

# Therapeutic Effects of Modified Gengnianchun Formula on Stress-Induced Diminished Ovarian Reserve Based on Experimental Approaches and Network Pharmacology

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**Aim:** To verify the effects of modified Gengnianchun formula (MGNC), a traditional Chinese medicine, on a stressed diminished ovarian reserve (DOR) animal model and predict the underlying mechanisms through network pharmacology strategies.

**Methods:** Sexually mature female C57BL/6 mice were allocated to five groups, abbreviated as the control (C) group, stress manipulated model (M) group, stress with normal saline gavage (N) group, stress with low-dose MGNC gavage (L) group, and stress with high-dose MGNC gavage (H) group. Body weight and the estrous cycle were monitored during the stress and gavage process. Serum stress hormones and reproductive hormones were evaluated by ELISA. Ovarian follicle counts were calculated, and ovarian follicle-stimulating hormone receptor (FSHR) and anti-Müllerian hormone (AMH) expression were assessed by Western blotting and immunohistochemistry. Network pharmacology strategies included active compound screening, drug and disease target analysis, gene ontology analysis, pathway analysis, and visualization of results.

**Results:** MGNC treatment significantly decreased serum corticosterone (CORT) and follicle-stimulating hormone (FSH) levels and increased testosterone (T) levels in the H group compared with the M and N groups. Primordial and preantral follicle counts and ovarian AMH and FSHR expression were significantly increased in the H group compared to those in the M and N groups. Through pharmacokinetic screening, we found 244 active compounds in MGNC. A total of 186 candidate intersection target genes of disease and MGNC were further screened to construct the interaction network. Gene ontology and KEGG pathway enrichment analysis ultimately unveiled a series of key targets that mainly mediated the effects of MGNC on DOR induced by chronic stress. The PI3K-Akt pathway may serve as the critical pathway underlying this therapeutic mechanism.

**Conclusion:** MGNC is a promising formula to treat DOR induced by chronic stress, and the PI3K-Akt pathway may play an essential role in this effect.

**Keywords:** modified Gengnianchun formula, diminished ovarian reserve, stress, follicle development, network pharmacology, target gene

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## Introduction

Diminished ovarian reserve (DOR) is defined by a decreased quantity or quality of ovarian follicles in women. It is a condition between normal reproductive physiology and premature ovarian insufficiency (POI). Although the criteria of DOR remain unresolved and are adopted differently in various regions,<sup>1</sup> it is basically characterized

by an elevated serum follicle-stimulating hormone (FSH) level, decreased anti-Müllerian hormone (AMH) level and diminished antral follicle count on ultrasonic examination. According to US data, the prevalence of DOR increased from 19% to 26% from 2004 to 2011 among assisted reproductive treated patients.<sup>2</sup> Among various causes of DOR, psychological factors have emerged as an important one in recent years.<sup>3–5</sup> While acute or brief stressors only cause limited damage to women's fertility, chronic stressors can be detrimental to ovarian reserve, leading to adverse fertility outcomes.<sup>4,6</sup> Our previous animal study also indicated that chronic unpredictable stress (CUS) can induce a typical and long-lasting DOR phenotype in C57BL/6 mice.<sup>7</sup>

The Western treatment of DOR is reproductive hormone-based therapy, which does not help to attenuate the pathology of DOR and stress conditions. There are also side effects that limit its clinical application.<sup>8</sup> Traditional Chinese medicine (TCM) has promising effects on treating gynecological endocrinology dysfunctions.<sup>9–11</sup> Gengnianchun (GNC) is a previously widely reported formula in the treatment of menopausal syndrome<sup>12</sup> and menopause-related diseases such as Alzheimer's.<sup>13</sup> According to TCM theories, DOR shares a similar etiology with menopause, which is deficient in the kidney. With a significant effect of tonifying the kidney, GNC has also been used to treat patients with DOR and has shown a satisfactory effect in improving ovarian reserve, balancing reproductive endocrinology conditions, and improving the pregnancy rate and live birth rate. The modified Gengnianchun formula (MGNC), which constitutes three more herbs than GNC, was intended to better manage stress symptoms in patients with DOR.<sup>14</sup> Here, we plan to investigate the effects of MGNC in a stress-induced DOR animal model.

Network pharmacology emerges as a novel method to integrate information from bioinformatics, systems biology, and polypharmacology.<sup>15</sup> With a “disease-gene-target-drug” network model, it evaluates the molecular mechanism of TCM formulas from a multidimensional perspective. This thinking coincides with the holistic and systemic characteristics of TCM.<sup>16</sup> By visualizing the multitarget, multi-gene, and multipathway interactions, the mechanisms of MGNC were expected to be unveiled and further explored.

In this work, an *in vivo* experiment was carried out to observe the effects of MGNC on a stress-induced DOR mouse model. Serum corticosterone (CORT) was tested, and body weight was monitored to evaluate the effects of MGNC on stress. Serum FSH, luteinizing hormone (LH), testosterone (T), estradiol (E2), and AMH were analyzed;

protein analysis of ovarian AMH and FSH receptor (FSHR) was undertaken to observe the endocrine regulation effects of MGNC. Furthermore, network pharmacology was applied to explore the active compounds and multitarget regulation of MGNC. Underlying mechanisms and signaling pathways were further suggested by GO and KEGG analysis to unveil the effects of MGNC against stress-induced DOR.

## Materials and Methods

### Animals and Treatment

The animal experiment was based on a previously reported DOR mouse model induced by CUS.<sup>7</sup> All animal experimental procedures were approved by the Animal Ethics Committee of Fudan University. Seventy-five female C57BL/6 mice aged 6 to 8 weeks were randomly and equally divided into five groups. All mice were housed in the facility for 1 week before the experiment. Mice in the control (C) group were raised without intervention for 8 weeks. Mice in the model (M) group were subjected to 8 weeks of CUS. Mice in the other three groups were administered the 8-week CUS and oral administration of normal saline (N), a low-dose (2.23 g/kg body weight) of MGNC (L), and a high-dose (8.92 g/kg body weight) of MGNC (H). Mice were sacrificed after the 8-week manipulation. Sample collection was carried out from 8 am to 10 am when serum CORT was at the base level of daily fluctuation. The body weight of mice from each group was measured every week. The experiment was repeated three times.

### Preparation of MGNC Decoction

The MGNC formula was prepared by mixing water extracts of 15 crude herbs purchased from Tianjiang Pharmaceutical Limited Company (Jiangyin, China) and dissolved in 100°C water to make the desired concentration for gavage. The products were manufactured with rigid quality control protocols following rigid specifications of the Pharmacopeia of China. The ingredients' names and herbal information of MGNC are listed in [Table 1](#). The formula information of MGNC and the conversion of water extract weight and crude herbs provided by the manufacturer are listed in [Table 1](#).

### High-Performance Liquid Chromatography (HPLC)

A total of 34.85 g of granule mix was resolved in 336 mL of hot water to make a solution that equals 0.5 g of dry ingredients per milliliter. Samples were filtered through

**Table I** Modified Gengnianchun Formula (MGNC)

English Name (Chinese Name)	Latin Name	Plant Part	Processing Method	Crude Herb (g)	Water Extract (g)	Lot Number
Radix Rehmanniae (Shengdi)	<i>Rehmannia glutinosa (Gaertn.) DC</i>	Root	Dried	15	4.5	1,803,033
Epimedium Brevicornums (Yinyanghuo)	<i>Epimedium acuminatum Franch</i>	Root	Dried	12	0.6	1,804,138
Radix Paeoniae Alba (Baishao)	<i>Paeonia lactiflora Pall</i>	Root	Dried	12	1.2	1,804,029
Fructus Lycii (Gouqizi)	<i>Lycium barbarum L.</i>	Fruit	Dried	12	4.8	1,804,052
Plastri Testudinis (Guiban)	<i>Carapax et plastrum Testudinis</i>	Carapax	Stir-Baking with Vinegar	15	0.75	1,811,057
Rhizoma Anemarrhenae (Zhimu)	<i>Anemarrhena asphodeloides Bunge</i>	Root	Dried	15	3.75	1,802,153
Semen Cuscutae (Tusizi)	<i>Cuscuta australis R.Br.</i>	Seed	Dried	12	0.6	1,803,027
Moridae Officinalis (Bajitian)	<i>Morinda officinalis F.C.How</i>	Root	Dried	12	3.6	1,803,168
Cistanche Salsa (Congrong)	<i>Cistanche deserticola Y.C.Ma</i>	Stem	Dried	12	3.6	1,804,041
Cortex Phellodendri Amurensis (Huangbai)	<i>Phellodendron chinense Schneid</i>	Bark	Dried	9	0.75	1,804,026
Rhizoma Coptidis (Huanglian)	<i>Coptis chinensis Franch.</i>	Rhizome	Dried	3	0.5	1,803,165
Poria (Fuling)	<i>Poria cocos (Schw.) Wolf</i>	Sclerotium	Dried	9	0.9	1,804,133
Radix Bupleuri (Chaihu)	<i>Bupleurum abchasicum Manden.</i>	Root	Dried	9	1.5	1,803,134
Angelica Sinensis (Danggui)	<i>Angelica sinensis (Oliv.) Diels</i>	Root	Dried	12	4.8	1,804,210
Ligusticum Wallichii (Chuanxiong)	<i>Conioselinum chinense (L.) Britton, Sterns &amp; Poggenb</i>	Root	Dried	9	3	1,804,003

a 0.45- $\mu$ m membrane filter before HPLC analysis. The HPLC machine was an Agilent S1200 system equipped with a Zorbax SB-C18 column (Agilent Technologies, Santa Clara, CA, USA). The detection wavelength was set at 210 nm, and the flow rate was 1.0 mL/min. The injection volume was 10  $\mu$ L. The column temperature was maintained at 30°C. The mobile phases were acetonitrile (A) and water-phosphoric acid (B, 0.01%, v/v). The eluting conditions were as follows: 0–19 min, 10–18% (A); 19–25 min, 18% (A); 25–56 min, 18–35% (A). According to the Pharmacopeia of China, mangiferin, paeoniflorin, hyperin, and icariin are the most important therapeutic constituents of Zhimu, Baishao, Tusizi, and Yinyanghuo, respectively. Ferulic acid is the main therapeutic constituent of Danggui and Chuanxiong. These six ingredients also play a key role in regulating ovarian function and ameliorating emotional problems according to the traditional

application of each ingredient. Therefore, for quality control, these five standard references were adopted to evaluate the quality and to establish HPLC fingerprints of MGNC. Standard references were purchased from the National Institutes for Food and Drug Control.

## Sample Collection and Analysis

### Estrous Cycle Analysis

Smears of vaginal epithelial cells were carried out every day at the same time in the last 3 weeks before sacrifice to monitor the estrous cycle of mice. The principle of defining the stage of the estrous cycle was based on a previous report.<sup>17</sup>

### Serum Hormone Assay

Mouse blood was collected from the heart after the intervention, and serum was separated by centrifugation. Hormone markers of chronic stress, CORT (LDN, AR E-8100) and

reproductive hormone LH (Shibayagi, AKRLH-010), FSH (Bioss, bsk00462), AMH (Enzyme-linked Biotechnology, YX-011308M), T (LDN, AR E-8000), and E2 (LDN, FR E-2000) were analyzed by enzyme-linked immunosorbent assay (ELISA) kits following the manufacturer's instructions. For each assay, there were 6–12 samples in each group with two replicates. The inter- and intra-assay variations of each kit were lower than 15%.

### Ovary Serial Section and Follicle Counting

One of the ovaries of mice in each group was harvested and fixed in paraformaldehyde for 48 h. The other ovary of each mouse was stored at  $-80^{\circ}\text{C}$  for further usage. After embedding in paraffin, ovaries were serially cut into 4- $\mu\text{m}$ -thick sections and stained with hematoxylin and eosin (H&E) for follicle counting based on previous methods.<sup>18</sup> Total numbers of follicles and corpus luteum per ovary were calculated by adding the counts of every fifth section throughout the entire ovary. The follicles were classified into four developing stages according to the modified Oktay system.<sup>19</sup> The counting was carried out by two different persons.

### Western Blot

Frozen ovaries were homogenized in lysis buffer. The lysates were boiled for 3 min, mixed with 5X loading buffer, and subjected to SDS-PAGE. After electrophoresis, the protein was transferred onto a polyvinylidene fluoride membrane (Millipore, USA) and blocked with 5% defatted milk in Tris-buffered saline Tween (TBST) at room temperature for 1 hour. The membranes were incubated with primary antibodies against FSHR (Thermo Fisher Scientific, PA5-50963) or AMH (Abcam, ab103233) overnight at  $4^{\circ}\text{C}$  and then washed with TBST three times, followed by incubation with goat anti-rabbit IgG antibody (Cell Signaling Technology, 4414) for 1 hour at room temperature. After washing three times, the immunoblots were detected by the ECL method using the ECL Western Blotting Substrate (Millipore, WBKLS0500). The quantification of bands was performed by ImageJ 1.51k (National Institutes of Health, USA).

### Immunohistochemistry

Ovarian sections were deparaffinized in xylene, rehydrated in a graded ethanol series, and boiled in 10 mM sodium citrate for antigen retrieval. Nonspecific binding sites were blocked by incubating in a 0.3%  $\text{H}_2\text{O}_2$  solution and blocking with 10% normal goat serum. Sections were incubated with anti-FSH receptor antibody (Abcam,

ab150557) or anti-AMH antibody (Abcam, ab24542) at  $4^{\circ}\text{C}$  overnight. After washing in PBS three times, the sections were incubated for 45 min with HRP-conjugated horse anti-rabbit IgG at room temperature. Finally, the sections were washed again in PBS three times, stained with DAB and counterstained with hematoxylin. After dehydration in an ethanol gradient and sealing with neutral gum, sections were observed under a microscope.

### Network Pharmacology Analysis MGNC Compound Library Construction

Information on herbal compounds and potential compound targets was retrieved from the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP). The information of *Plastri Testudinis* was obtained from other databases, including TCMID,<sup>20</sup> TCM-ID,<sup>21</sup> TCM-Mesh,<sup>22</sup> and text mining.<sup>23,24</sup> To screen out the potential ADME-favorable molecules from the large number of active components, the oral bioavailability (OB) threshold was set at 30%, and the drug likeness (DL) threshold was set at 0.18.<sup>25</sup> When compounds met these two criteria, their relevant targets were adopted as candidate compounds and drug targets.

### Collection of Potential Targets of Stress-Induced DOR

Genes associated with DOR were identified with GeneCards ([www.genecards.org](http://www.genecards.org)), a database that provides comprehensive information on all annotated and predicted human genes, and the Online Mendelian Inheritance in Man database ([www.omim.org](http://www.omim.org)), a comprehensive and authoritative database of human genes and genetic phenotypes on all known Mendelian disorders. The keywords used in searching were “diminished ovarian reserve” and “decreased ovarian reserve”. Intersection genes of drug targets and disease targets were used for further analysis.

### Gene Name Identification and Network Construction

The gene names of the selected drug targets were standardized using the UniProt Knowledge database ([www.uniprot.org](http://www.uniprot.org)). Gene sets of disease and MGNC were analyzed and visualized by RStudio 1.2.5001. The ingredient-compound network and compound-target network were constructed by Cytoscape 3.7.2. A target protein–protein interaction (PPI) network was constructed by STRING ([string-db.org](http://string-db.org)) with the species set to ‘homo sapiens’ and confidence score set to  $>0.9$ , which was further edited and



visualized by Cytoscape 3.7.2. Finally, GO analysis of candidate targets was conducted by WebGestalt 2019.<sup>26</sup> KEGG enrichment analysis was generated by clusterProfiler package<sup>27</sup> and RStudio 1.2.5001.

## Statistical Analysis

All data are presented as the mean value  $\pm$  standard error (SEM) from at least three independent experiments. SPSS 22.0 software was used for statistical analysis. Comparisons between multiple groups were analyzed by ANOVA. The comparison of body weight changes between two groups was conducted using repeated measurement analysis of variance. P-values of less than 0.05 were considered statistically significant. Network centrality analysis was assessed by two topological measurements called degree and betweenness.<sup>28</sup> The degree is the number of links to other nodes in a network. It is associated with the topological robustness of biological networks. Betweenness is the number of shortest paths that pass through each node, which functions as a “gatekeeper” to control the flow of interactions in the network. Topological data were generated from Cytoscape 3.7.2 software.

## Results

### HPLC Result of MGNC

To confirm the authenticities of the ingredients, the chromatogram of MGNC was examined and contrasted with five standard references (Figure 1). The constituents of MGNC were well separated under the optimized conditions. Five components of MGNC were identified as mangiferin, paeoniflorin, ferulic acid, hyperin, and icariin. According to the Pharmacopoeia of China, these components represent the most important therapeutic contents belonging to six ingredients out of the fifteen ingredients of the MGNC formula. The six ingredients were selected as promising ingredients that contribute most to the effect of chronic stress-induced DOR therapy.

### Mice

Body weight is one of the primary indexes to monitor the efficacy of stressors. As shown in Figure 2A, mice in the model, NS and MGNC-treated groups exhibited significantly lower body weight gain than mice in the nonstressed control group during the eight-week stress paradigm. Mice subjected to both stressors and gavage treatment showed a slightly lower body weight than model mice. The mean body weight of mice administered a high-dose of MGNC was higher than that of mice in the NS group, but the

difference was not significant. Furthermore, serum CORT, the most crucial chronic stress hormone, was markedly elevated in the M and N groups but was significantly down-regulated in the high-dose MGNC-treated group (Figure 2B,  $P < 0.05$ ). Another prominent effect of chronic stress is on female menstruation. We observed a significantly lengthened diestrus phase in model mice and NS-treated mice during the last 3 weeks of experiments (Figure 2C). This abnormality was not completely recovered to regular lengths but was attenuated in the MGNC-treated groups, with a better effect in the high-dose MGNC-treated group (Figure 2C).

Figure 2D shows that primordial follicles ( $P < 0.05$  in the M and N groups), primary follicles ( $P < 0.05$  in the M and N groups), secondary follicles ( $P < 0.05$  in the M group) and antral follicles ( $P < 0.05$  in the N group) declined in the M and/or N group compared to the C group, while primordial follicle and preantral follicle numbers were significantly improved in the H group compared to the M and/or N group ( $P < 0.05$ ). No corpus luteum was observed in the M, N, L, or H groups, indicating disturbed ovulation.

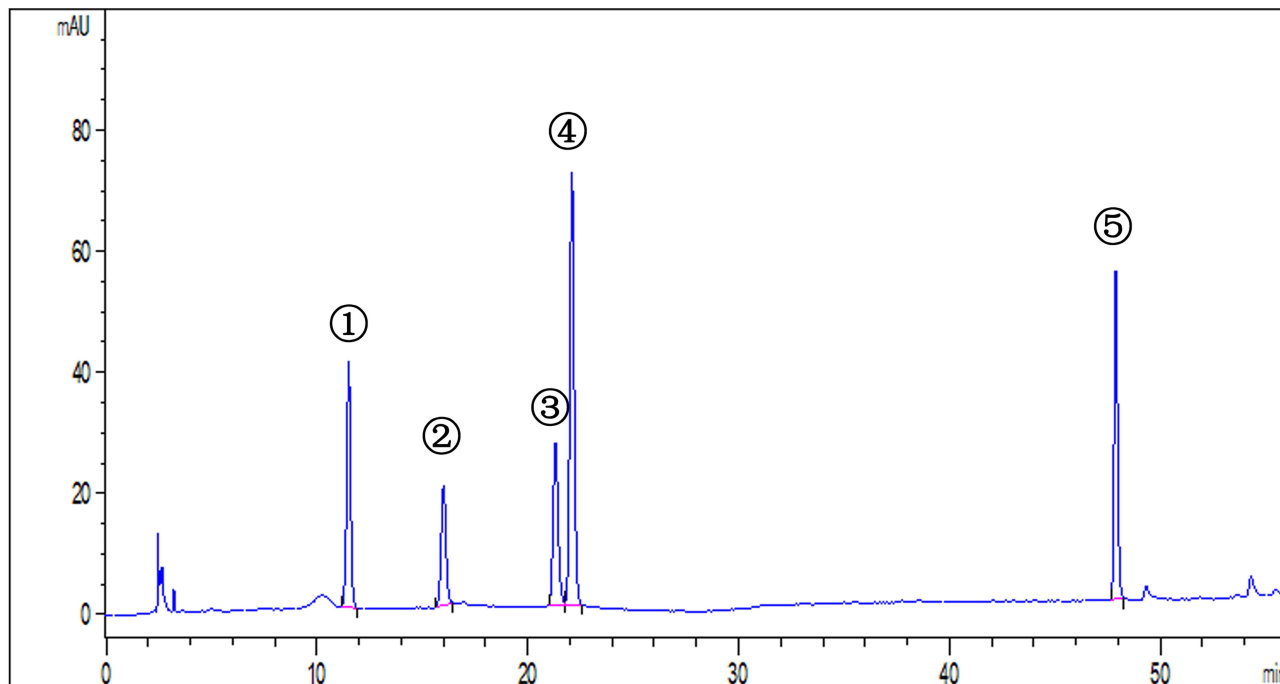
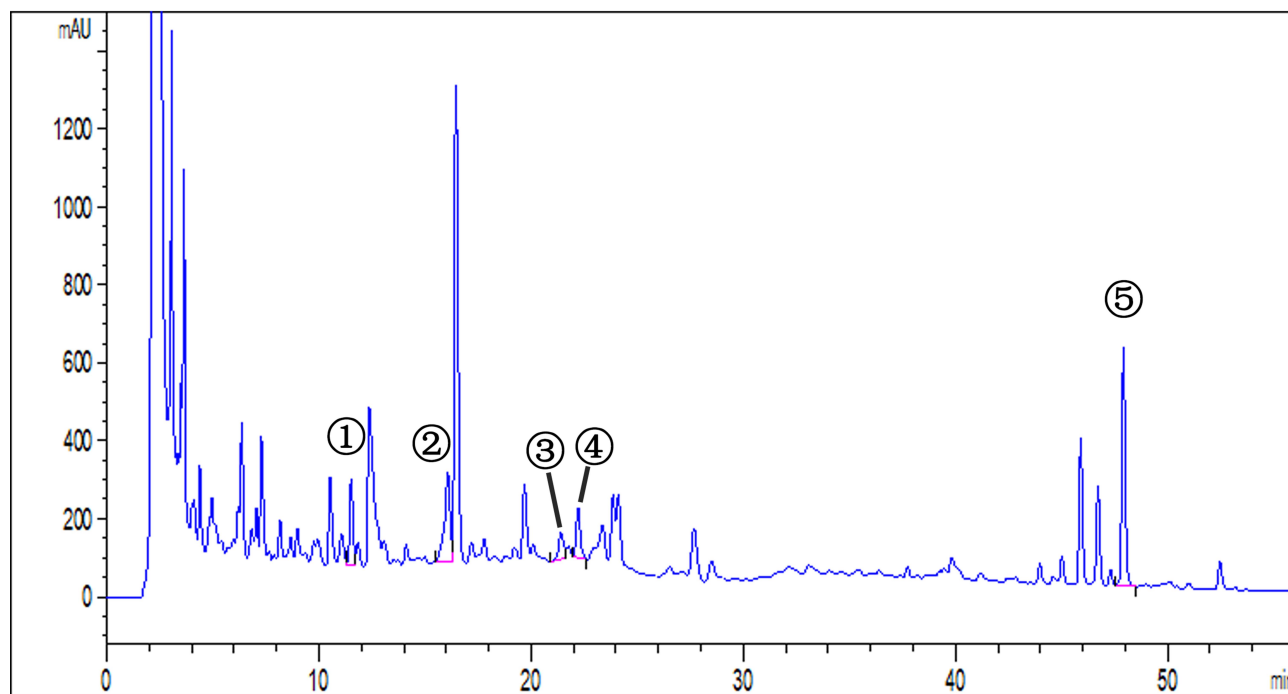
### MGNC Balances Abnormal

### Endocrinology Dysfunction in Stress-Mice

Reproductive endocrinology dysfunction is one of the primary symptoms of DOR. As Figure 3 shows, significantly elevated FSH levels and significantly decreased LH, AMH, E2, and T levels were observed in the M and N groups ( $P < 0.05$ ). With MGNC treatment, the abnormal FSH and T levels were attenuated compared to mice in the N group ( $P < 0.05$ ). While no significant recovery in E2, LH, and AMH levels was observed, serum LH and AMH levels tended to be elevated in the high-dose MGNC-treated group. The effect of MGNC showed a dose-dependent trend in the above results.

### MGNC Treatment Increases Ovarian AMH and FSHR Expression

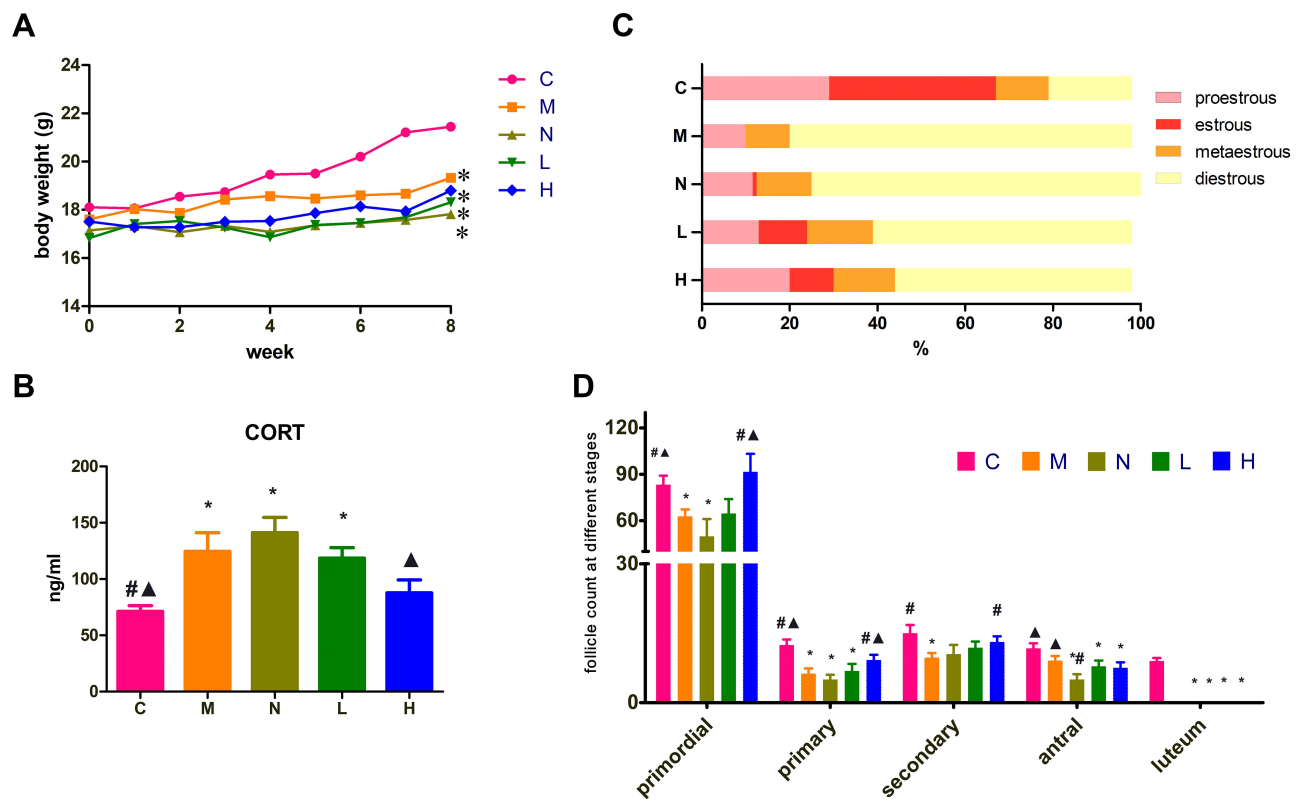
While serum AMH results can be inaccurate because of dissatisfactory ELISA kits, ovarian protein analysis can better reveal the status of follicle AMH expression. Immunohistochemistry and Western blot analysis were carried out to assess ovarian AMH and FSHR expression. In Figure 4A, typical images of AMH-positive preantral and antral follicles are shown. Deeply stained follicles were observed in the C and H groups. The immunoblot analysis results were consistent with the histological images

**A****B**

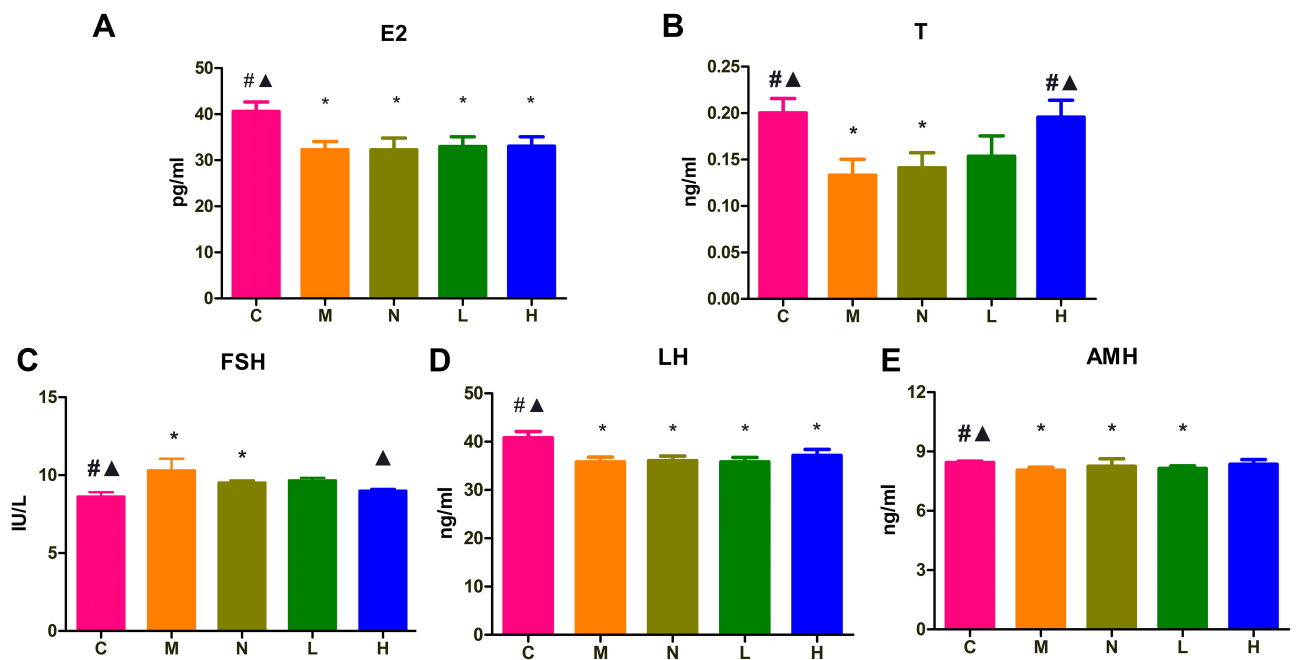
**Figure 1** HPLC results of standard references and MGNC. **(A)** HPLC result of five standard references. **(B)** The HPLC fingerprints of MGNC sample. Indicated four peaks were: ①mangiferin; ②paeoniflorin; ③ferulic acid; ④hyperin; ⑤icariin.

(Figure 4D). Quantitative analysis showed that MGNC treatment significantly increased ovarian FSHR expression by immunohistochemistry (Figure 4B,  $P < 0.05$ ) and increased

AMH protein expression by Western blotting (Figure 4F,  $P < 0.05$ ) compared to the NS or Model groups, yet the immunohistochemical AMH data were not significant between the



**Figure 2** Model effect validation and MGNC effects on stress management and ovarian reserve. (A) Average body weight of mice in different groups changes during the 8-week CUS stress paradigm. (B) Serum CORT level of mice in each group. (C) The percentage of time (days) at four estrous phases in the last three weeks of the stress paradigm. (D) The number of follicles at different stages in mice ovaries. Primordial: primordial follicles; primary: primary follicles; secondary: secondary follicles; antral: antral follicles; atretic: atretic follicles; luteum: corpus luteum. \*: P<0.05 compared with C group, #: P<0.05 compared with M group, ▲: P<0.05 compared with N group.



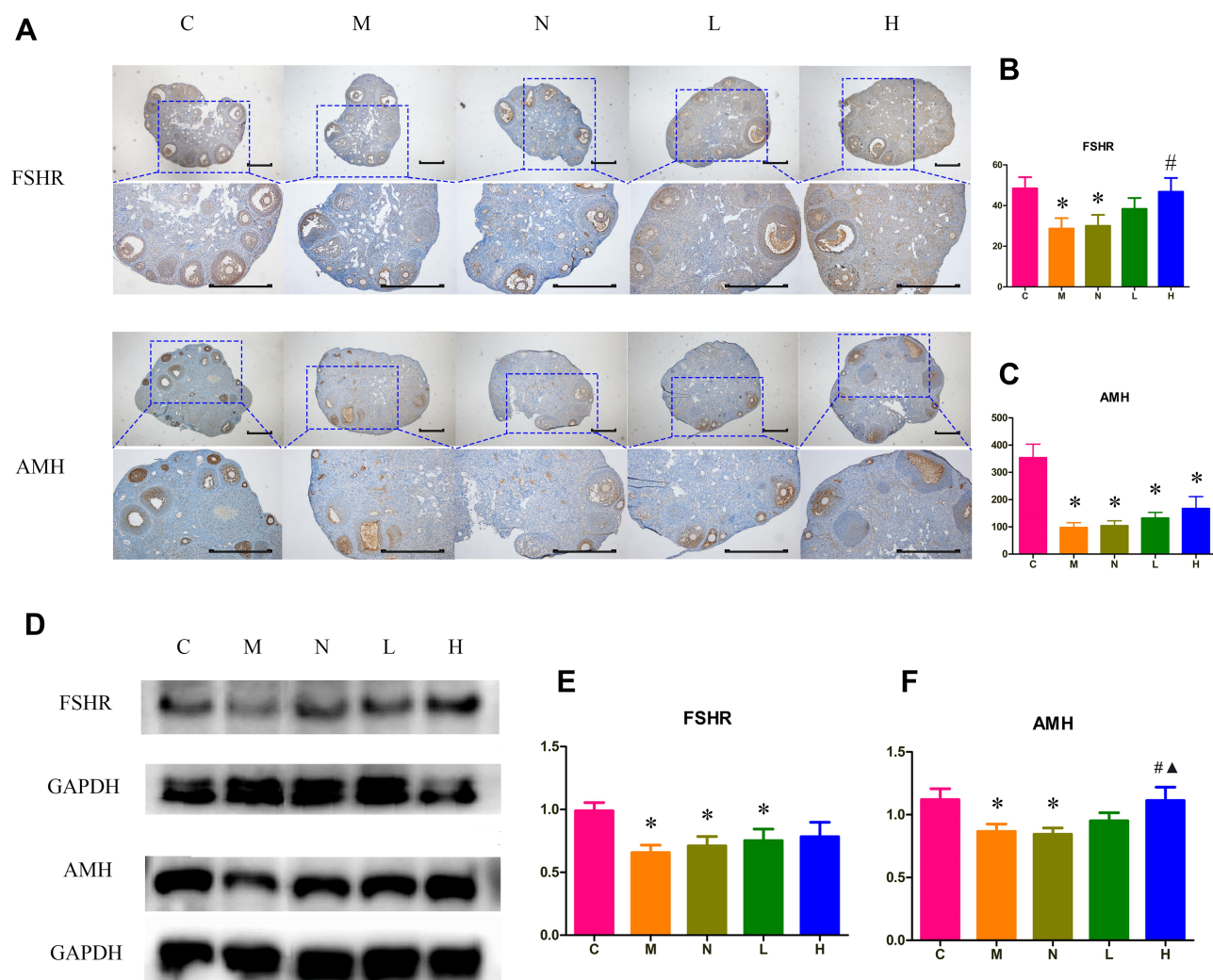
**Figure 3** ELISA analysis of serum reproductive hormones. (A)–(E) shows ELISA analysis results of serum E2, T, FSH, LH, and AMH level of mice in different groups. \*: P<0.05 compared with C group, #: P<0.05 compared with M group, ▲: P<0.05 compared with N group.

H and M/N groups, nor were the Western blotting data for FSH (Figure 4C and E). Considering that both methods are semiquantitative, it can be speculated that MGNC therapy improved ovarian AMH and FSHR protein expression.

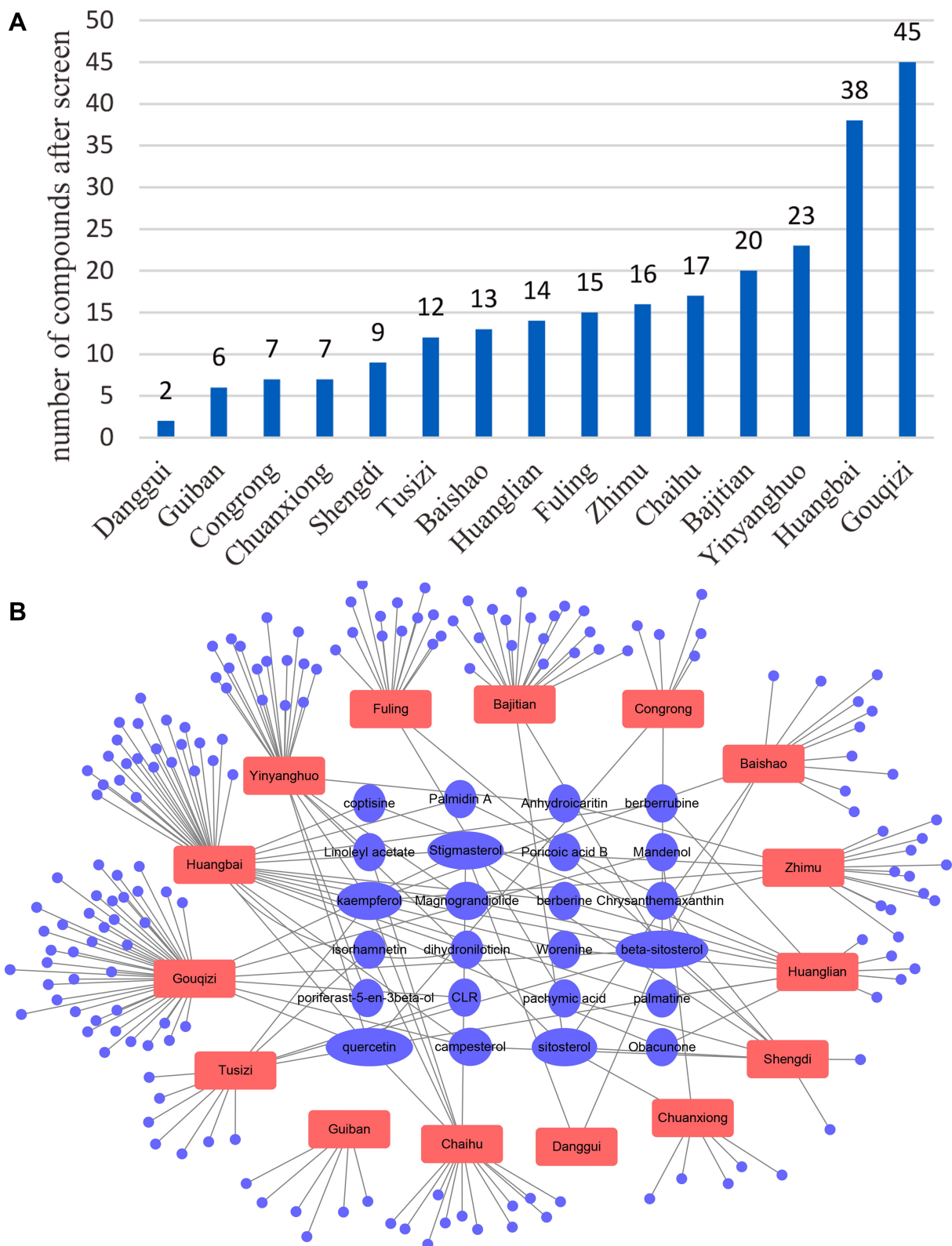
## Database Miming and Compound Network Analysis Revealed the Active Compounds in the MGNC Formula

After ADME screening, a total of 244 active compounds from 15 ingredients of MGNC were selected to compose a compound library for further study (Supplementary Table S1). The numbers of compounds from each herb are shown in Figure 5A. Figure 5B shows a network

between herbs and the compounds they contain. Common compounds shared by at least two ingredients were placed inside the circle of ingredient nodes labeled by the compound names. Except for Guiban, an animal-derived ingredient, all other 14 ingredients in the formula share at least one active compound. Twenty-four compounds were shared by at least two ingredients from the formula. The top five compounds shared by ingredients were beta-sitosterol, stigmasterol, quercetin, kaempferol, and sitosterol, with network degrees ranging from 5 to 8. All five compounds are flavonoids, a class of substances that widely exists in many kinds of plants and is well known for their antioxidant and anti-aging effects.



**Figure 4** Protein analysis of ovarian FSHR and AMH in different groups. (A) Immunohistochemistry analysis of ovary sections in different groups. (B) and (C) are quantitative analysis of the positive stained part in follicles. (D) Images of the Western-blot results in different groups. (E) and (F) are quantitative analysis of the blot bands. \*:  $P < 0.05$  compared with C group, #:  $P < 0.05$  compared with M group, ▲:  $P < 0.05$  compared with N group. Magnification: 400X. Scale bar: 25 $\mu$ m.



**Figure 5 (A)** Compound number after ADME screen of each herb in MGNC. **(B)** Compound network of MGNC. Compounds shared by at least two herbs are pictured in blue eclipse and encircled by herb names labeled in red squares. Other blue circles without label represent unique compounds owned by related herbs. Width of compound nodes represents the degree of the compounds in the network.



## Compound-Target Network Analysis Determined the Most Relative Compounds of MGNC and Disease Targets

Since the effect of MGNC on chronic stress-induced DOR is our main concern, “stress” and “DOR” were combined as a disease set. Relationships between targets of this disease set and the drug targets were further investigated. Through the online databases, we managed to find a total of 267 drug targets related to the 244 active compounds ([Supplementary Table S2](#)), 981 DOR-related targets, and 1953 stress-related targets ([Supplementary Table S3](#)). One hundred eighty-six intersection genes were shared by the disease set and MGNC ([Figure 6A](#)). One hundred eighty-six shared targets and 135 compounds directed to these targets were further used to construct a compound-target network ([Figure 6B](#), [Supplementary Table S4](#)). Notably, the top ten compounds with the highest degrees are quercetin (degree=108), kaempferol (degree=48), luteolin (degree=45), 51095-85-3 (degree=37), beta-sitosterol (degree=34), isocorypalmine (degree=28), (S)-canadine (degree=26), atropine (degree=26), isohamnetin (degree=26), and stigmasterol (degree=25). These ten compounds include the four most shared compounds among ingredients, as described in the former section, indicating a collaborative effect of the ingredients of MGNC on stress-induced DOR. The top ten targets with the highest degrees are PTGS2 (degree=73), PTGS1 (degree=49), PGR (degree=47), SCN5A (degree=35), PRKCA (degree=30), ADRB2 (degree=27), AR (degree=26), HTR2A (degree=26), NR3C2 (degree=24), and ESR1 (degree=23). PGR, AR, and ESR1 are three important reproductive hormone receptors and are involved in various stages of follicle development.

## PPI Network Construction and Validation Denote “Hub Nodes” in the Biological Function of MGNC in Treating Chronic Stress-Induced DOR

One hundred eighty-six intersection genes were considered candidate targets and were used to generate a PPI network ([Figure 7A](#)). Since there were only three extra herbs added to MGNC, the targets of MGNC and GNC were supposed to be similar to a large extent. Therefore, targets of the GNC formula in our previously reported literature were also denoted in the network to validate

the reliability of network pharmacological analysis. These targets were APP, IL1B, TNF, NFKB1A, Akt, CASP3, BAX, PARP1, IL6, IL2, INFG, MAPK1, and SOD1. Additional targets in the network not directly verified but probably related to the effect of GNC in previously reported work are also marked in [Figure 6](#). The network was degree visualized by two topological parameters: degree and betweenness. Statistical analysis results of these two parameters are shown in [Figure 7B](#) and C. A few proteins occupied high centrality in the PPI network. Proteins such as AKT1, APP, TP53, JUN, TNF, MAPK1, CXCL8, IL6, and EGFR have both a high degree and betweenness. Other proteins worth noting were ESR1 and a cluster of CYP family proteins, which are essential for steroidogenesis.

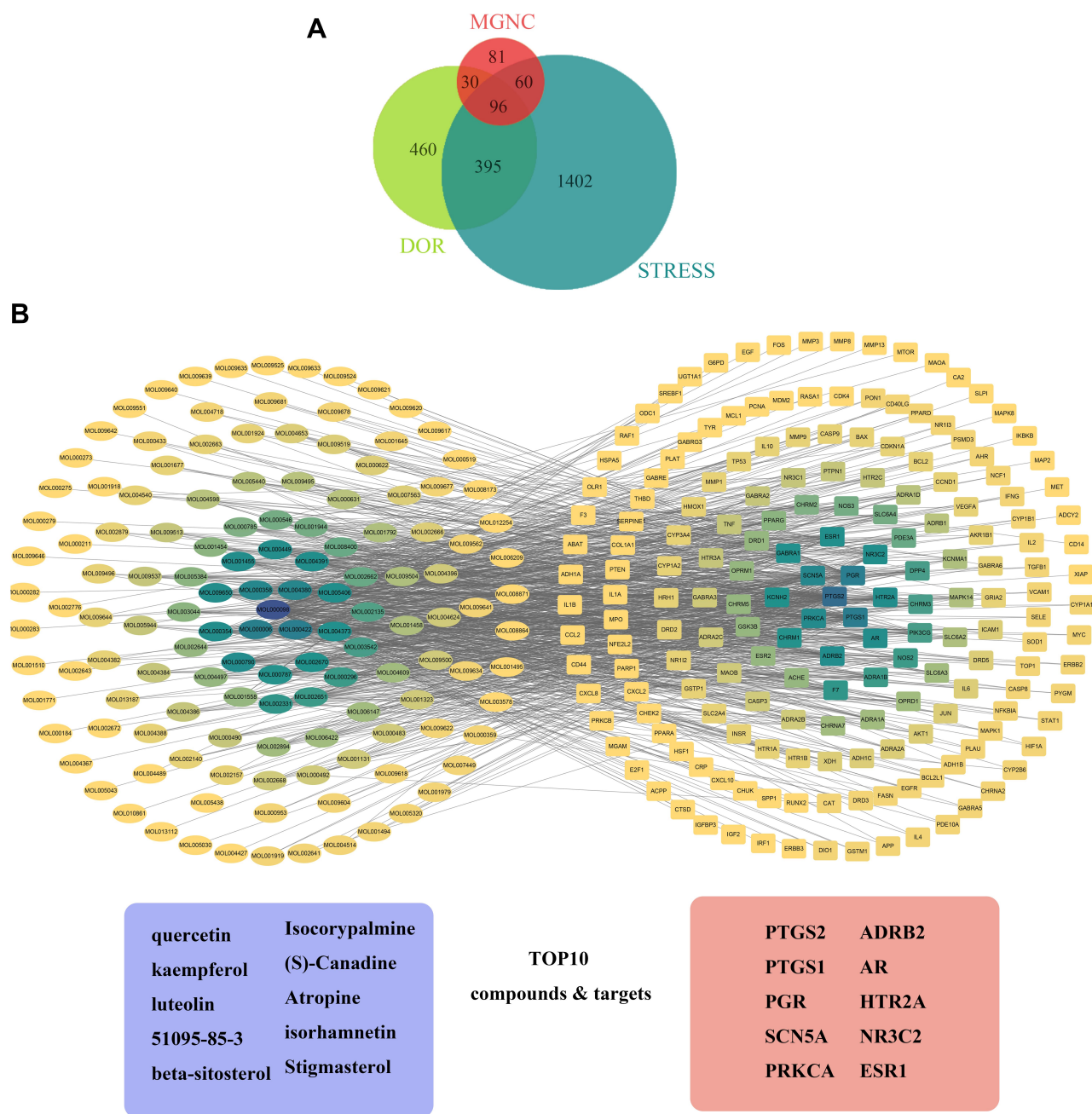
## GO and KEGG Enrichment Results Predict the Probable Mechanism of MGNC

Based on 186 identified targets, GO analysis was conducted, and the most enriched GO terms of biological process (BP), cellular components (CC) and molecular functions (MF) are exhibited in [Figure 8A](#). The most enriched GO terms of BP were “response to stimulus,” “biological regulation,” “metabolic process multicellular organismal process,” “cell communication,” “developmental process,” “cellular component organization,” “cell proliferation,” “multiorganism process,” and “reproduction and growth.” The most enriched GO term of CC was “membrane,” with a gene ratio that exceeds that of other GO terms by a lot. The most enriched GO terms of MF were “protein binding,” “ion binding”, and “molecular transducer activity.”

As shown in [Figure 8B](#) and C, the PI3K-Akt pathway was the pathway enriched with the most target genes. Pathways with the highest adjusted p values also include the “AGE-RAGE signaling pathway in diabetic complications,” inflammatory and anti-pathogen-related pathways, and cancer-related pathways. [Figure 8D](#) shows a schematic diagram of the PI3K-Akt pathway. It is easily observed that the great majority of pathway molecules were covered by MGNC targets.

## Discussion

DOR is one of the leading causes of infertility, abortion, and advanced menopause. Due to the fast pace of modern society, chronic stress has emerged as an unneglectable

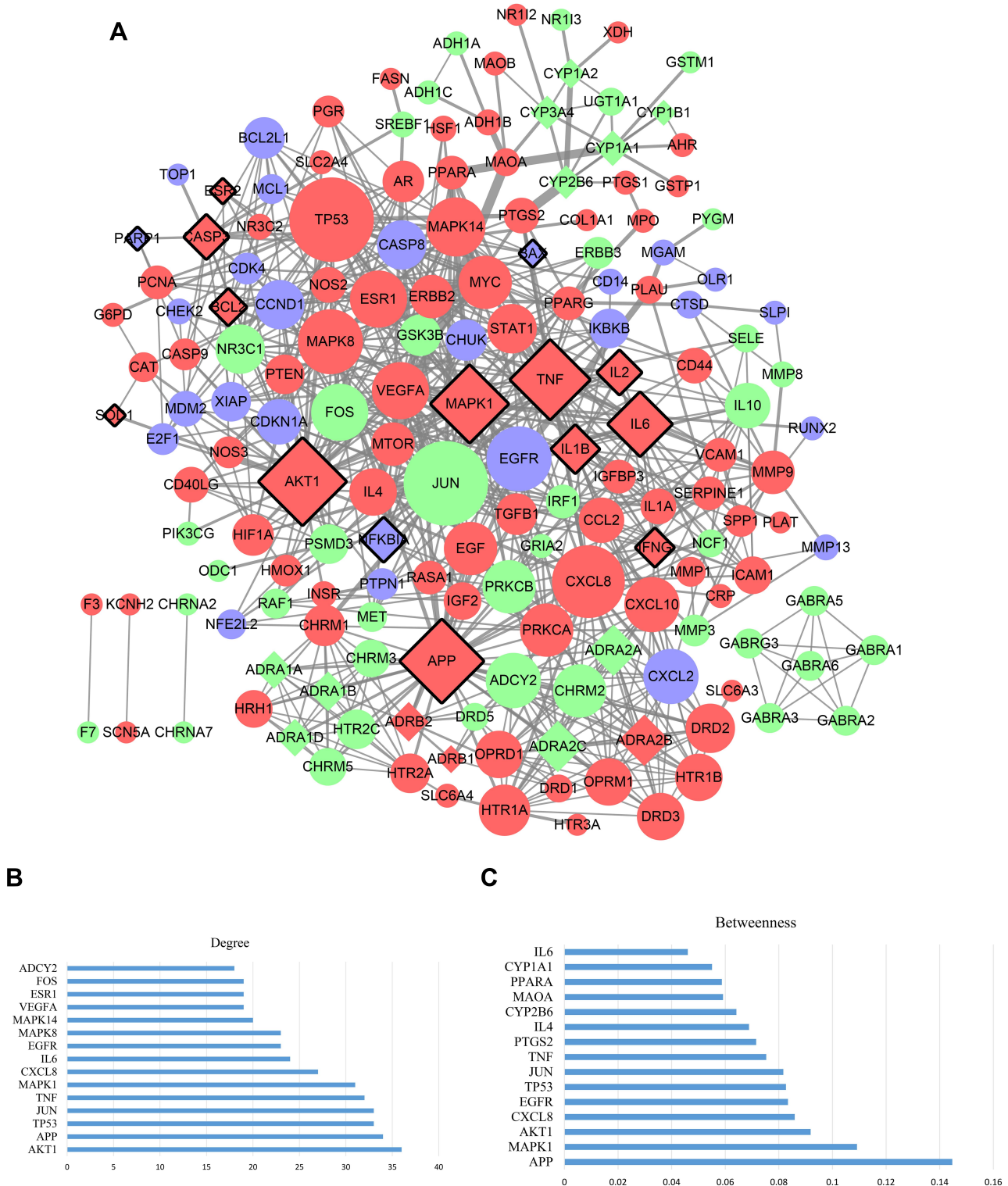


**Figure 6** Disease and MGNC target analysis and Compound-Target network of MGNC on stress-induced DOR. **(A)** Venn diagram of target numbers shared by MGNC formula, DOR, and psychological stress. **(B)** Compound-Target network of intersection targets and related active compounds. Ellipse represents active compounds, and rectangle represents intersection targets of MGNC, stress, and DOR. The depth of color represents degree of each node. The deeper degree is, the deeper the color is. The downside of the network listed nodes of compounds and targets with top 10 degree numbers.

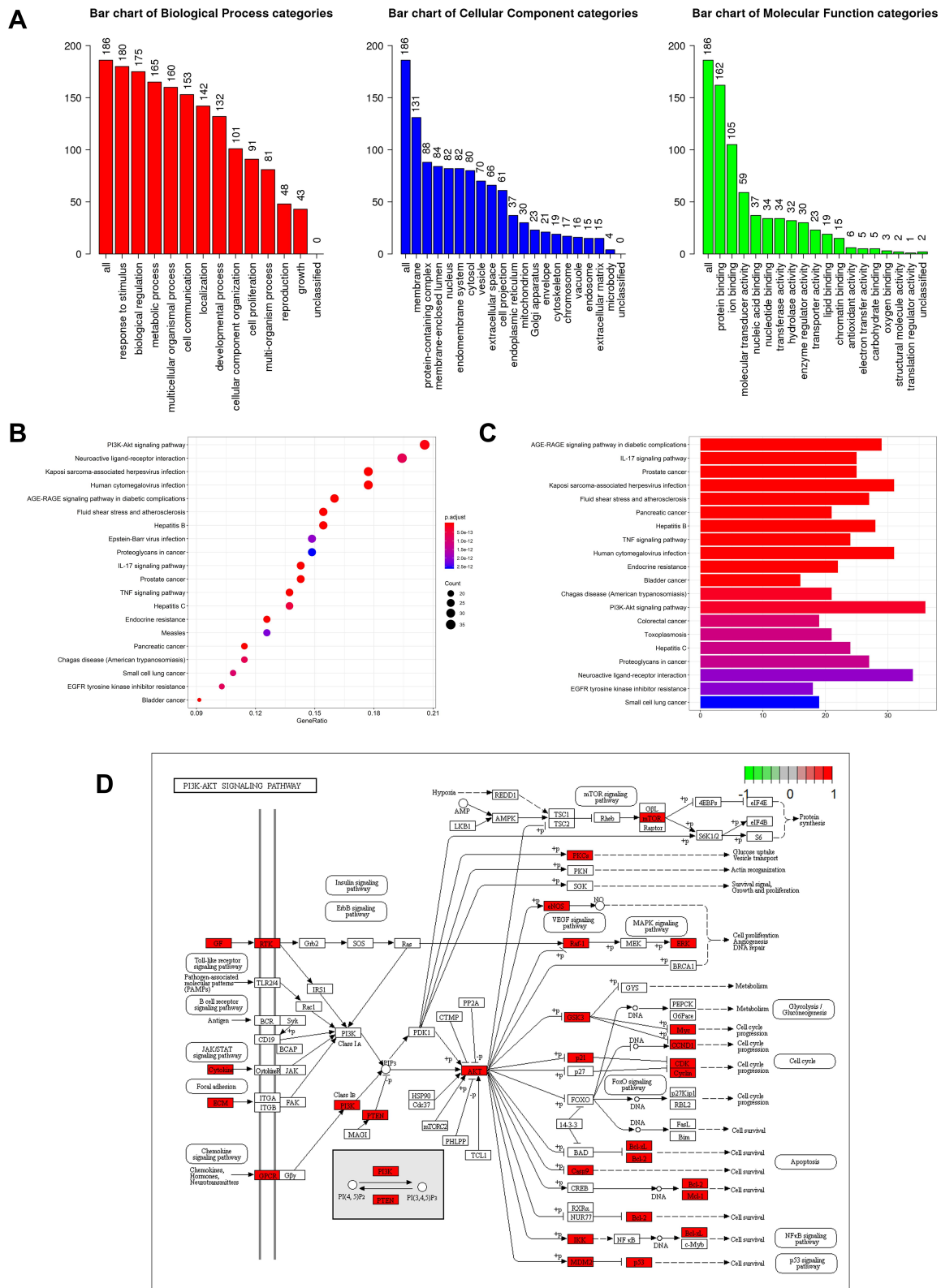
cause of DOR. Compared to simple hormone therapy adopted to treat DOR in Western medicine, TCM has outbalanced multitarget and multipathway regulation effects. Through animal model verification and network pharmacology exploration, the results of the current study revealed that a TCM formula, MGNC, exerted promising effects in improving ovarian reserve induced by chronic

stress, and this effect is probably mediated by a series of key targets and the PI3K-Akt pathway.

GNC is a formula mainly applied clinically to alleviate perimenopausal syndrome and ovarian aging-related diseases, such as Alzheimer's disease, which was previously widely studied by our group.<sup>12,13,29,30</sup> Ovarian aging shares similar phenotypes with DOR, as both feature



**Figure 7** Target PPI network analysis of MGNC formula. **(A)** PPI network of the 186 intersection targets shared by MGNC, DOR, and psychological stress. Green circles represent the 30 unique targets shared by MGNC and DOR. Blue circles represent the 60 unique targets shared by MGNC and psychological stress. Red circles represent the 96 common targets of three sets. The size of circles represents the degree of targets in the network. Diamond nodes with black border indicates previously reported targets of GNC formula. Diamond nodes without black border indicate targets verified indirectly related to GNC formula. The edge between circles represents protein interactions. Width of edges stands for the betweenness in the network. **(B)** Bar chart of targets with top 15 degree. **(C)** Bar chart of targets with top 15 betweenness.



**Figure 8** Functional enrichment analysis of target genes. **(A)** Three ontology subset results of GO enrichment analysis. **(B)** Bubble chart result of KEGG enrichment analysis. **(C)** Bar chart result of KEGG enrichment analysis. **(D)** Schematic diagram of PI3K-Akt pathway, the pathway with most gene enriched. Molecules labeled in red represent targets covered by MGNC formula.



a decreased follicle pool and hypogonadal endocrine status. Thus, GNC has also been used to treat patients with DOR. In TCM applications, formulas should be flexibly used according to the patient's general conditions, such as appetite, sleeping habits, and mental status. According to TCM theory, stress is a liver depression syndrome. Chaihu, danggui, and chuanxiong are three ingredients that belong to the liver meridian. They can play the role of soothing the liver to relieve depression, nourishing blood and promoting blood circulation, and regulating emotions. Studies have found that chaihu attenuated CUS-induced depression-like syndrome, increased brain-derived neurotrophic factor (BDNF) expression,<sup>31</sup> and regulated neurotransmitters of the hippocampus and frontal cortex.<sup>32</sup> Danggui<sup>33</sup> and chuanxiong<sup>34</sup> have also been proven to have an antidepressant effect, and this was partly mediated by the effect of hematological and cardiovascular system regulation.<sup>33,35</sup> Therefore, these three ingredients were added to GNC to form MGNC and garner comprehensive effects in clinical practice to treat patients with DOR with obvious stress symptoms. In this study, based on a stress-induced DOR animal model established in our previous work,<sup>7</sup> we further validated its effects and investigated the mechanism by network pharmacology.

To validate the stress effect, we monitored body weight throughout the stress paradigm and serum CORT level.<sup>36,37</sup> We found a significantly decreased body weight growth rate and elevated CORT level after stress in the model and NS groups. However, mice in the MGNC-treated group exhibited slightly higher body weight data than those in the NS group. Although the body weight of mice remained significantly lower than that of the control group, serum CORT was significantly downregulated to almost normal levels in the high-dose MGNC-treated group. This indicates that MGNC alleviates stress levels and thus indirectly decreases stress damage to the ovary.

Ovarian reserve constitutes a group of growing follicles and another group of resting primordial follicles.<sup>21</sup> Although only the former part of follicle reserve is often referred to as ovarian reserve clinically, it represents the functional ovarian reserve (FOR) and is only a small fraction of "total ovarian reserve (TOR)"<sup>38</sup> The total pregnancy possibility also depends on the resting follicles. Western hormone therapy only targets growing follicles and improves the quality of growing follicles. However, our results show that MGNC has significant effects not only in increasing preantral follicles and generating more regular estrous cycles but also in improving primordial

follicle counts in mouse ovaries. These results indicate that MGNC has a protective effect on the ovarian follicle pool while maintaining regular follicle recruitment.

Serum reproductive hormone analysis revealed a significant elevation of the T level, as well as unchanged E2 and LH levels, under MGNC formula treatment. T is the precursor of E2 biological synthesis. Lower serum T levels are prevalent in the DOR population<sup>39</sup> and impair pregnancy outcome.<sup>40</sup> While FSH is usually believed to stimulate late antral follicles to become preovulating follicles, androgen receptors appear in the highest concentrations in immature preantral and early antral follicles.<sup>41</sup> Androgen supplementation is beneficial in improving the quality of early-stage follicle maturation, suggesting that androgen plays an essential role in primary to secondary follicle transformation.<sup>42</sup> Among all androgens, T and its metabolite 5 $\alpha$ -dihydrotestosterone are the most potent naturally occurring agonists.<sup>43</sup> These studies support that MGNC improves ovarian reserve by improving serum T levels, which supports early follicle growth and leads to improvement in FOR.

AMH and FSH are two main diagnostic criteria of DOR. Although we did not observe significantly elevated serum AMH levels in mice under MGNC treatment compared to those in the model or NS group, protein analysis revealed AMH upregulation in mouse ovaries. A recent study indicates that women with DOR may have more follicles than their AMH levels imply.<sup>44</sup> Even in women with extremely low AMH levels, the probability of pregnancy remains.<sup>45</sup> We observed a significantly decreased serum FSH level and upregulated ovarian FSHR after MGNC treatment under stress conditions. These results imply a more favorable status of granulosa cells to proliferate under FSH stimulation and improved follicle quality. Altogether, these results demonstrate that MGNC is effective in attenuating reproductive abnormalities and improving ovarian responsiveness to hormone stimulation in stress-induced DOR mice.

In further network analysis, we tried to determine what kind of compounds play a critical role in the MGNC treatment of stress-induced DOR and what kind of biological targets, processes, and pathways could be involved in the mechanism of this TCM therapy. Taking advantage of database information and topological analysis strategy, we identified five flavonoid compounds as the most shared compounds in 15 herbs of the MGNC formula. Flavonoids widely exist in natural products and have been profoundly studied in recent years for their wide biological effects including anti-inflammation, metabolism



regulation, antioxidant, and anticancer.<sup>46–50</sup> In ovarian biology, they have been reported to be effective in activating primordial follicles,<sup>51</sup> improving oocyte quality,<sup>52</sup> promoting follicle growth<sup>53</sup> (Santos, Monteet al 2019), promoting DNA damage repair,<sup>54</sup> and inhibiting aromatase.<sup>55</sup> These results correspond with our observations of significantly improved preantral follicle counts and significantly elevated T levels rather than E2 levels after MGNC treatment (Figure 3A and B).

In the PPI network analysis, we used intersection targets shared by disease targets and MGNC therapy targets to depict the multitarget regulation of MGNC. In this network, we found many targets that were the same as targets in our previously reported study of GNC therapy in the regulation of a series of neuroendocrinology network malfunctions. For example, the APP, IL1B, and TNF proteins and the NF- $\kappa$ B pathway are related to the immunoregulatory effects of GNC against Alzheimer's disease.<sup>29</sup> The IL6, IL2, and INFG proteins are involved in GNC's effect on improving learning and memory ability in ovariectomized rats.<sup>56</sup> The MAPK pathway mediates the antiapoptotic effects of GNC in PC-12 cells.<sup>30</sup> The Akt, CASP3, BAX, and PARP1 proteins are PI3K-Akt pathway targets found to be involved in the antiapoptosis effect of GNC in the VCD-induced DOR model.<sup>57</sup> SOD1 was the target molecule of the antioxidant effect of GNC in a *Caenorhabditis elegans* study.<sup>58</sup> With only three herbs added, MGNC was supposed to share many targets with GNC. This target network not only exhibits targets that coincide with our previous work, which validated the reliability of network pharmacology, but also provides many more targets and potential biological processes that are worth further exploration.

Gene ontology enrichment and KEGG analysis helped to clarify the mechanism underlying the effectiveness of MGNC. The enriched GO term "controlling stress stimulus" conformed to the expectation of stress management and mobilizes the cell response to signaling molecules. The most enriched GO term of CC was cell membrane, where cell receptors are mostly found. The most significantly enriched GO term of MF was "protein or ion binding", and "molecular transducer activity" also implies the importance of signaling molecules. The PI3K/Akt pathway was the most enriched pathway in our study and had unparalleled significance in ovarian function.<sup>59</sup> It is a critical pathway in regulating ovarian functions, including quiescence, activation and survival of primordial follicles, granulosa cell proliferation and differentiation and meiotic maturation of oocytes.<sup>59</sup> Almost all upstream and

many downstream molecules of this pathway were enriched, which supports the multitarget effects and functional efficacy of MGNC in stress-induced DOR therapy. In addition, in our previous work with GNC, we found that GNC can protect primordial and primary follicles of rat ovaries in a DOR model induced by 4-vinylcyclohexene diepoxide (VCD), an industrial chemical.<sup>60</sup> The therapeutic effects involve the activation of the PI3K-Akt signaling pathway and decreased downstream apoptosis-related protein expression.<sup>57</sup> These results indirectly validated that the PI3K-Akt pathway plays a vital role in the effect of MGNC. Furthermore, FSHR also belongs to the GPCR family and is pivotal in follicle development regulation, which was found to be upregulated under MGNC treatment (Figure 4).

## Conclusion

In this study, we first verified the treatment effect of MGNC formula on a stress-induced DOR animal model. Then, based on network pharmacology, we identified five flavonoids as the most common active compounds of MGNC, pinpointed multiple potential therapeutic targets, and discovered that the PI3K-Akt pathway was the most promising pathway to mediate the effect of MGNC. The combined data provide a preliminary understanding of the pharmacological mechanism of MGNC and generate more leads for further mechanistic research.

## Abbreviations

MGNC, Modified Gengnianchun; GNC, Gengnianchun; TCM, Traditional Chinese Medicine; DOR, Diminished ovarian reserve; CORT, Corticosterone; FSH, Follicle-stimulating hormone; FSHR, FSH receptor; LH, Luteinizing hormone; AMH, Anti-Müllerian hormone; E2, Estradiol; T, Testosterone; CUS, Chronic Unpredictable stress; H&E, Hematoxylin and eosin; TBST, Tris-buffered saline tween; OB, Oral bioavailability; DL, Drug likeness; ADME, Absorption, distribution, metabolism, and excretion; NS, Normal saline; PPI, Protein-protein interaction; GO, Gene ontology; BP, Biological process; CC, Cellular components; MF, Molecular functions; KEGG, Kyoto encyclopedia of genes and genomes; PI3K, Phosphatidylinositol-4,5-bisphosphate 3-kinase; AKT, Protein kinase B; AGE, Advanced glycation end products; RAGE, Receptor of advanced glycation end products; PTEN, Phosphatase and tensin homolog; GPCR, G protein-binding receptor; NF- $\kappa$ B, Nuclear factor kappa b; POI, Primary ovarian insufficiency; FOR, Functional ovarian reserve; TOR, Total ovarian

reserve; VCD, 4-vinylcyclohexene diepoxide; APP, Amyloid beta precursor protein; IL1B, Interleukin 1 beta; IL2, Interleukin 2; IL6, Interleukin 6; TNF, Tumor necrosis factor; INFG, Interferon gamma; MAPK, Mitogen-activated protein kinase 1; CASP3, Caspase 3; BAX, BCL2 associated x, apoptosis regulator; PARP1, Poly (adp-ribose) polymerase 1; SOD1, Superoxide dismutase 1; CREB, CAMP response element binding protein; BDNF, Brain-derived neurotrophic factor.

## Ethics Approval

All experimental protocols were carried out according to the National Institutes of Health (NIH) guidelines. The research was approved by the Institutional Animal Care and Use Committee of Animal Ethics of the School of Traditional Chinese Pharmacy, China Pharmaceutical University.

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## Disclosure

The authors report no conflicts of interest in this work.

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