Drug Design, Development and Therapy

Dovepress
open access to scientific and medical research

Open Access Full Text Article

ORIGINAL RESEARCH

Study on the Mechanism of Jiawei Shengjiang Powder in Improving Male Asthma-Induced Asthenospermia Based on Network Pharmacology and Bioinformatics

This article was published in the following Dove Press journal:
Drug Design, Development and Therapy

Ji-sheng Wang[1,2,*]
Xue-feng Gong[3,4,*]
Jun-long Feng[1,2,*]
Hai-song Li[2]
Xiao Li[5]
Sheng Deng[1,2]
Pei-zhong Ren[3,4]
Jia-mei Wang[3,4]
Ming-sheng Lv[3,4]
Rui-feng Jin[3,4]
Qiu-yi Chen[3,4]
Bin Wang[2]
Hong-sheng Cui[4]

[1]First Clinical Medical College, Beijing University of Chinese Medicine, Beijing, 100029, People's Republic of China; [2]Andrology Department, Dongzhimen Hospital, Beijing University of Chinese Medicine, Beijing, 100700, People's Republic of China; [3]The Third Affiliated Hospital of Beijing University of Chinese Medicine, Beijing, 100029, People's Republic of China; [4]Pneumology Department, The Third Affiliated Hospital of Beijing University of Chinese Medicine, Beijing, 100029, People's Republic of China; [5]Department of Andrology, The First Affiliated Hospital of Henan University of Chinese Medicine, Zhengzhou, 450046, People's Republic of China

*These authors contributed equally to this work

Correspondence: Bin Wang
Andrology Department, Dongzhimen Hospital, Beijing University of Chinese Medicine, Beijing, 100700, People's Republic of China
Email dayiwangbln@sina.com

Hong-sheng Cui
Pneumology Department, The Third Affiliated Hospital of Beijing University of Chinese Medicine, Beijing, 100029, People's Republic of China
Email Hshcui@sina.com

Background: Jiawei Shengjiang Powder (JWSJP) is a classical Chinese medicinal formula, which has been widely applied in the treatment of asthma and complications for many years due to its curative effect.

Aim: To verify the effect of JWSJP in improving abnormal sperm motility caused by asthma and to explore its potential mechanism.

Materials and Methods: The active compounds of JWSJP were obtained from high performance liquid chromatography tandem mass spectrometry and the Traditional Chinese Medicine System Pharmacology. The key active components and targets of JWSJP were predicted based on network pharmacological analysis and bioinformatics research. Rats were randomly divided into normal, model and treatment groups. The rat model of allergic asthma was induced by intraperitoneal injection of ovalbumin solution. The experiment judged improvement of semen quality by evaluating sperm motility, and detected the expression of related proteins in testicular tissue of Sprague-Dawley rats by RT-qPCR and Western blot methods. Hematoxylin and eosin (HE) staining was used to observe the changes in testicular tissue structure in rats.

Results: Through the analysis of network pharmacology and bioinformatics, it was found that beta-sitosterol, quercetin, gallic acid, pelargonidin and kaempferol were the key active components of Jiawei Shengjiang Powder. Tumor necrosis factor (TNF), interleukin-6 (IL-6) and insulin (INS) genes are crucial targets of JWSJP in the treatment of spermatogenic dysfunction caused by acute asthma. After 8 weeks of intervention, compared with the model group, the treatment group had significantly improved sperm motility ($P < 0.05$). There were significant differences in TNF, IL6, and INS proteins in the treatment group, and the HE staining of testicular tissue structure in the treatment group was significantly improved.

Conclusion: JWSJP can improve the abnormal sperm motility induced by asthma, and its mechanism may be related to the expression of related proteins and mRNA of TNF, IL6, and INS.

Keywords: Jiawei Shengjiang Powder, asthmatic attack, asthenospermia, network pharmacology

Introduction

Asthma is a common respiratory disease with high morbidity and mortality. The disease affects nearly 300 million patients worldwide.[1,2] Among patients with severe asthma, the activation of apoptosis pathways induced by hypoxia has attracted much attention.[3] Some literature studies have showed that testicular

temperature rises, testicular morphology changes, the interstitial space between cells increases, the seminiferous epithelium thins, vacuoles appear, and the number of spermatozoa decreases significantly under the condition of hypoxia.[4–6] At present, the treatment has no obvious effect on the improvement of spermatogenic dysfunction caused by asthma,[7] while traditional Chinese medicine is playing an increasingly important role in the treatment of male infertility as an indispensable part of Chinese excellent traditional culture in recent years, making it a safe and effective treatment.[8] In recent years, traditional Chinese medicine has been verified to be effective in the study of multi-component, multi-target and multi-pathway treatment of infertility.[9]

Shengjiang Powder is a famous formula for febrile disease in traditional Chinese medicine, widely used by clinical physicians of traditional Chinese medicine.[10] It is mainly composed of four kinds of medicine, namely *Illicium verum* Hook.f (pinyin name Bai Jiang Can; Latin name *Bombyx batryticatus*), *Saussurea involucrata* (Kar. and Kir.) Sch.Bip (pinyin name Chan Tui; Latin name *Cicadae periostracum*), *Curcuma aromatica* Salisb. (pinyin name Jiang Huang; Latin name *Curcumae longae rhizoma*) and *Rheum palmatum* L. (pinyin name Da Huang; Latin name *Radix et Rhizoma Rhei*), which is believed by traditional Chinese medicine to have the function of relieving depression, removing stagnation, and promoting penetration, as well as reducing fever and relieving heat.[11–13] Pharmacological and experimental studies show that Shengjiang Powder has curative effects of anti-inflammation, anti-virus, anti-convulsion, anti-tumor, anticoagulation.[14,15] This unique formula can also regulate multiple organs including heart, liver, spleen, lung, kidney, while positively influencing other diseases, especially asthma and infertility.[16,17] The Jiawei Shengjiang Powder (JWSJP) in our study is the improved version of Shengjiang Powder. Network pharmacology is an interactive network based on the concept of "disease-gene-target-drug", which treats the intervention and effect of drugs on the disease network from a systematic and comprehensive perspective, so as to reveal the complex mechanism of drugs on the human body. The systematic nature of this strategy is consistent with the holistic view of traditional Chinese medicine and the synergistic mechanism of multi-components, multi-pathways and multi-objectives in the formulation of traditional Chinese medicine. Our research planned to screen out the relevant components of JWSJP through multiple databases and obtain potential targets through target capture. Then, relevant targets of asthma and asthenospermia were selected by integrating a multi-source database. Based on the matching results of JWSJP's potential and disease targets, a protein-protein interaction (PPI) network is established to analyze the interaction between these targets, and screen targets through topology calculations. In addition, the bioinformatics resources of DAVID were used to obtain the enrichment analysis of gene ontology (GO), biological process (BP), cellular component (CC), molecular function (MF) and Kyoto Encyclopedia of Genes and Genomes (KEGG).

Therefore, we assume that Jiawei Shengjiang Powder may treat asthma-induced asthenospermia by inhibiting inflammation and improving hypoxia. This study combined network pharmacology with experimental verification. After predicting the potential mechanism of JWSJP on asthenospermia caused by asthma, *in vivo* experiments were conducted to verify the improvement of JWSJP on asthma-induced asthenospermia, and its key active components and targets (Figure 1).

Materials and Methods

Identification of Chemical Constituents

High performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) was used to separate and identify the substances of JWSJP. The equipments used are ultra performance liquid chromatography (Thermo Fisher Scientific, USA), high resolution mass spectrometry (Thermo Fisher Scientific, USA) and chromatographic column (Agilent Technologies, USA). And JWSJP's chemical constituents were also obtained from the Traditional Chinese Medicine System Pharmacology (TCMSP, https://tcmspw.com/tcmsp.php).[18] TCMSP is a unique platform for systematic pharmacology of Chinese herbal medicine, through which the relationship between drugs, targets and diseases can be discovered. A chemical composition list was generated from relevant literature and the TCMSP database. Then based on the drug likeness (DL) (> 0.18) and oral bioavailability (OB) (> 30%),[19,20] research hotspots, literature mining, and the preliminary research basis of the laboratory were used to excavate and sort a large number of literatures, thus further screening for the chemical components of JWSJP. Finally, the above two methods were superimposed to establish the active chemical components of JWSJP.

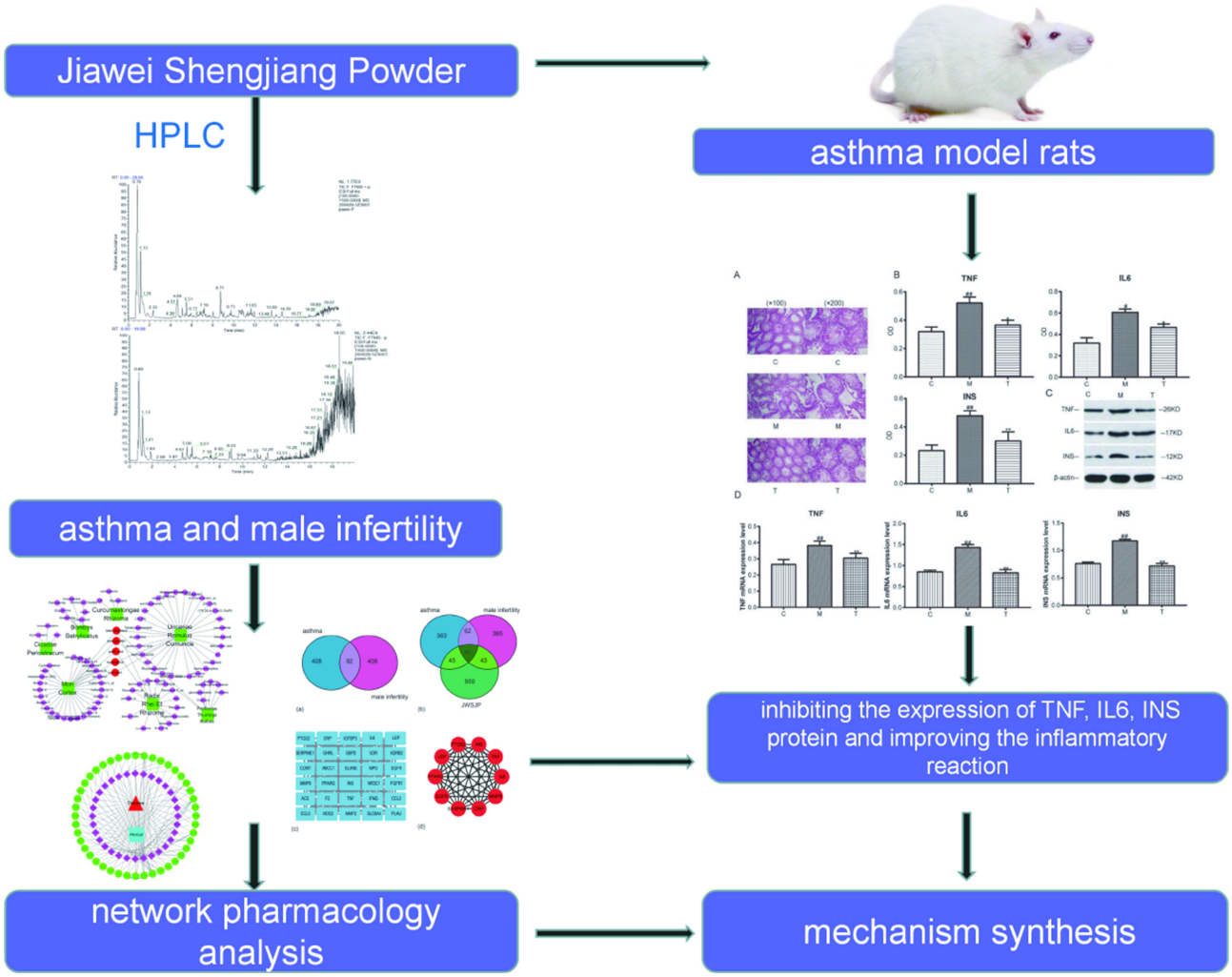

Figure 1 Overall process based on network pharmacology and animal experiments.

Target Collection

We used the GeneCards database[21] (https://www.genecards.org/) and the Online Mendelian Inheritance in Man database[22] (OMIM, https://omim.org/) to obtain the related targets of asthma and infertility. Then relevant targets of JWSJP's active components were collected from HPLC-MS/MS, TCMSP (https://tcmspw.com/tcmsp.php), PubChem (https://pubchem.ncbi.nlm.nih.gov/) and Swiss Target Prediction (http://www.swisstargetprediction.ch/). For further network establishment and analysis, the targets of asthma were overlapped with that of infertility and JWSJP.

Construction of the PPI Network

We used the STRING database (https://STRING-db.org/)[23] to identify the possible protein-protein interaction (PPI). In order to improve data reliability, PPI with a minimum interaction score below 0.40 was further filtered out, and the remaining PPI was used for network construction and analysis.

Network Construction and Analysis

Cytoscape software (version 3.7.1, https://cytoscape.org/)[24] was applied to build an ingredient network of Chinese herbal medicine, PPI network and herbal-compound-target-disease network. Afterwards, the PPI network was further analyzed using cytohubba, a Cytoscape plug-in, to determine its indispensable targets.

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

The Database for Annotation, Visualization and Integrated Discovery (version 6.8, DAVID, https://david.ncifcrf.gov/)[25]

provides mainframe computers with systematic and comprehensive biological function annotation information about genes or proteins, which can identify the most crucial and abundant biological annotations. We imported the targets of JWSJP into the DAVID database to ensure that the selected identifier can be set as the official gene symbol, gene list as list type, and *Homo sapiens* as qualified species. Also, enrichment analysis of GO and KEGG pathways on the targets of JWSJP was carried out.

Chemicals and Reagents

JWSJP used in chemical and reagent experiments was all purchased by the Pharmacy Department of the Third Affiliated Hospital of Beijing University of Traditional Chinese Medicine. The therapeutic drugs were traditional Chinese medicine formula granules (Beijing Kangrentang Pharmaceutical Co., Ltd, Beijing, China): *Illicium verum* Hook.f (20009911), *Saussurea involucrata* (Kar. and Kir.) Sch.Bip (19015871), *Curcuma aromatica* Salisb. (19009461), *Rheum palmatum* L. (19004941), *Fritillaria thunbergii* Miq (20000331), *Uncaria rhynchophylla* (Miq.) Miq (19045041), and *Morus alba* L. (19042131). According to the dosage calculated based on the surface areas of human and animal bodies, the dose of rats per kg is 6 times that of adult humans. In addition, the dosage form is all-component formula granules of traditional Chinese medicine (Beijing Kangrentang Pharmaceutical Company Ltd). The specific herbs and dosage were as follows: 12 g of *Bombyx batryticatus*, 12 g of *Periostracum cicadae*, 10 g of *Radix curcuma*, 6 g of *Rheum officinale*, 10 g of bulb of *Fritillaria thunbergii*, 10 g of *Uncaria*, and 10 g of Cortex Mori (daily dose for adults). Each dose was ground into fine powder and fully dissolved with aseptic double distilled water into 20 mL suspension. The suspension was stored in refrigerator at 4° C after being fully mixed in cases of intragastric administration.

Ovalbumin (OVA, No.A5503) was purchased from Sigma Aldrich Co., Ltd (St. Louis, MO, USA), and aluminum hydroxide gel (No. 77161) was purchased from Pierce Co., Ltd (SJ, CN). Pentobarbital sodium was purchased from Sinopharm Group Chemical Reagent Co., Ltd (SJ, CN). All primers were acquired from Invitrogen Biotechnology Co., Ltd (SJ, CN). We also purchased a range of assay kits from Bioss (Woburn, MA, USA), including tumor necrosis factor (TNF) kit (No. BS-10802R), interleukin-6 (IL-6) kit (No. BS-20393R), an insulin gene (INS) kit (No. BS-0862R). IgG (H+L)/Biotin, HRP (bs-0295G) was purchased from Bioss (Woburn, MA, USA). Beta-actin mouse anti IgG (YM3028) was purchased from Immunoway (Plano, TX, USA).

Animals

Thirty male SPF Sprague-Dawley (SD) rats aged 4–5 weeks were prepared, with an average weight of about 120–130 g, all of which were purchased from Sibeifu Biotechnology Co., Ltd (licence No. SCXK Beijing 2016–0002, Beijing, China). The rats were raised in the SPF-grade animal room of Beijing University of Chinese Medicine in an environment with daily illumination of 10–12 h, indoor humidity of 55–60%, and temperature of 22–26 °C.

Firstly, animals were fed with deionized water and solid feed for 7 days' adaptation. The experimental scheme was approved by the Experimental Animal Ethics Committee of Beijing University of Chinese Medicine (Ethical number: BUCM-4-2019091105-3064) and followed the guidelines for the ethical review of laboratory animal welfare People's Republic of China National Standard GB/T 35892-2018.

Construction and Grouping of Asthma Model

The 30 SD rats after 7-day adaptation were later numbered, weighed and recorded. Following the random number table method, 6 rats were randomly selected into the blank control group, and the remaining 24 rats were used for modeling. In the first 3 weeks of the experiment, rats in the blank control group were injected (via the intraperitoneal route) with 1 mL of sterile saline every day. On days 0, 7, and 14, rats in the asthma modeling group were sensitized by injecting 1 mL of mixable solution containing 100 mg OVA, 10 mg of aluminum hydroxide gel (via the intraperitoneal route). From day 22 of the experiment, the rats in blank control group were given sterile saline to inhale through a compression atomizer (DL, CN) at a rate of 0.2 mL/min for 30 min, while the rats of the asthma modeling group were given 1% OVA (atomization) at a rate of 0.2 mL/min over the course of 30 min. Both groups underwent surgery once a day for 3 weeks. After 3 weeks, we assessed the rats of the asthma modeling group in terms of behaviors, presence of upright fur, shortness of breath, nose scratching, incontinence, and by monitoring

abdominal breathing, as described previously. Finally, in 24 rats, 20 rats successfully constructed animal models.

After the successful establishment of the above animal models, 12 asthma modeling rats were randomly divided into the model group (group M, n=6), the treatment group (group T, n=6), and blank control group (group C, n=6), which we have mentioned above. The intervention methods were as follows: group C and group M were fed with deionized water respectively, while group T was fed with suspension of JWSJP 1.59 g/kg by gavage (6 times the human dosage). All the experimental rats were fed routinely, with the medicine being given at 10:00 per day, and the corresponding doses were given according to the standard of 1 mL/100 g body weight. The experiment lasted for 2 weeks. During the experiment, the dose was adjusted by corresponding weight, and calculated according to the ratio of body weight to dose of the Pharmacological Experimental method.[26]

Determination of Sperm Quality

The epididymal tissues were excised to remove excess fat, and the WLJY-9000 Weili sperm quality detection system (Xingrong Technology Co, Ltd, Beijing, China) was used for testing. The system was debugged and the temperature was set to 37°C, according to the manufacturer's instructions. Then, for semen specimen testing, the counting plate was placed on the thermostat for heating. After semen liquefaction, semen analysis specimens were prepared, and 5 μL of the quantified semen was dripped onto the semen pool of the clean counting plate with a micropipette and then covered with a cover glass. The microscope was mounted on a thermostatic plate on the stage and analyzed. Specific observation methods were conducted according to WHO criteria:[27] 10 fields of view were randomly selected and the numbers of sperm in 10 large squares were counted. The average value was 10^6 per mL.

Histological Analysis

The left testicle was cut open along the largest surface, half of which was taken out to be fixed into 4% paraformaldehyde fixative for 24 h. Then the fixed tissue was removed and trimmed, and testis tissue was cut along the transverse axis of the testicular tissue, and continue to fix for 12 h. After 12 h, it was rinsed with flowing tap water for 24 h, then rinsed with deionized water for 2 h, and dehumidified with absorbent paper after washing. The tissue was then embedded with wax blocks and made into slices with a thickness of 4 μm. Finally, HE staining was performed to observe and analyze the pathological changes of testicular tissue of experimental rats.

Detection of TNF, IL6, and INS Proteins by Western Blot

A portion of the cavernous tissues of each rat was removed, and then RIPA lysis buffer was added (including 1 mmol/L of PMSF). The sample was lysed on ice for 30 min, followed by centrifugation at 12000 r/min for 15 min, and collection of the supernatant. Next, samples containing equal amounts of protein (50 μg) were boiled in protein loading buffer for 10 min, separated on 12% SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were blocked with 3% BSA in Tris-buffer saline. Then the membranes were incubated at 4°C overnight with β-actin antibody (1: 1000). After washing, the membranes were incubated with a horseradish peroxidase conjugated secondary antibody (mouse anti-rabbit IgG, 1: 3000; rabbit anti-goat IgG, 1: 3000) for 1 h at room temperature. Then PVDF membrane was put into the automatic chemiluminescence imaging system (Tanon; 5200, SH, CN) to read the data. The optical density of the target band is analyzed with GIS software.[28]

mRNA of TNF, IL6, and INS Detected by RT-qPCR

The tissue (100 mg) was added to the homogenizer for full grinding, so as to centrifuge the supernatant with 250 μL trichloromethane added. The supernatant was centrifuged for 3 min and placed at −20°C for 15 min. After centrifugation at 4°C for 10 min, the white precipitate at the bottom of the tube was RNA. Next, 20 μL of dissolved RNA without RNA enzyme was added. In accordance with the instructions of cDNA reverse transcription kit, the PCR amplification conditions were pre-denatured at 95°C for 2 min, denatured at 95°C for 15 s, elongated at 55–68°C for 30 s, cycled 35–45 times, and annealed at 45°C for 20 s. The sequence of RT-qPCR primers is listed (Table 1).

The number of cycles (Ct value) experienced by the fluorescence signals in each reaction tube when it reached the set threshold was recorded. The difference in the expression of the gene was determined by computing the multiple of the target gene relative to the reference, with the relative quantification (RQ) method ($RQ = 2^{-\Delta\Delta Ct}$).

Statistical Analysis

Statistical analysis of the data was conducted using SPSS version 20.0 (SPSS Inc, Chicago, Illinois, USA). Data

Table 1 The Primer Sequences for RT-qPCR

Primer Name		Primer Pairs (5'to3')	Fragment Length (bp)	Annealing Temperature (°C)	Position
TNF	Forward	CAGCCAGGAGGGAGAAC	93	63.9	11
	Reverse	GTATGAGAGGGACGGAACC		63.9	103
IL6	Forward	CTCACGCACCGATGTCT	109	64.2	4034
	Reverse	AGGCTGTGGGCTCAATC		64.3	4142
INS	Forward	TCTGTCAACTTTGCCGACT	114	64.3	736
	Reverse	AACCGTGTCTTCGTCCAG		64.4	849
β-actin	Forward	CCTCACTGTCCACCTTCCA	120	66	1117
	Reverse	GGGTGTAAAACGCAGCTCA		65	1236

were presented in the form of mean ± SD (standard deviation). For the measurement data that conformed to the normal distribution, independent sample *t*-tests were employed for comparison between the two groups. $p < 0.05$ was considered statistically significant.

Results

Screening of Active Compounds of JWSJP

One hundred and eighty-one JWSJP medicine compounds were identified by HPLC-MS/MS, among which 104 compounds were identified only under positive ion mode, while 69 compounds were identified only under negative ion mode, with 8 ingredients identified under both positive and negative ion mode. Details are listed in Table S1 and S2. Total ion flow charts for JWSJP under positive and negative ion modes were obtained (Figure 2). A total of 447 chemical constituents were retrieved from TCMSP database. According to our screening, 60 active compounds were screened out, among which 11 compounds (18.3%) were *Bombyx batryticatus*, 3 compounds (5.0%) were *Cicadae periostracum*, 3 compounds (5.0%) were *Curcumae longae rhizoma*, 6 compounds (10.0%) for Radix et Rhizoma Rhei, 4 compounds (6.7%) were Fritillariae Thunbergii Bulbus, 17 compounds (28.3%) were Uncariae ramulus cum Uncis, and 16 compounds (26.7%) were Cortex Mori. The network diagram of active components of Chinese herbal medicine was established (Figure 3). In the network diagram, the effective compounds were analyzed and arranged in descending order. The first five active compounds were β-sitosterol, quercetin, Gallic acid, geranium and kaempferol (Table 2).

Targets Collection, Construction of PPI Network, and Topology Analysis

After collection, 500 male infertility related targets and 500 asthma related targets were obtained from GeneCards, and the targets were obtained after calculating intersection (Figure 4A). A total of 677 JWSJP targets were obtained from TCMSP, PubChem and Swiss Target Prediction. PPI analysis was performed on 30 overlapping targets (Figure 4B) through the STRING database, all of which were associated with JWSJP and male infertility caused by asthma. These 30 targets participating in PPI were used to build the network further by using Cytoscape software (Figure 4C). Through cytohubba analysis, the first five cytotoxicity parameters were defined as key targets, including INS (rank 1), TNF (rank 2), IL6 (rank 3), EGFR (rank 4), PTGS2 (rank 5) (Table 3 and Figure 4D). For the target with score ≥ 22, the experimental verification was carried out. Besides, Cytoscape software was used to construct a herbal-compound-target-disease network (Figure 5).

Enrichment Analysis of GO and KEGG

The enrichment analysis of 30 targets using DAVID 6.8 revealed that JWSJP was involved in 82 biological processes (BP), 7 cellular components (CC), 14 molecular functions (MF), and 36 signal pathways. The first five results of GO and KEGG analysis were selected according to the P value for display (Figure 6).

Verification of the Asthma Model

Rats in the asthma model group exhibited anxiety, upright fur, shortness of breath, obvious abdominal breathing, nose

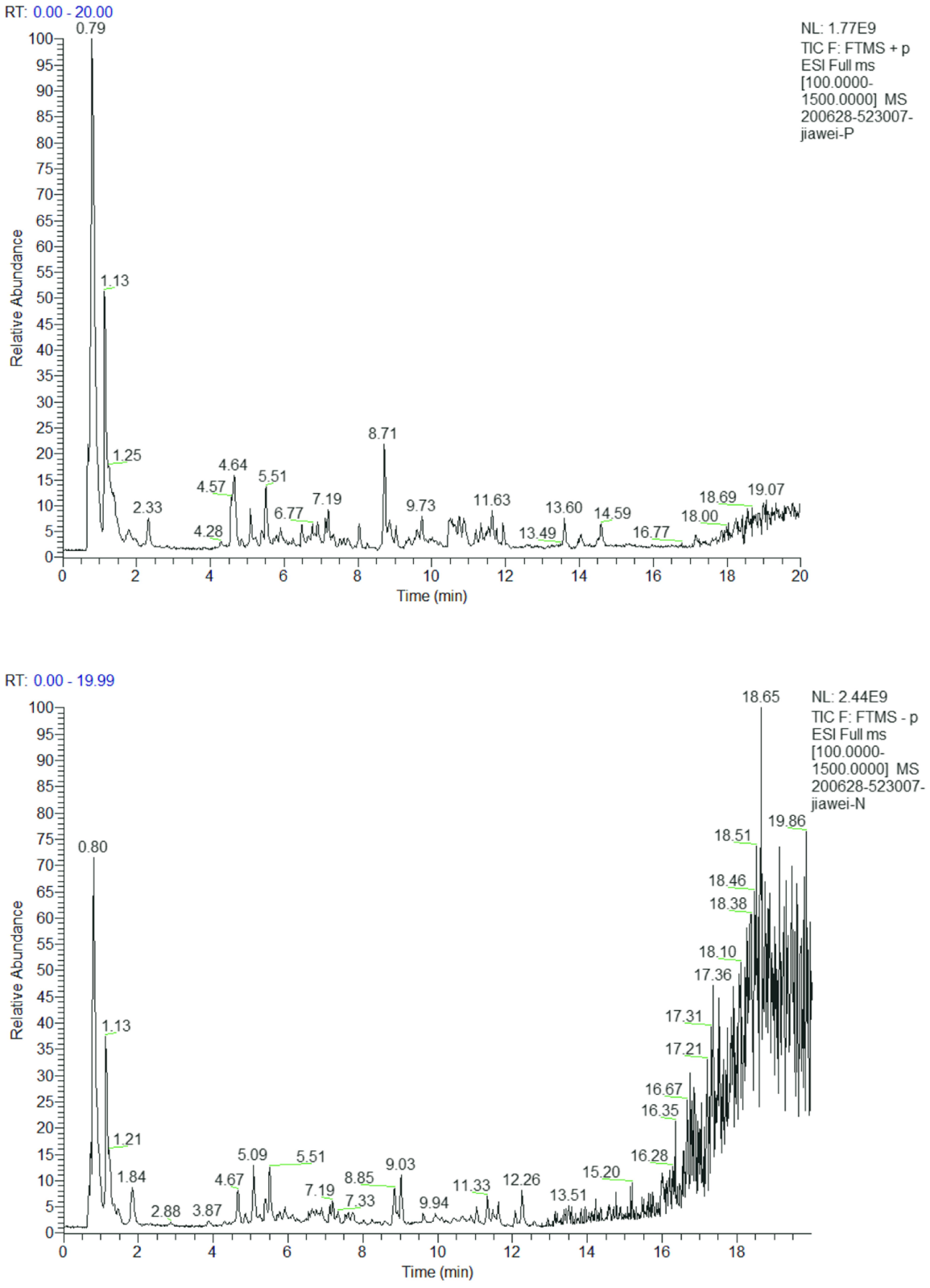

Figure 2 Total ion chromatography (TIC) of JWSJP based on HPLC-MS/MS.

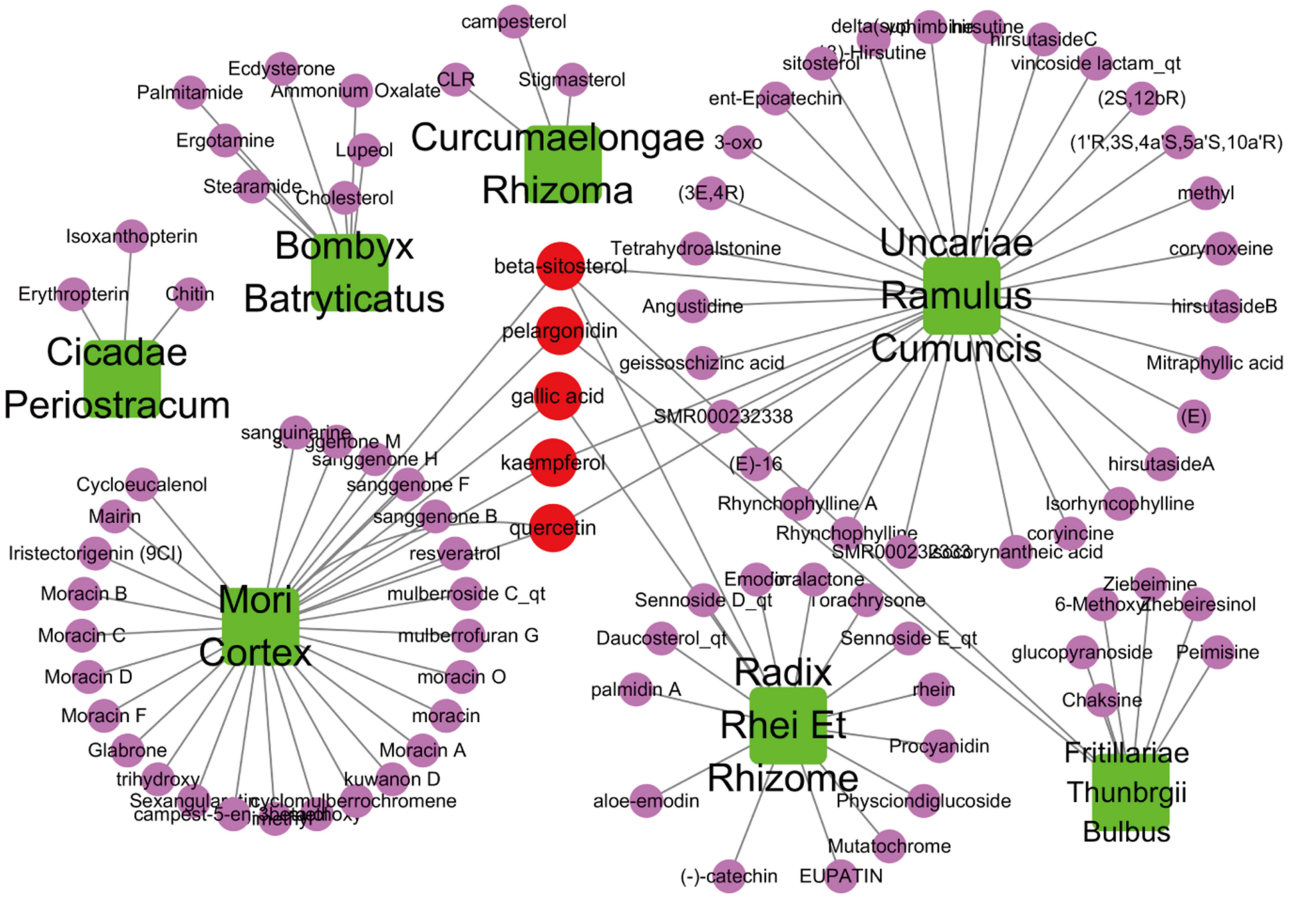

Figure 3 The network construction for herbs-active ingredient. Green nodes represent herbs, red nodes represent key active ingredients after network analysis, and Purple nodes represent other active ingredients.

scratching and incontinence. Rats in the asthma model group showed evidence of 6 different types of symptoms; none of the control rats exhibited any symptoms.

Analysis of Sperm Quality

The sperm concentration in group M was significantly different from that in the blank group ($P < 0.05$, *t*-test), and the sperm motility decreased significantly ($P < 0.01$, *t*-test). Besides, compared with group M, the difference in sperm concentration in group T was statistically significant ($P < 0.05$, *t*-test), and the sperm motility increased significantly ($P < 0.05$, *t*-test, Table 4).

Table 2 Information on Five Key Active Ingredients

Molecule Name	Molecule ID	Degree
Beta-sitosterol	MOL000358	4
Quercetin	MOL000098	3
Gallic acid	MOL000554	2
Pelargonidin	MOL001004	2
Kaempferol	MOL000422	2

Analysis of HE Staining Results

Through HE staining and observation under light microscope, it was found that seminiferous tubules in group C had normal structure and orderly arrangement, and a large number of normal sperm and spermatogenic cells at all levels could be seen in the lumen. The cell structure of each layer was complete, orderly and clear, with no obvious morphological abnormality. Ingroup M, the abnormal structure of seminiferous tubules increased significantly with irregular arrangement, the lumen of seminiferous tubules shrank, the epithelium was degenerated partially and a mass of vacuoles could be observed. Moreover, the proportion of abnormal sperm and sperm cells increased obviously. Many spermatogenic cells at all levels in the seminiferous tubules were lost, the layers were disordered, and lots of spermatogenic cells fell off. As for group T, the pathological changes, degree and extent of lesions were significantly less

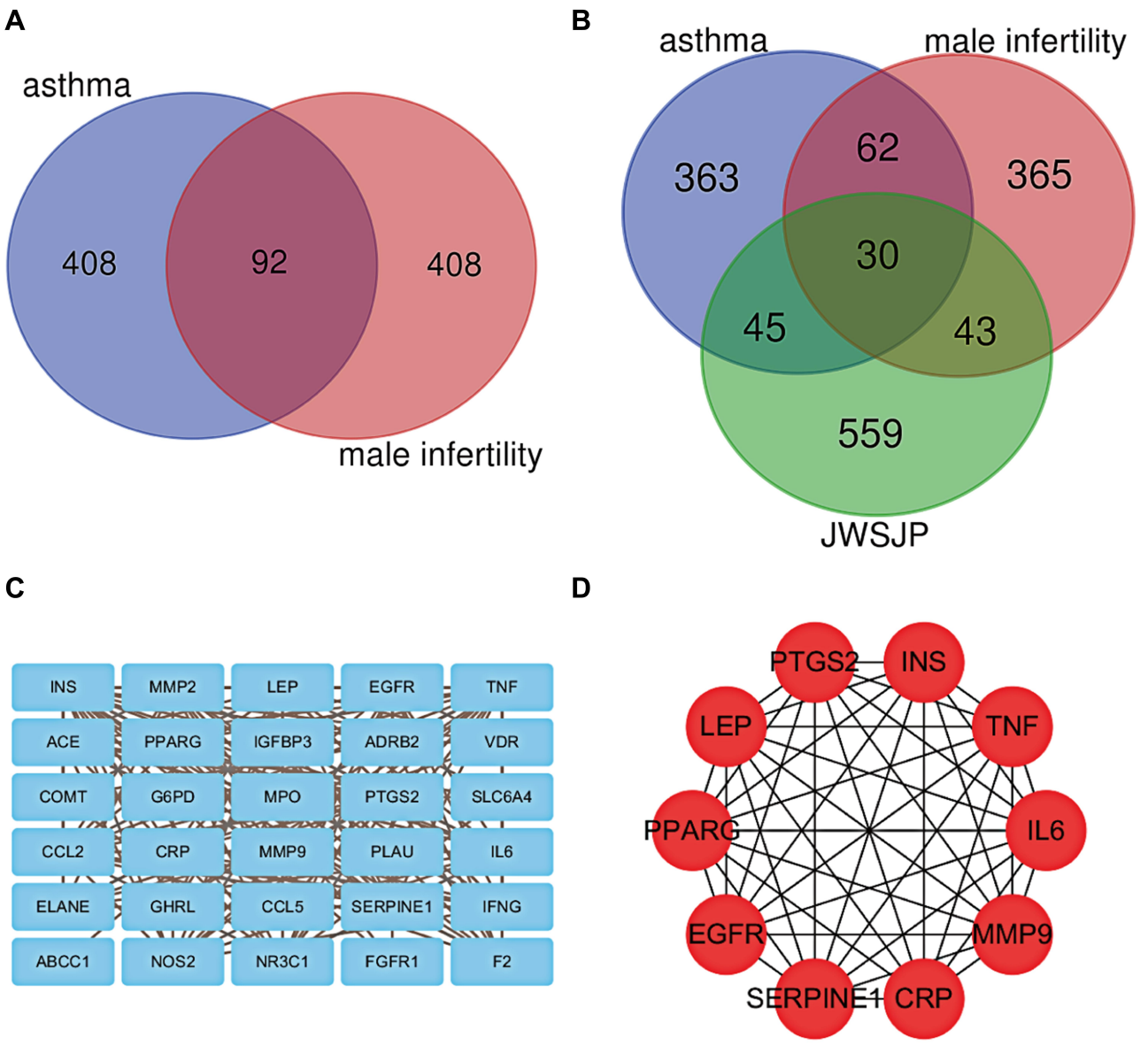

Figure 4 (**A**) Intersection of targets for asthma and targets for male infertility; (**B**) intersection of JWSJP targets, asthma targets, and erectile male infertility target; (**C**) PPI network built by Cytoscape (3.7.1); (**D**) PPI network processed by Cytoscape (3.7.1) plug-in (cytohubba).

than those in group M. The lumen of seminiferous tubules was enlarged significantly, spermatozoa in seminiferous tubule was increased obviously. Some seminiferous tubules returned to normal, but there was still a certain gap compared with group C (Figure 7A).

Table 3 Information on Five Key Target

Rank	Target Name	Score
1	INS	26
2	TNF	24
3	IL6	22
4	EGFR	21
4	PTGS2	21

Detection of TNF, IL6, and INS Proteins in Testicular Tissues of Rats by Western Blot

After 8 weeks of intervention, the level of TNF protein in group M was markedly higher than group C ($P < 0.01$, *t*-test), while the expression of TNF protein in group T was much lower than group M ($P < 0.05$, *t*-test). The expression of IL6 protein in group M was far higher than group C ($P < 0.05$, *t*-test). The expression of IL6 protein in group T was significantly lower than group M ($P < 0.05$, *t*-test). The expression of

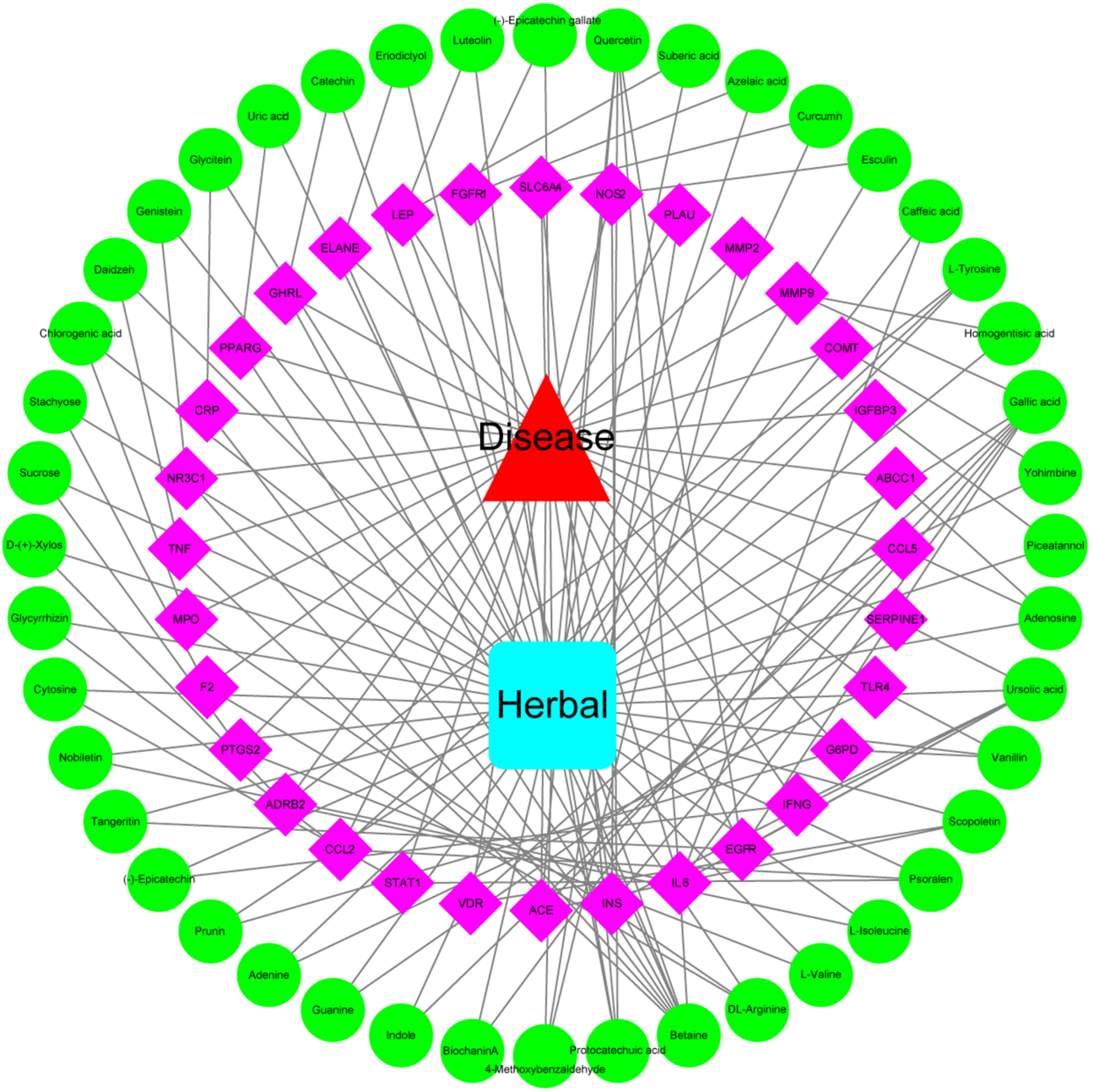

Figure 5 The network construction for herbal-compound-target-disease.

INS in group M was significantly higher than that in group C ($P < 0.01$, *t*-test). The expression of INS protein in group T was notably lower than group M ($P < 0.05$, *t*-test, Figure 7B and C).

The mRNA Expression of TNF, IL6, and INS in Testicular Tissue of Rats by RT-qPCR Assay

With analysis of the expression value with real-time quantitative PCR, the expression of TNF mRNA in group M was significantly higher than that in group C ($P < 0.01$, *t*-test). After drug intervention, the mRNA expression of TNF in group T was markedly lower than group M ($P < 0.01$, *t*-test). The mRNA expression of IL6 in group M was higher than group C ($P < 0.01$, *t*-test). After drug intervention, the mRNA expression of IL6 in group T was much lower than group M ($P < 0.01$, *t*-test). The mRNA expression of INS- in group M was shown to be far higher than group C ($P < 0.01$, *t*-test). After drug intervention, the mRNA expression of INS in group T was much lower than group M ($P < 0.01$, *t*-test, Figure 7D).

studies on asthma-induced asthenospermia, and the

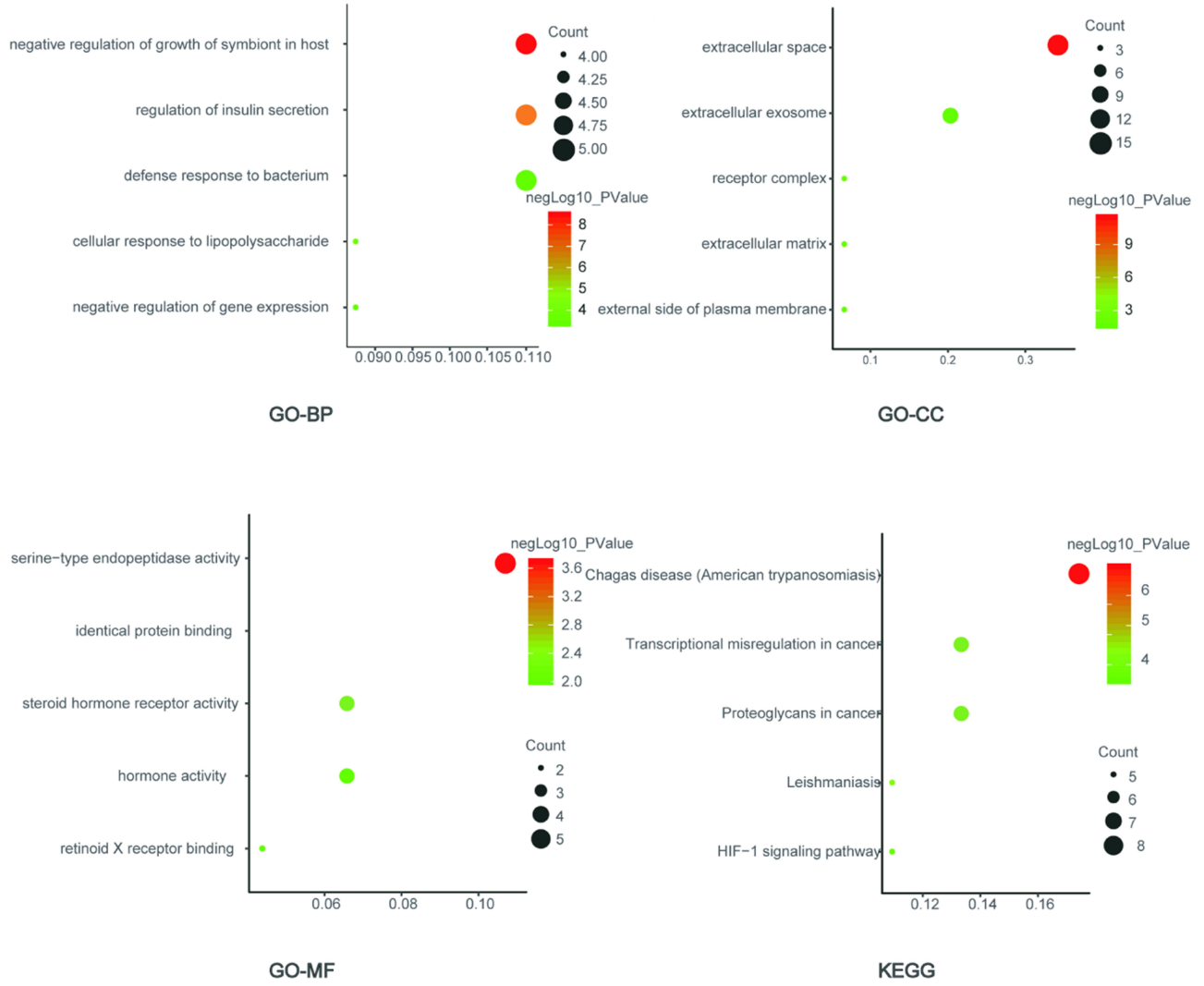

Figure 6 GO and KEGG Path Enrichment Analysis. Y-axis is the name, X-axis is the rich factor, the size of the node is proportional to the number of genes, and the color of the node is proportional to the P value.

Discussion

The results are basically consistent with our previous scientific conjecture. In this study, we found that JWSJP could significantly improve the sperm motility of rats with infertility induced by asthma. At present, there are few mechanism is still unclear. Hence, we put forward the possible mechanism hypothesis combined with the predicted targets of network pharmacology and the analysis of animal experiment results for further study.

Table 4 Sperm Count and Sperm Motility of Rats from the C, M, T Groups. 10^6/Ml, %, n=6, $\bar{X} \pm S$

Group	Sperm Concentration	Sperm Motility
Blank control group	38.54±9.21	56.79±8.65
Model group	19.58±5.79$^{\#}$	20.04±6.40$^{\#\#}$
Treat group	32.61±7.09*	43.79±5.51*

Notes: Differences with P < 0.05 were considered statistically significant. $^{\#}$P < 0.05 and $^{\#\#}$P < 0.01, the model group versus the blank control group; *p < 0.05, the treatment group versus the model group.

There are many clinical drugs for the treatment of male infertility. However, on the whole, the drug treatment of male infertility is still in the exploratory stage, and there is no reliable evidence to support which drug has what exact clinical effect on the treatment of male oligoasthenospermia. Therefore, there is no suitable positive control drug for this research.[29,30] In this experiment, we found that the sperm motility of rats in group M was markedly lower than that in group C. HE staining also verified that the lumen of seminiferous tubules in the testicular tissue contracted, and the proportion of abnormal sperm and sperm

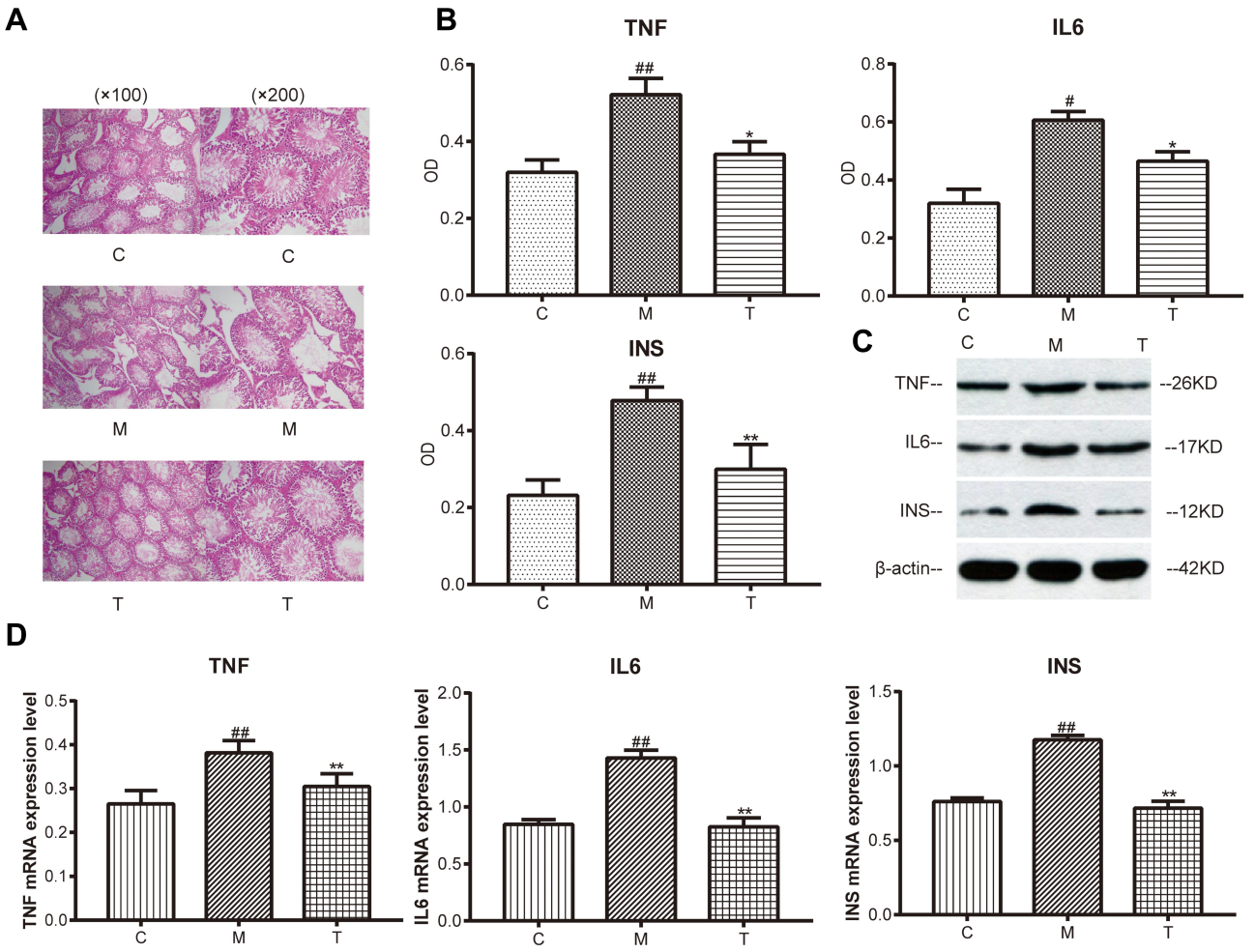

Figure 7 (**A**) HE staining of testicular tissues of rats from the blank control, model and treatment group; (**B**) the expression levels of three proteins (TNF, IL6, INS) in rats from the C, M, T groups determined using Western blotting. (**C**) Electrophoretogram of three proteins (TNF, IL6, INS) in rats from the C, M, T groups. (**D**) The mRNA expression levels of three proteins (TNF, IL6, INS) in rats from the C, M, T groups determined using Western blotting. Data are expressed as the mean ± SEM. Differences with $P < 0.05$ were considered statistically significant. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, the M group versus the C group; $^{*}p < 0.05$ and $^{**}P < 0.01$, the T group versus the M group.

cells increased significantly, which is a direct reason that asthma could lead to asthenospermia. After treatment with JWSJP, the sperm motility was increased obviously, the lumen of seminiferous tubule was significantly enlarged, the number of spermatozoa was also increased significantly, and some of the seminiferous ducts returned to normal, indicating that JWSJP could greatly improve the pathological tissue of testis.

TNF, IL-6, and INS are the key targets of PPI network analysis, which was found in the treatment of asthenospermia caused by asthma with JWSJP. TNF-α is a pro-inflammatory cytokine involved in airway inflammation in asthma. Many studies have shown that TNF-α may play a crucial role in the pathogenesis of asthma. The activation of monocyte cells will produce TNF-α, which will cause damage and irreversible obstruction in respiratory tissues to aggravate the attack of asthma and airway inflammation.[31,32] The increase of TNF-α caused by asthma decreases sperm motility. Mauduit et al.[33] found that TNF-α receptors are also present in Sertoli cells and Leydig cells in testes, so TNF-α can regulate its function. However, the level of TNF-α in seminal plasma is negatively correlated with sperm motility. That is to say, high levels of TNF-α can reduce sperm motility.[34] In this experiment, the results of WB and RT-qPCR test also showed that the expression of TNF protein increased markedly in group M, but decreased significantly in group T, which again verified that the high expression of TNF protein would reduce sperm motility and lead to asthenospermia.

IL-6 is a multifunctional cytokine that can promote the proliferation and differentiation of many kinds of cells. IL-6 plays a vital role in inflammation of asthma. Under the

action of IL-6, IL-4 can induce a large amount of IgE, enhance the production of monocyte chemoattractant and endothelial cell adhesion molecules, and promote the accumulation of inflammatory factors in infection and inflammatory sites.[35] The increase of IL-6 caused by an acute attack of asthma leads to a decrease of sperm motility. The experiments of Potashnik et al.[36] showed that IL-6 plays a critical role in the male reproductive system. IL-6 can reduce sperm penetration, antioxidation, and the activity of antioxidants. Besides, this protein directly reduces sperm parameters and affects long-term inflammation of the male reproductive system. In this experiment, the expression of IL-6 protein was greatly increased in group M and decreased significantly in group T, proving that IL-6 exacerbates asthma, and further reduces sperm motility that leads to asthenospermia.

It is interesting to note that INS protein is the key target of JWSJP in the treatment of asthenospermia caused by asthma, ranking the first in the network pharmacological analysis. The expression of INS protein increased in group M and decreased notably in group T, which also verified that the high expression of INS protein would weaken the sperm motility in the experiment. However, there is no related literature to discuss the specific mechanism, which is worthy of further study in the future.

β-sitosterol, quercetin and gallic acid are the critical active compounds of JWSJP in the treatment of asthenospermia caused by asthma through the analysis of the Chinese herbs-active compounds network. β-sitosterol is a good antioxidant with good free radical scavenging ability,[37] this being one of the main reasons for its capability to improve sperm motility. For example, Zhao et al.[38] found that β-sitosterol could improve the capacitation and fertilization of porcine spermatozoa effectively *in vitro*, and speculated that this phenomenon was related to its antioxidant properties. In addition, studies have shown that β-sitosterol has an obvious anti-asthma effect and can inhibit the increase of TNF-α level.[39] Based on the fact that the high level of TNF-α is negatively correlated with sperm motility, β-sitosterol can protect sperm through this pathway. Quercetin has a wide range of pharmacological effects, and can improve sperm motility by reducing inflammation. Diao et al.[40] collected semen samples from 56 infertile men with leukocytosis and 44 normal fertile men.

Quercetin has a protective effect on antioxidative damage of infertile males' spermatozoa mediated by leukocytes according to sperm motility (10 umol/L) before and after incubation of quercetin measured using computer-assisted semen analysis (CASA). Some studies have confirmed that 50 μmol/L quercetin can significantly inhibit the production of inflammatory factors such as TNF-α and IL-6,[41] thus further reducing the inflammatory reaction, and protecting spermatozoa. Gallic acid can reduce infiltration of inflammatory cells, improve airway hyperresponsiveness, and thus inhibit asthma exacerbation.[42] At the same time, gallic acid also improves sperm motility to some extent. Mehraban et al.[43] confirmed that gallic acid could improve the reproductive toxicity of cyclophosphamide in NMRI mice, thus improving the antioxidant capacity of testicular tissues.

At present, the relationship between asthma and asthenospermia is rarely discussed in the literature. Considering this situation, we preliminarily explain the basic mechanism of asthenospermia caused by asthma through the prediction of network pharmacology and the verification of experimental results, which forms our innovation. We also find that the HIF-1 signal pathway is one of the key pathways of JWSJP in the treatment of asthenospermia caused by asthma through network pharmacological analysis.

Limitation and Future Research Plan

However, we have not detected the indicators of related hormones, since the endocrine disorders caused by hypoxia will also have a certain impact on sperm motility, which is a deficiency in our study. Besides, this study is the first attempt at drug treatment of asthma induced infertility research, which is still in the preliminary stage of exploration. The lack of dose-dependent research is indeed a deficiency, which will be the focus of our further research. We prepare to test the relevant indicators in the future and conduct more in-depth research on the mechanism of asthenospermia caused by asthma, so as to provide certain guidance for the clinical treatment of asthenospermia caused by asthma.

Conclusion

To sum up, we find that JWSJP can improve the sperm motility of asthenospermia rats by inhibiting the expression of TNF, IL6, and INS proteins and improving the inflammatory reaction, which probably is one of its mechanism to improve sperm motility.

Abbreviations

BP, biological process; BUCM, Beijing University of Chinese Medicine; CC, cellular component; DL, drug likeness; GO, gene ontology; HPLC-MS/MS, high performance liquid chromatography tandem mass spectrometry; HE, hematoxylin and eosin; IL-6, interleukin-6; INS, insulin; JWSJP, Jiawei Shengjiang Powder; KEGG, Kyoto Encyclopedia of Genes and Genomes; MF, molecular function; OVA, ovalbumin; OB, oral bioavailability; PPI, protein-protein interaction; RQ, relative quantification; SD, Sprague-Dawley; TCMSP, Traditional Chinese Medicine System Pharmacology; TNF, tumor necrosis factor.

Ethics Approval and Consent to Participate

The experimental scheme was approved by the Experimental Animal Ethics Committee of Beijing University of Chinese Medicine (Ethical number: BUCM-4-2019091105-3064) and followed the guidelines for the ethical review of laboratory animal welfare People's Republic of China National Standard GB/T 35892-2018.

Funding

This study was supported by the National Natural Science Foundation of China (grant Number: 81774320) and university-level project of Beijing University of Chinese Medicine (NO.2019-XS-ZB04).

Disclosure

The authors report no conflicts of interest in this work.

References

1. Zhang J, Paré PD, Sandford AJ. Recent advances in asthma genetics. *Respir Res*. 2008;9(1):4. doi:10.1186/1465-9921-9-4
2. Eder W, Ege MJ, von Mutius E. The asthma epidemic. *N Engl J Med*. 2006;355(21):2226-2235. doi:10.1056/NEJMra054308
3. Spinozzi F, de Benedictis D, de Benedictis FM. Apoptosis, airway inflammation and anti-asthma therapy: from immunobiology to clinical application. *Pediatr Allergy Immunol*. 2008;19(4):287-295. doi:10.1111/j.1399-3038.2007.00668.x
4. Gasco M, Rubio J, Chung A, Villegas L, Gonzales GF. Effect of high altitude exposure on spermatogenesis and epididymal sperm count in male rats. *Andrologia*. 2003;35(6):368-374. doi:10.1046/j.0303-4569.2003.00593.x
5. Farias JG, Bustos-Obregón E, Orellana R, et al. Effects of chronic hypobaric hypoxia on testis histology and round spermatidoxidativemetabolism. *Andrologia*. 2005;37(1):47–52. doi:10.1111/j.1439-0272.2004.00651.x
6. Farías JG, Bustos-Obregón E, Reyes JG. Increase in testicular temperature and vascularization induced by hypobaric hypoxia in rats. *J Androl*. 2005;26(6):693–697. doi:10.2164/jandrol.05013
7. Umławska W, Gąszczyk G, Sands D. Physical development in children and adolescents with bronchial asthma. *Respir Physiol Neurobiol*. 2013;187(1):108–113. doi:10.1016/j.resp.2013.02.012
8. Zhou SH, Deng YF, Weng ZW, Weng HW, Liu ZD. Traditional Chinese medicine as a remedy for male infertility: a review. *World J Mens Health*. 2019;37(2):175-185. doi:10.5534/wjmh.180069
9. Liu H, Zeng L, Yang K, Zhang G. A network pharmacology approach to explore the pharmacological mechanism of Xiaoyao powder on anovulatory infertility. *Evid Based Complement Alternat Med*. 2016;2016:2960372. doi:10.1155/2016/2960372
10. Chang B. Clinical application of Shengjiang Powder. *Guangming Traditional Chine Med*. 2014;29(4):863–864.
11. Yılmaz N, Seven B, Timur H, et al. Ginger (zingiber officinale) might improve female fertility: a rat model. *J Chin Med Assoc*. 2018;81(10):905–911. doi:10.1016/j.jcma.2017.09.012
12. Banihani SA. Effect of ginger (Zingiber officinale) on semen quality. *Andrologia*. 2019;51(6):e13296. doi:10.1111/and.13296
13. Arach K, Fathiazad F, Mohammad N, et al. The effects of Ginger on spermatogenesis and sperm parameters of rat. *Int J Rep BioMed*. 2009;7(1):7–12.
14. Liu WJ, Xue YX, Hu DP. Research progress on modern pharmacological mechanism of Sheng-Jiang powder. *Beijing Zhongyiyao*. 2012;31:939–943.
15. Li J, Zhang YM, Li JY, et al. Effect of Sheng-Jiang powder on obesity-induced multiple organ injuries in rats. *Evid Based Complement Alternat Med*. 2017;2017:6575276. doi:10.1155/2017/6575276
16. Huang YL, Chen JR, Huang BL, Ai J. clinical progress of Shengjiang Powder. *Guangxi Traditional Chine Med*. 2014;37(03):7–9.
17. Cui HS, Wang XQ, Wu WP. Clinical observation of Shengjiang powder in the treatment of acute attack of bronchial Asthma. *Emergency Traditional Chine Med*. 2000;03:96–97.
18. Ru J, Li P, Wang J, et al. TCMSP: a database of systems pharmacology for drug discovery from herbal medicines. *J Cheminform*. 2014;6(1):13. doi:10.1186/1758-2946-6-13
19. Ahmed SS, Ramakrishnan V. Systems biological approach of molecular descriptors connectivity: optimal descriptors for oral bioavailability prediction. *PLoS One*. 2012;7(7):e40654. doi:10.1371/journal.pone.0040654
20. Ursu O, Rayan A, Goldblum A, Tudor I. Understanding drug-likeness. *Wires Comput Mol Sci*. 2011;1(5):760–781. doi:10.1002/wcms.52
21. Rappaport N, Fishilevich S, Nudel R, et al. Rational confederation of genes and diseases: NGS interpretation via GeneCards, MalaCards and VarElect. *Biomed Eng Online*. 2017;16(Suppl 1):72. doi:10.1186/s12938-017-0359-2
22. Hamosh A, Scott AF, Amberger JS, Bocchini CA. Online Mendelian Inheritance in Man (OMIM), a knowledgebase of human genes and genetic disorders. *Nucleic Acids Res*. 2005;33:D514–D517. doi:10.1093/nar/gki033
23. Szklarczyk D, Morris JH, Cook H, et al. The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible. *Nucleic Acids Res*. 2017;45(D1):D362-D368. doi:10.1093/nar/gkw937
24. Shannon P, Markiel A, Ozier O, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res*. 2003;13(11):2498-2504. doi:10.1101/gr.1239303
25. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc*. 2009;4(1):44–57. doi:10.1038/nprot.2008.211
26. Wang X, Gao Y, Yang Q, Fang X, Li Z. Pingchuanning decoction attenuates airway inflammation by suppressing autophagy via phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin signaling pathway in rat models of asthma. *J Cell Biochem*. 2019;120(3):3833–3844. doi:10.1002/jcb.27665

27. Institute of Science and Technology, National population and Family Planning Commission. *Manual of the World Health Organization Laboratory for Human Semen Examination and Treatment*. 5th edition. Beijing: people's Health Publishing House; 2011: 1–192.
28. Stavrova T, Borel A, Daujeard C, Vettese D. A GIS based approach to long bone breakage patterns derived from marrow extraction. *PLoS One*. 2019;14(5):e0216733. doi:10.1371/journal.pone.0216733
29. Li H. Problems related to drug treatment of idiopathic male infertility. *Chine J Family Planning*. 2013;21(04):286–288.
30. Zhang X, Li H. Drug treatment of idiopathic male infertility. *Chine J Andrology*. 2008;1(10):939–942.
31. Huang H, Nie W, Qian J, et al. Effects of TNF-α polymorphisms on asthma risk: a systematic review and meta-analysis. *J Invest Allergol Clin Immunol*. 2014;24(6):406-417.
32. Fu WC. Effects of nebulized inhalation of budesonide combined with salbutamol on tumor necrosis factor-α and hypersensitive C-reactive protein in patients with acute exacerbation of bronchial asthma. *J Pract Hospital Clin*. 2012;9(5):109–111.
33. Mauduit C, Besset V, Caussanel V, Benahmed M. Tumor necrosis factor alpha receptor p55 is under hormonal (follicle-stimulating hormone) control in testicular Sertoli cells. *Biochem Biophys Res Commun*. 1996;224(3):631–637. doi:10.1006/bbrc.1996.1077
34. Qian L, Sun G, Zhou B, et al. Study on the relationship between different cytokines in the semen of infertility patients. *Am J Reprod Immunol*. 2011;66(2):157–161. doi:10.1111/j.1600-0897.2010.00980.x
35. Miao P, Lu MS, Zhang DQ. New progress in the study of the relationship between IL-6/IL-6 receptor and rheumatoid arthritis. *J Immunol*. 2011;27(4):355–360.
36. Potashnik H, Elhija MA, Lunenfeld E, et al. Interleukin-6 expression during normal maturation of the mouse testis. *Eur Cytokine Netw*. 2005;16(2):161–165.
37. Liu HQ, Guo SH, Shen YS, Li XM. Study on antioxidation of β-sitosterol. *J Guangdong Coll Pharm*. 2004;20(4):281–283.
38. Zhao Z, Xu J, Xiang RK, et al. Effects of β-sitosterol and icariin on in vitro capacitation of porcine spermatozoa. *Chine J Vet Med*. 2010;30(7):996–999.
39. Mahajan SG, Mehta AA. Suppression of ovalbumin-induced Th2-driven airway inflammation by β-sitosterol in a guinea pig model of asthma. *Eur J Pharmacol*. 2011;650(1):458-464. doi:10.1016/j.ejphar.2010.09.075
40. Diao R, Gan H, Tian F, et al. In vitro antioxidation effect of Quercetin on sperm function from the infertile patients with leukocytospermia. *Am J Reprod Immunol*. 2019;82(3):e13155. doi:10.1111/aji.13155
41. Lee JY, Park W. Anti-inflammatory effect of wogonin on RAW 264.7 mouse macrophages induced with polyinosinic-polycytidylic acid. *Molecules*. 2015;20(4):6888-6900. doi:10.3390/molecules20046888
42. Wang X, Zhao H, Ma C, Lv L, Feng J, Han S. Gallic acid attenuates allergic airway inflammation via suppressed interleukin-33 and group 2 innate lymphoid cells in ovalbumin-induced asthma in mice. *Int Forum Allergy Rhinol*. 2018;8(11):1284-1290. doi:10.1002/alr.22207
43. Mehraban Z, Ghaffari Novin M, Golmohammadi MG, et al. Protective effect of gallic acid on testicular tissue, sperm parameters, and DNA fragmentation against toxicity induced by cyclophosphamide in adult NMRI mice. *Urol J*. 2020;17(1):78–85. doi:10.22037/uj.v0i0.4858