiens," To make the PPI network more reliable, we set with a minimum interaction score of 0.7, ensuring the reliability of this study. The remaining parameters were set to their default values, and the results were stored in TSV format. The TSV files were imported into Cytoscape3.7.2 for network analysis (Cytoscape→Tools→Network Analyzer→Network Analysis→Analyze Network). The network analysis results were saved, with node size and color reflecting the Degree of interaction; the larger the node, the higher the Degree value. The thickness of the edges was used to reflect the combined score, and the thicker the edge, the higher the combined score. Core targets were selected for the protein interaction network. (Note: The String database is a database that searches for interactions between known proteins and predicts interactions between proteins.)

GO enrichment analysis and KEGG pathway analysis

The drug-disease intersection genes were uploaded to the DAVID database (https://david.ncifcrf.gov/summary.jsp), with the gene identifier set to OFFICIAL_GENE_SYMBOL, and the species set to "Homo sapiens." Using DAVID 6.8 GO gene function, the role of target proteins involved in the treatment of diminished ovarian reserve using Tonifying Yang Formula in gene functions was annotated in terms of biological processes (BP), cellular components (CC), and molecular functions (MF). KEGG pathway enrichment analysis was performed to clarify the role of the target proteins in signal pathways with respect to diminished ovarian reserve. The top 10 entries for BP, CC, and MF were selected from the GO function, along with 20 KEGG pathway entries related to diminished ovarian reserve ($P < .01$), to predict the main gene functions and signal pathways involved in the treatment of diminished ovarian reserve using Tonifying Yang Formula. (Note: DAVID is a bioinformatics database that integrates biological data and analytical tools to provide systematic and comprehensive biofunctional annotation information for large-scale gene or protein lists, helping users extract biological information from them.)

Molecular docking

In general, the higher the Degree value of a node in a network, the more important its position. Therefore, the proteins with higher degree values in the protein-protein interaction network play important roles in the treatment of ovarian reserve decline using Tonifying Yang Formula. AutoDock Vina (1.1.2) was used for molecular docking of the active ingredients in the drug and key targets to verify their interaction activity. The specific steps are as follows: (1) Download compounds in Mol2 format from the TCMSP website, then import them into Chembio3D for energy minimization, and then import them into AutodockTools-1.5.6 to add hydrogen, compute charges, assign charges, and set rotatable bonds before saving as "pdbqt" format; (2) Download the key target protein from the PDB database (http://www.rcsb.org/), with human proteins given priority and those with high structural similarity to the original ligand and the active ingredient to be docked selected, and those with high resolution chosen; (3) Import the protein into PyMoL (2.3.0), remove the original ligand and water molecules, and then import the protein into AutoDocktools (v1.5.6) for adding hydrogen, computing charges, assigning charges, and specifying atomic types before saving as "pdbqt" format; (4) Use the original ligand of the protein as the center of the docking box. If there is no original ligand, use the nearby key amino acid residues as the docking area. Based on past experience, The grid box size is set to 60×60×60 (the spacing between each grid point is 0.375 Å), and the remaining parameters are set to default settings. It is more conducive to conduct small molecule docking. (5) Use PyMOL and Ligplot for interaction pattern analysis. (Note: AutoDock is an open source protein-small molecule automatic docking software. The PDB database is the most important database to collect the structure of biological acromolecules. It is a three-dimensional structure database of proteins, polysaccharides, nucleic acids, viruses and other biological macromolecules determined by X-ray single crystal diffraction, nuclear magnetic resonance, electron diffraction and other experimental means.)

Experiment validation

According Guideline on Network Pharmacological Evaluation Methods,[10] we conducted the experiment validation. The rats were divided into three groups: normal group, model group, and stigmasterol group, with 6 rats in each group. The model group and the stigmasterol group constructed the DOR rat models. We chose 3-month-old female SD rats, dissolved deoxyethylcyclohexene (VCD) in sesame oil, and injected VCD 80 mg/kg intraperitoneally once a day for 15 consecutive days. All other rats were intraperitoneally injected with VCD 80 mg/kg except for the normal group. The normal group was intraperitoneally injected with an equal volume of sesame oil once a day for 15 consecutive days. Starting from the 16th day, rats in the stigmasterol group were given 40mg/ml of stigmasterol (dissolved in corn oil) daily, once a day, for a total of 15 days. The normal group and the model group were given 10 g/kg physiological saline by gavage once a day for a total of 15 days. After 15 days of administration, the rats were euthanized, and ovarian tissues were taken by laparotomy to detect the following indicators. All experimental protocols involving animals were approved by the Ethics Committee of Hangzhou Ninth People's Hospital (Hangzhou, China).

Ovarian morphology

We made ovarian paraffin sections with thickness of 5 μm and used the HE staining kit to observe the morphological changes of rat ovarian tissues under a microscope. Ovarian tissues of rats were first immersed into the dehydrator, and dehydrated with gradient alcohol, followed by wax dipping and tissue embedding in the embedding machine. Then, microtome knives were then installed on the holder of the microtome, and the sliced paraffin sections were gently laid on the water surface of the water bath. The paraffin sections floated in the warm water and unfolded naturally and

smoothly after the heating. Then, the slices were temporarily dried at the room temperature and baked in a 40°C constant temperature oven for 0.5-2h. The sections were dewaxed by treatment with Xylene I and xylene II (Xylene I is the same substance as xylene II. Xylene I means first using xylene to dewax the slices by one container, and xylene II means second using xylene to dewax the slices by another container), hydrated by gradient alcohol, stained with Hematoxylin, rinsed with tap water, differentiated with the differentiation fluid, immersed in the tap water, stained with eosin, washed with tap water and dehydrated with the gradient alcohol, followed by transparent treatment with xylene I and xylene II and neutral gum sealing. The sections were dried in an oven at 60°C and observed under a microscope. Finally, we randomly selected the visual fields and took photos (200×).

PCR detection of gene levels

We used PCR to detect PI3K/AKT signaling pathway factors in rat oocytes, including PI3K, Akt1, and mTOR expression. We used a glass grinder to grind ovarian tissues, extracted RNAs from the tissues, and measured RNA concentration. After reverse transcription into cDNA, GAPDH was used as the internal reference to detect RNA expression. The Ct value was obtained by amplifying the curve, using $2^{-\Delta\Delta Ct}$ method for relative quantitative analysis.1 mL of RNAios Plus was added to the tissue samples that were ground with the liquid nitrogen. The samples were kept on the vortex oscillator for 30 s and incubated at the room temperature for 5 min. After addition of 200 μL of chloroform, the samples were mixed upside down, shook violently, and then incubated at the room temperature for 5 min. After centrifugation at 4°C and 13000 rpm for 15 min, the supernatant was transferred to a new eppendorf tube. Thereafter, the samples were mixed upside down with an equal volume of isopropanol and placed at the room temperature for 5 min. The supernatant was discarded, after centrifugation at 4°C and 13000 rpm for 15 min. The samples were mixed upside down with 1 mL of 75% ethanol, and placed at the room temperature for 10 min, followed by centrifugation at 4 °C and 13000 rpm for 10 min to precipitate the RNA and remove the ethanol. After 5 min of ventilation, RNA was dissolved into 50 μL of DEPC water. The PCR reaction solution was prepared for real-time PCR reaction. After the reaction was set up, amplification curve and melting curve of real-time PCR was determined and the 2- Δ Δ ct value was calculated. Calculation method used was: 2- Δ Δ ct value could be deduced from Δ Ct value (Δ Ct =Ct target gene - Ct internal reference gene) and Δ Δ Ct value (Δ Ct experimental group - Δ Ct control group).

Statistical analysis

SPSS 24.0 software was used for data processing. Differences between multiple groups were examined with one-way analysis of variance for those that complied with a homogeneity of variance post hoc test. Non-parametric test was adopted for those that did not comply with homogeneity of variance. $P < .05$ was considered to indicate a statistically significant difference.

RESEARCH RESULTS

Prediction of active ingredients and targets of Tonifying Yang Formula

Through TCMSP retrieval, 17 ingredients of Morinda officinalis, 5 ingredients of Dipsacus asper, 12 ingredients of Dioscorea opposita, 21 ingredients of Epimedium, and 17 ingredients of Codonopsis pilosula were screened and collected with OB ≥ 30%, DL ≥ 0.18, and excluding invalid components. Since no ingredients of Psoralea corylifolia were included after TCMSP screening, 14 ingredients were obtained through the BATMAN-TCM database under the condition of Scorecutoff > 20. In total, 78 drug ingredients were obtained.

Related targets of diminished ovarian reserve

By searching with the keywords "decreased ovarian reserve" and "diminished ovarian reserve," 1661 and 1527 targets were obtained through GeneCards and DisGeNET databases, respectively. After de-duplication, a total of 1663 targets related to decreased ovarian reserve were obtained, and the Uniprot database corrected the obtained genes. After taking the intersection of the drug target and the target genes related to decreased ovarian reserve, 164 intersection target genes related to the treatment of decreased ovarian reserve by drugs were obtained (Figure 1).

Prediction results of drug-ingredient-target

Using drug-ingredient-target data, the "network.xlsx" and "type.xlsx" files were constructed, and the files were imported into Cytoscape 3.7.2 for plotting (Figure 2). The network consisted of 237 nodes and 639 edges. The top five components ranked by degree value were quercetin, luteolin, beta-sitosterol, Stigmasterol, and kaempferol.

Figure 1. Drug-disease Venn diagram

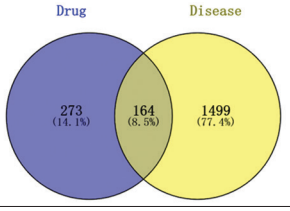


Figure 2. Drug-ingredient-target diagram

Note: rectangular nodes represent targets, oval nodes represent drug components, and diamond nodes represent traditional Chinese medicine. This analysis indicated that the main effective components of Tonifying Yang Formula may be quercetin, luteolin, beta-sitosterol, Stigmasterol, and kaempferol.

Core Targets and Network Interactions

After taking the intersection of the drug target and the target genes related to decreased ovarian reserve, 164 intersection target genes related to the treatment of decreased ovarian reserve by drugs were obtained. These 164 intersection target genes were imported into the String (https://string-db.org/) database for protein-protein interaction prediction, setting the species to HomoSapiens and confidence level to 0.7. We saved the network file in TSV format and imported it into Cytoscape 3.7.2 software for drawing the protein-protein interaction network. We selected nodes with degree value greater than 5 and obtained a network with 91 nodes and 1085 edges. We performed topological analysis on the network, using degree values to reflect target size and color, and combined score values to reflect edge thickness, thus constructing the protein-protein interaction network shown in Figure 3. The core targets were AKT1, TNF, JUN, TP53, IL6, IL1B, EGFR, VEGFA, INS, and CASP3. This analysis suggested that Tonifying Yang Formula may improve ovarian reserve function mainly through these targets.

Biological Function Enrichment Analysis

GO Enrichment Analysis. We took the intersection of drug-disease target genes and performed GO gene function enrichment analysis using the DAVID database. We selected 568 GO entries and used $P < .01$ as the filtering criteria. Significant enrichment of biological processes (BP) for the treatment of ovarian reserve function decline by Tonifying Yang Formula mainly included 438 entries, involving positive regulation of gene expression, negative regulation of cell apoptosis process, signal transduction, positive regulation of cell proliferation, response to external stimuli, inflammatory response, negative regulation of gene expression, cell apoptosis process, positive regulation of cell apoptosis process, and hypoxia response. There were 51 entries related to cellular components (CC), including cytoplasm, plasma membrane, nucleus, cytosol, nucleoplasm, extracellular space, extracellular region, plasma membrane component, extracellular exosomes, and chromatin. Among them, there were 79 entries related to molecular function (MF), including protein binding, identical protein binding, enzyme binding, protein homodimerization activity, DNA binding, zinc ion binding, macromolecular complex binding, protein heterodimerization activity, receptor binding, and protein kinase binding (Figure 4-5).

KEGG Pathway Enrichment Analysis. The DAVID database was used for pathway enrichment analysis. A total of 135 pathways related to the decline in ovarian reserve were filtered out with a significance level of $P < .01$. The pathways associated with the decline in ovarian reserve include pathways in cancer, the AGE-RAGE signaling pathway in diabetic complications, prostate cancer, lipid and atherosclerosis, fluid shear stress and atherosclerosis, IL-17 signaling pathway, tumor necrosis factor signaling pathway, Kaposi sarcoma-associated herpesvirus infection, pancreatic cancer, Chagas disease, hepatitis B, HIF-1 signaling pathway, alcoholic liver

Figure 3. Protein-Protein Interaction Network

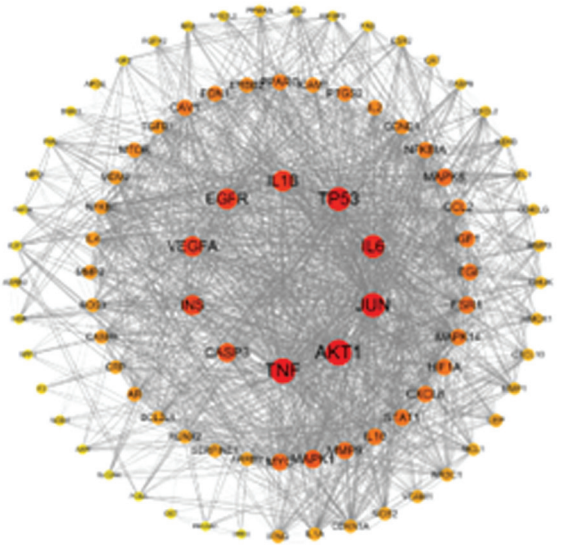


Figure 4. GO Bar Chart

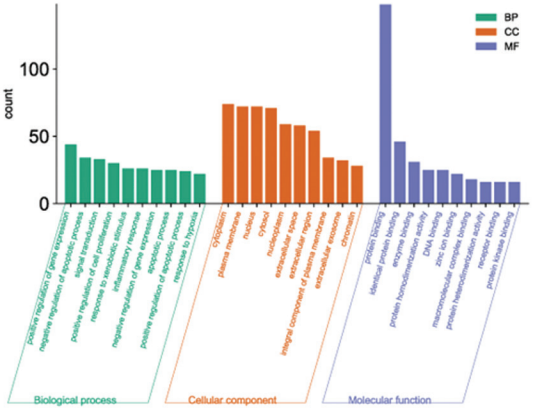


Figure 5. GO Bubble Chart

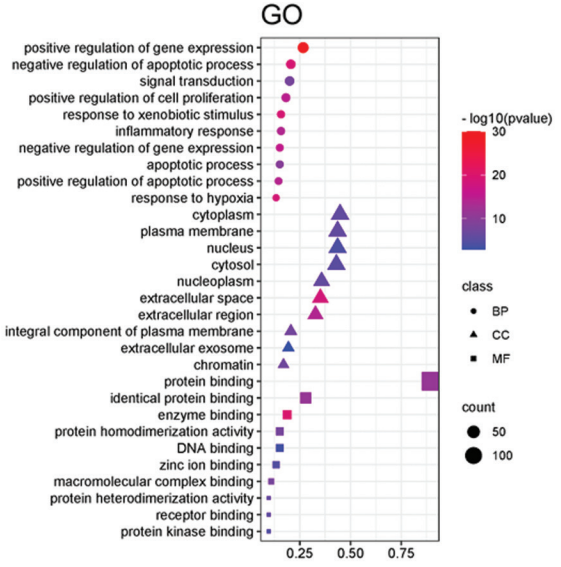


Figure 6. KEGG bar chart

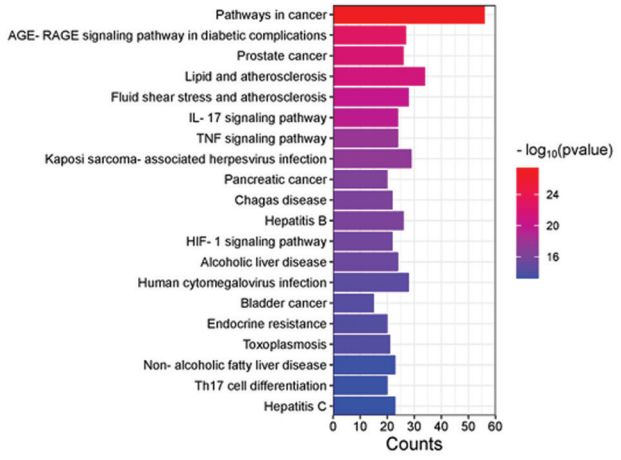
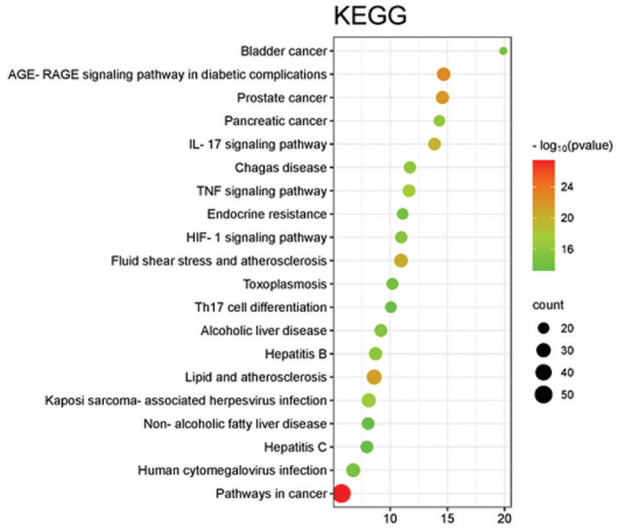


Figure 7. KEGG bubble chart



Note: The size of the circles represents the data of enriched genes in the corresponding pathway, with a gradual decrease in *P* values from green to red.

Table 1. Docking results of small molecules with core target proteins.

Target	PDB ID	Compound	Binding energy (kcal/mol)
IL6	1ALU	MOL000006 (luteolin)	-6.9
		MOL000098 (quercetin)	-7
		MOL000358 (beta-sitosterol)	-6.8
		MOL000422 (kaempferol)	-6.8
		MOL000449 (Stigmasterol)	-6.4
AKT1	1UNQ	MOL000006 (luteolin)	-6.4
		MOL000098 (quercetin)	-6.1
		MOL000358 (beta-sitosterol)	-7.1
		MOL000422 (kaempferol)	-5.9
		MOL000449 (Stigmasterol)	-7.2
TP53	1UOL	MOL000006 (luteolin)	-7.1
		MOL000098 (quercetin)	-6.5
		MOL000358 (beta-sitosterol)	-6.6
		MOL000422 (kaempferol)	-6.2
		MOL000449 (Stigmasterol)	-6.8
TNF	2E7A	MOL000006 (luteolin)	-8.9
		MOL000098 (quercetin)	-9.1
		MOL000358 (beta-sitosterol)	-8
		MOL000422 (kaempferol)	-9
		MOL000449 (Stigmasterol)	-8.7
JUN	5T01	MOL000006 (luteolin)	-6
		MOL000098 (quercetin)	-5.3
		MOL000358 (beta-sitosterol)	-7.1
		MOL000422 (kaempferol)	-5.8
		MOL000449 (Stigmasterol)	-7.2

disease, human cytomegalovirus infection, bladder cancer, endocrine resistance, toxoplasmosis, nonalcoholic fatty liver, Th17 cell differentiation, and hepatitis C (Figure 6-7).

The top 20 KEGG metabolic pathways were selected based on their P-values and plotted on a bubble chart. The x-axis represents the number of enriched genes in each pathway, the size of the bubbles represents the quantity of enriched genes, and the color depth represents the significance level. This provides a visual representation of the significant enrichment information.

Molecular Docking Results

The top 5 important targets and compounds with high degree values were selected based on the previous analysis for semi-flexible docking. The binding affinity was used to assess the binding between small molecules and target proteins. A binding affinity value less than 0 indicates a strong binding possibility.

The docking results showed that the small molecules could enter the target protein's active site. The best docking poses of each protein with the selected small molecules were displayed as follows: Stigmasterol formed a hydrogen bond with Glu95 (A) of AKT1, with a hydrogen bond length of 2.87Å; quercetin formed hydrogen bonds with Gln175 (A), Arg30 (A), Asp34 (A) of IL6, with hydrogen bond lengths of 2.91Å, 3.19Å, 2.96Å, and 2.74Å, respectively; Stigmasterol formed a hydrogen bond with Lys285 (B) of JUN, with a hydrogen bond length of 3.19Å; quercetin formed hydrogen bonds with Arg98 (C), Arg98 (B), Ser99 (B), Pro100 (B), Asn112 (A), Gln102 (C) of TNF, with hydrogen bond lengths of 3.02Å, 2.96Å, 2.84Å, 2.90Å, 2.83Å, 3.17Å, and 3.15Å, respectively; luteolin formed hydrogen bonds with Asp268 (A), Ser269 (A), Phe113 (A), His115 (A) of TP53, with hydrogen bond lengths of 3.04Å, 3.05Å, 3.06Å, 3.02Å, 2.92Å, and 3.14Å, respectively. These small molecules also formed strong hydrophobic interactions with surrounding amino acid residues. The molecular docking results showed that the binding energy between the key active ingredients and the core target is less than -5kcal/mol, indicating good binding activity between them. For detailed docking results, refer to Table 1 and Figures 8-12.

Figure 8. Interaction pattern analysis of Stigmasterol with AKT1 protein

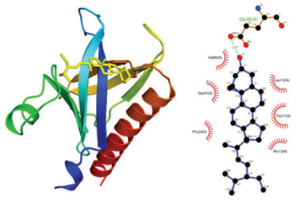


Figure 9. Interaction pattern analysis of quercetin with IL6 protein

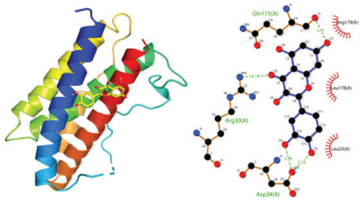


Figure 10. Interaction pattern analysis of Stigmasterol with JUN protein

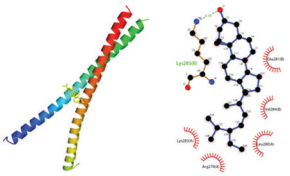


Figure 11. Interaction pattern analysis of quercetin with TNF protein

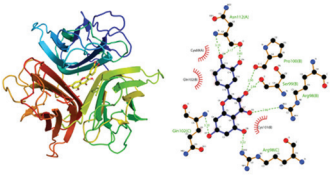


Figure 12. Interaction pattern analysis of luteolin with TP53 protein

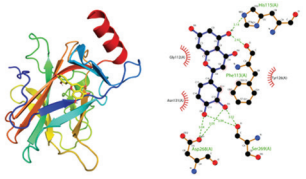


Figure 13. HE staining results of ovarian tissues of the three different groups

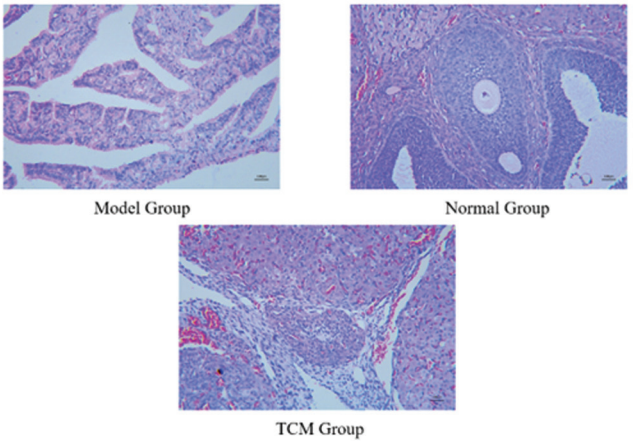
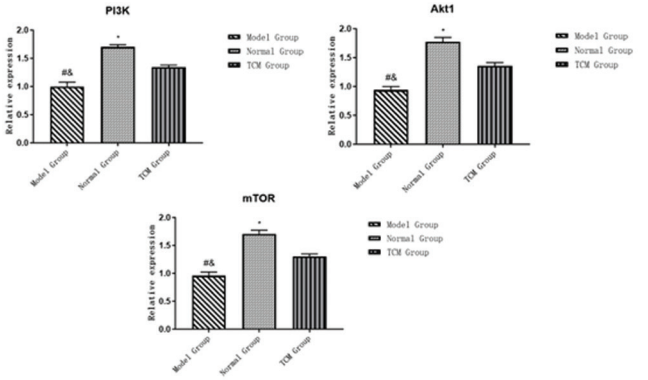


Figure 14 The levels of ovarian genes of the three different groups



Note: (1) The normal group *vs.* the model group, $^{*}P < .05$; (2) The normal group *vs.* the TCM group, $^{\&}P < .05$; (3) The model group *vs.* the TCM group, $^{\#}P < .05$.

Experiment validation

Ovarian morphology as observed by HE staining. (Figure 13) The model group exhibited decreased ovarian volume and reduced quantities of growing follicles, indicating degraded reserve function of ovaries. However, compared with the model group, the TCM group exhibited increased ovarian volume and enhanced quantities of growing follicles, suggesting that Tonifying Yang Formua could prevent the decline of ovarian reserve function to a certain extent.

Gene expression. (Figure 14) The levels of PI3K, Akt1 and mTOR in ovaries of the normal group were significantly higher than those of the model group ($P < .05$). However, compared with the model group, the TCM group exhibited significantly elevated levels of PI3K, Akt1 and mTOR ($P < .05$). The differences were statistically significant. It suggested that Tonifying Yang Formula could significantly activate PI3K/AKT signaling pathway of rat oocytes.

Through experimental verification, it was further confirmed that Tonifying Yang Formula could treat diminished ovarian reserve through PI3K/AKT signaling pathway, which was consistent with network pharmacological analysis.

DISCUSSION

With the implementation of the two-child policy, the age at which women are trying to conceive has increased. External factors such as environmental pollution and social pressure have also led to a decrease in ovarian reserve function, resulting in a lower clinical pregnancy rate and an increasing number of infertility patients caused by diminished ovarian reserve (DOR). Therefore, actively improving the ovarian reserve function of patients and slowing down ovarian aging is very important.[11-13] The advantage of Traditional Chinese Medicine in treating DOR is to restore ovarian ovulation, establish a natural cycle, and have less side effects, providing a good prospect in terms of safety, effectiveness, and low toxicity. The sequential therapy of nourishing Yin and tonifying Yang is to use nourishing Yin therapy in the late period and tonifying Yang therapy in the pre-menstrual phase to treat DOR and other diseases sequentially. It has satisfactory clinical efficacy. Tonifying Yang Formula is composed of Morinda officinalis, Dipsacus asper, Psoralea corylifolia, Epimedium, Codonopsis pilosula, and Chinese yam rhizome. It can maintain corpus luteum function, prepare for pregnancy, and has the function of tonifying the kidney, supporting yang, and invigorating Qi.

The main chemical components in Tonifying Yang Formula for treating DOR, as obtained through screening, include quercetin, luteolin, beta-sitosterol, stigmasterol, kaempferol, etc., suggesting that they may play a key role in

the treatment of DOR. Quercetin can significantly improve ovarian reserve function, regulate hormone secretion, improve the quality of follicles, and inhibit granulosa cell apoptosis, among other characteristics.[14-16] Luteolin can effectively promote the secretion of estradiol by ovarian granulosa cells without affecting the normal differentiation of cells, but has a certain impact on their proliferation. It may promote granulosa cell synthesis of estradiol by affecting the CAMP signaling pathway and aromatic enzymes related to estradiol synthesis in cells.[17-18] Beta-sitosterol can also act on ovarian granulosa cells to increase estradiol levels and improve bone loss associated with low estradiol levels.[19] Kaempferol promotes the activation of primitive follicles through the phosphatidylinositol 3-kinase/protein kinase B signaling pathway and reduces DNA fragmentation in sheep pre-antral follicles in vitro culture.[20] Stigmasterol may act on MAPK3 and PRKACA targets to regulate estrogen signaling pathways and exert anti-inflammatory effects.[21]

Molecular docking results showed that the core components in Tonifying Yang Formula have higher docking energy with the targets AKT1, IL6, JUN, TNF, TP53, indicating stable binding. The PI3K/AKT signaling pathway is believed to be involved in the activation of primordial follicles, influencing the proliferation and differentiation of granulosa cells, maintaining the function of the ovarian corpus luteum, and playing a regulatory role in various stages of follicular development. This is related to the involvement of PI3K/AKT in processes such as cell proliferation, differentiation, and apoptosis.[22-23] The IL-related pathway is involved in the occurrence and development of DOR by affecting inflammation, cell proliferation, and angiogenesis. IL-6, as a multifunctional cytokine, promotes cell proliferation and differentiation.[24] The JUN signaling pathway plays a crucial regulatory role in cell apoptosis and proliferation, and it has an important regulatory role in the growth and development of follicles.[25] The TNF signaling pathway plays an important role in oxidative stress, inflammation, cell proliferation, differentiation, and apoptosis, and it is involved in ovarian function-related signaling pathways.[26] TP53 is involved in cell apoptosis and cell cycle regulation and participates in processes such as proliferation and apoptosis of granulosa cells in the ovary.[27]

This study used network pharmacology methods to screen 164 key targets of Tonifying Yang Formula in treating DOR, with AKT1, TNF, JUN, TP53, IL6, IL1B, EGFR, VEGFA, INS and CASP3 as core targets. GO enrichment analysis found that tonifying Yang formula in the treatment of DOR mainly includes positive regulation of gene expression, negative regulation of apoptosis process, signal transduction, positive regulation of cell proliferation, response to exogenous stimuli, inflammatory response, negative regulation of gene expression, apoptosis process, positive regulation of apoptosis process, and hypoxia response. There were 51 entries related to cellular components (CC), including cytoplasm, plasma membrane, nucleus, cytosol, nucleoplasm, extracellular space, extracellular region, plasma membrane component, extracellular exosomes, and chromatin. Among them, there were 79 entries related to molecular function (MF), including protein binding, identical protein binding, enzyme binding, protein homodimerization activity, DNA binding, zinc ion binding, macromolecular complex binding, protein heterodimerization activity, receptor binding, and protein kinase binding KEGG pathway enrichment analysis mainly involves pathways in cancer, the role of AGE-RAGE signaling pathway in diabetic complications, prostate cancer, lipid and atherosclerosis, fluid shear stress and atherosclerosis, IL-17 signaling pathway, tumor necrosis factor signaling pathway, Kaposi sarcoma-associated herpesvirus infection, pancreatic cancer, Chagas disease, hepatitis B, HIF-1 signaling pathway, alcoholic liver disease, human cytomegalovirus infection, bladder cancer, endocrine resistance, toxoplasmosis, nonalcoholic fatty liver disease, Th17 cell differentiation, hepatitis C.

In this study, through core targets and network interactions, AKT1 is most likely the main target of Tonifying Yang Formulain treating DOR. Therefore, this study selected the PI3K/AKT signaling pathway for validation. Through experimental research, we found that Tonifying Yang Formula could improve ovarian reserve function by activating the PI3K/AKT signaling pathway. This study also had some shortcomings, and the active ingredients in Tonifying Yang Formula had not been screened and verified, which needed to be further studied.

In summary, the potential mechanism of Tonifying Yang Formula therapy for DOR may be related to the influence of Chinese herbal compounds on pathways such as AKT1, IL6, JUN, TNF, TP53, regulating the proliferation and apoptosis of granulosa cells, maintaining the function of the ovarian corpus luteum, regulating the secretion of related hormones, and alleviating ovarian tissue inflammation. Network pharmacology analysis results confirm the advantage of the Chinese herbal compound targeting multiple targets in the treatment of DOR and provide ideas and theoretical basis for further verification of its specific mechanisms through basic experiments.

ETHICS APPROVAL

All experimental protocols involving animals were approved by the Ethics Committee of Hangzhou Ninth Peoples Hospital (Hangzhou, China).

CONSENT TO PUBLISH

All authors consent to publish this article.

AVAILABILITY OF DATA AND MATERIALS

The analyzed datasets generated during the study are available from the corresponding author upon reasonable request.

COMPETING INTERESTS

The authors declare that they have no conflict of interest.

ETHICAL STATEMENT

Not applicable.

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AUTHORS' CONTRIBUTIONS

MQW, Wu XZ, Tan Y, Li W and Chen DD conceived and designed research. Wu JF, Shen MX, Guo Q and Ye CS carried out the bioinformatic analysis. MQW wrote the manuscript. All authors read and approved the manuscript.

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