

Original Article

Study on the mechanism of Yishen Tongluo Formula in improving asthenospermia based on mitochondrial autophagy dysfunction induced by oxidative stress

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Abstract: Objective: This study explored the mechanism by which Yishen Tongluo Formula improves asthenospermia (AS) based on oxidative stress-induced mitochondrial autophagy dysfunction. Methods: Serum pharmacochemistry was used to identify the blood-entering components of Yishen Tongluo Formula. A tripterygium glycosides (TG)-induced rat model of AS was established, and rats were treated with low, medium, or high doses of Yishen Tongluo Formula. Western blotting and quantitative polymerase chain reaction were used to detect the protein and mRNA expression levels of oxidative stress- and mitophagy-related markers. Transmission electron microscopy (TEM) was used to observe the mitochondrial ultrastructure of Sertoli cells. Results: Eighty blood-entering components were identified, mainly including terpenoids and flavonoids. Yishen Tongluo Formula improved the gonadal index, sperm quality, and seminiferous tubule morphology in TG-induced AS rats. Compared with the model group, Yishen Tongluo Formula restored testicular mass, testicular index, testicular volume, epididymal mass, and epididymal index to varying degrees, increased sperm concentration, sperm motility, and number of progressively motile sperm, and decreased number of immotile sperm ($P<0.05$). It also increased the Johnsen score and seminiferous tubule diameter ($P<0.05$). Mechanistically, Yishen Tongluo Formula upregulated superoxide dismutase 2, nuclear factor erythroid 2-related factor 2 (Nrf2), and heme oxygenase-1 expression ($P<0.05$). Meanwhile, it downregulated PTEN-induced putative kinase 1 (PINK1), Parkinson disease protein (Parkin), microtubule-associated protein 1 light chain 3, and lysosomal-associated membrane protein 2 and upregulated sequestosome 1 ($P<0.05$), suggesting that it may inhibit excessive mitophagy. TEM showed improved mitochondrial ultrastructure. Conclusion: Yishen Tongluo Formula may improve sperm motility by regulating the Nrf2-mediated antioxidant defense system and the PINK1/Parkin-mediated mitophagy pathway, providing an experimental basis for treating AS with traditional Chinese medicine.

Keywords: Yishen Tongluo Formula, asthenospermia, oxidative stress, mitophagy, PINK1/Parkin pathway, serum pharmacochemistry

Introduction

Asthenospermia (AS) is one of the main causes of male infertility, accounting for 30% of the factors contributing to male infertility [1]. According to the World Health Organization, the global prevalence of infertility is 9%, and male factors are present in 50% of infertile couples

[2]. Recent studies have indicated that the concentration, total number, and motility of sperm from men in China have declined significantly [3]. In 2024, China's birth rate dropped to 6.77%, and the natural growth rate was -0.99%, highlighting the urgency of improving fertility. In traditional Chinese medicine, AS belongs to the categories of "jing cold" and "infertility", and is

based on kidney deficiency and is accompanied by damp-heat, blood stasis, and toxin pathogen. Ma et al. [4] summarized Professor Wang Qi's view that "kidney deficiency with damp-heat and stasis-toxin" is the core pathogenesis of male infertility. Lin et al. [5] reported that Jujing Zhuyu Decoction protects reproductive function in asthenozoospermia by regulating mitochondrial apoptosis. Chief Yuan Zhuojun integrated these academic ideas with Ye Tianshi's theory of "chronic disease entering collaterals", added blood-activating and collateral-dredging drugs to Jujing Zhuyu Decoction, and developed Yishen Tongluo Formula, which was clinically confirmed to significantly improve sperm motility.

Clinical practice has shown that Yishen Tongluo Formula is effective in treating AS. Due to the complex composition of traditional Chinese medicine contained in the compound and its many targets of action, it is difficult to elucidate its material basis and mechanism of action. With the progress of science and technology, mass spectrometry (MS) has been introduced into modern Chinese medicine research. MS has been widely used because of its high sensitivity, high selectivity, and rapidity [6], which has promoted the development of serum pharmacochimistry. The serum pharmacochimistry of traditional Chinese medicine is a research method related to metabolomics, which focuses on screening the pharmacodynamic material basis of transitional components in serum after oral administration of traditional Chinese medicine [7]. According to modern pharmaceutical theory, the condition for Chinese medicine to take effect is that the active ingredients are absorbed into the blood and then transported to different targets. The process of absorption and transport involves many complex biological processes, such as transmembrane transport, intestinal microbiota metabolism, and hepatic enzyme metabolism. In the process of metabolism, drugs are transformed into each other, resulting in some components that actually play a role being different from the inherent components of traditional Chinese medicine. Therefore, obtaining from serum the active substances that directly exert effects and tracing the precursor compounds can more comprehensively explain the material basis of the efficacy of traditional Chinese medicine. In addition to the prototype components, the active

substances also include metabolites, which may be classified as effective components as long as they are absorbed into the blood after oral administration.

According to modern medical research, the etiology of AS is quite complex. It is caused by a variety of pathogenic factors, including but not limited to infection [8], immune responses, endocrine disorders [9], varicocele [10], genetic factors, congenital defects [11], and other organic diseases. Modern medicine believes that the pathogenesis of AS is closely related to oxidative stress and mitophagy disorders [12]. Mitochondria, as the organelles retained by mature sperm, play an important role in maintaining the development and maturation of sperm. Mitochondria provide the vast majority of adenosine triphosphate (ATP) required to ensure cellular function and integrity. Mitochondrial dysfunction leads to the accumulation of reactive oxygen species (ROS), lipid peroxidation, and destruction of sperm ultrastructure and motility [13]. The degree of lipid peroxidation is usually quantified by the peroxidation product malondialdehyde (MDA). According to relevant studies abroad, there is a negative correlation between MDA and sperm concentration, morphology, and motility, but a positive correlation between malondialdehyde and sperm DNA damage, which further confirms that lipid peroxidation may be a key factor linking sperm DNA damage to sperm concentration, morphology, and motility [14]. Antioxidant proteins such as superoxide dismutase (SOD), glutathione peroxidase, and heme oxygenase-1 (HO-1) are regulated by nuclear factor erythroid 2-related factor 2 (Nrf2) under certain conditions. They can remove ROS in germ cells, thereby optimizing semen quality and enhancing semen activity [15]. The PTEN-induced putative kinase 1 (PINK1)/Parkinson's disease protein (Parkin) signaling pathway is a key pathway regulating mitophagy, which is closely related to the spermatogenesis and sperm development [16]. PINK1 is the initial signal that triggers mitophagy. Under normal conditions, PINK1 is transported from the outer mitochondrial membrane to the inner mitochondrial membrane and then degraded by proteolytic enzymes to maintain low expression levels, making it difficult to detect in testicular tissue [17]. When mitochondria are damaged, the mitochondrial membrane potential decreases,

and the transport of PINK1 is also hindered, which leads to the aggregation and stabilization of PINK1 on the outer mitochondrial membrane and attracts Parkin, which has mitophagy signal amplification ability, to migrate from the cytoplasm to the outer membrane. The increase in Parkin helps PINK1 further aggregate and activate more Parkin, thus forming a positive feedback loop [18]. In addition, Parkin contributes to the protein ubiquitination process on the mitochondrial membrane. When ubiquitinated proteins on mitochondria interact with selective autophagy linker protein 62 (p62), p62 aggregates on the mitochondrial membrane through voltage-dependent anion channel 1 protein. p62 binds to microtubule-associated protein 1 light chain 3 (LC3) to form p62-LC3 [19]. Subsequently, lysosomal-associated membrane protein 2 (LAMP2) participates in the fusion of autophagosomes with lysosomes to form autolysosomes, which are eventually degraded [20]. LC3 and LAMP2 are both marker proteins of mitophagy, and their expression levels directly reflect changes in autophagy levels, which are closely related to the number of autophagosomes, spermatogenic cell apoptosis, and decreases in sperm motility [21].

Although previous studies have confirmed the clinical efficacy of Yishen Tongluo Formula, the molecular mechanism by which it regulates mitophagy through the PINK1/Parkin pathway and the antioxidative stress response through the Nrf2 pathway has not been elucidated. Based on this, this study proposes that Yishen Tongluo Formula may regulate the removal of damaged mitochondria marked by PINK1, Parkin, p62, LC3, and LAMP2 and restore sperm motility by modulating the PINK1/Parkin pathway; it may increase the expression of antioxidant enzymes such as SOD and HO-1, reduce MDA levels, and reduce oxidative damage by regulating the Nrf2 pathway. ROS-related mitochondrial damage and antioxidant responses were taken as the research entry point to evaluate the reproductive system of rats. Sperm motility and the expression of oxidative stress-related markers MDA, SOD2, HO-1, and Nrf2 and mitophagy-related proteins PINK1, Parkin, p62, LC3, and LAMP2 in rat testicular tissue were detected. The purpose of this study is to reveal the mechanism by which Yishen Tongluo Formula improves sperm motility by regulating

mitochondrial function and antioxidant defense through multiple targets and to provide an experimental basis for the treatment of AS with traditional Chinese medicine.

In summary, this study intends to analyze the blood-entering components of Yishen Tongluo Formula through serum pharmacochimistry, so as to clarify the active components of this prescription and provide a basis for rational clinical drug use.

Materials and methods

Drugs and reagents

Tripterygium Glycosides Tablets (10 mg/tablet) were purchased from Yuanda Pharmaceutical Huangshi Feiyun Pharmaceutical Co., Ltd. (Sinopharm Z42021212); Yishen Tongluo Formula was composed of 18 medicinal materials, all purchased from the Pharmacy of the First Affiliated Hospital of Yunnan University of Traditional Chinese Medicine. Methanol (CNW Technologies), acetonitrile (CNW Technologies), formic acid (SIGMA), and L-2-chlorophenylalanine (Shanghai Hengbai Biotechnology Co., Ltd.) were used.

The crude drugs were decocted twice with a 10-fold volume of distilled water (v/w) for 1.5 h each. The combined filtrates were concentrated under reduced pressure to a final concentration of 2 g crude drug/mL. The concentrate was stored at 4°C and diluted with distilled water to the required concentrations before administration. The low-, medium-, and high-dose regimens (2.5, 5, and 10 g crude drug/kg body weight) were selected based on preliminary experiments and human-rat dose conversion (human clinical dose: 0.36 g/kg, converted to a rat equivalent dose of approximately 2.5 g/kg as the low dose) (see [Supplementary Table 1](#)).

Animals

Specific pathogen-free male Sprague-Dawley (SD) rats (n = 50), weighing 200-250 g, were purchased from Sberford (Beijing) Biotechnology Co., Ltd. (certificate number: SCXK (Beijing) 2019-0010). The rats were housed in an animal room at 22 ± 1°C and 55 ± 5% humidity under a 12-h light/dark cycle, with free access to food and water. All experimental conditions were in strict accordance with the

relevant provisions of animal ethics of Yunnan Provincial Hospital of Traditional Chinese Medicine. After 7 days of adaptive feeding, SD rats without obvious abnormalities were used for related experimental research.

Analysis of blood-entering components of Yishen Tongluo Formula

The extract of Yishen Tongluo Formula was prepared, and an appropriate amount was centrifuged at 12,000 rpm and 4°C for 15 min. Then, 300 µL of the supernatant was placed in an Eppendorf tube, and 1 mL of the extract solution (water:methanol = 1:4) was added; the mixture was vortexed for 30 s, ultrasonicated in an ice-water bath for 5 min, allowed to stand at -40°C for 1 h, and centrifuged at 12,000 rpm and 4°C for 15 min. The supernatant was filtered through a 0.22-µm microporous membrane to obtain a traditional Chinese medicine sample for subsequent detection. Eight SD rats were randomly divided into a blank group and an administration group, with four rats in each group. The blank group was given distilled water by gavage, and the administration group was given Yishen Tongluo Formula extract by gavage (8 g/kg). Gavage was performed once daily for 7 days. One hour after the last gavage on the 7th day, the rats were anesthetized, blood was collected from the abdominal aorta, and serum was obtained after standing and centrifugation. A 400-µL serum sample was taken, and 40 µL of hydrochloric acid (2 mol/L) was added. The sample was vortexed 1 min, allowed to stand at 4°C for 15 min, and this procedure was repeated four times; 1.6 mL of acetonitrile was added, and the sample was vortexed for 5 min and centrifuged at 12,000 rpm for 5 min; 1.8 mL of the supernatant was collected and dried under nitrogen. Then, 150 µL of 80% methanol (internal standard concentration: 10 µg/mL) was added for redissolution, followed by vortexing for 5 min and centrifugation at 12,000 rpm for 5 min. Finally, 120 µL of the supernatant was transferred to an injection vial for analysis. Chromatographic analysis was performed using ultra-performance liquid chromatography. Chromatographic conditions were as follows: Waters UPLC BEH C18 column (1.7 µm × 2.1 mm × 100 mm); the injection volume was 5 µL, mobile phase A was water, mobile phase B was acetonitrile, and 0.1% formic acid was added to both phases A and B. MS analysis

was performed under the control of Xcalibur software using a Q Exactive Focus mass spectrometer (OE-MS), which collected MS1 and MS2 data using the FullScan-ddMS2 function. The specific parameters were as follows: capillary temperature: 400°C; collision energy: 15/30/45 eV in normalized collision energy mode; sheath gas flow rate: 45 Arb; auxiliary gas flow rate: 15 Arb; spray voltage: 4.0 kV (positive mode) or -4.0 kV (negative mode); full MS resolution: 70,000; and MS/MS resolution: 17,500. XCMS software was used to import the raw MS data, correct the retention time, and identify, extract, integrate, and align the data peaks. The peaks of MS/MS data were identified by matching the corresponding fragmentation rules and using the tandem MS database.

AS model

TG tablets were prepared with purified water to a concentration of 10 mg/mL, and TG was administered by gavage at 20 mg/(kg·d) for 5 weeks. If sperm motility in the model group was at least 50% lower than that in the blank group, the model was judged to be successful.

Experimental grouping, treatment, and sample preparation

A total of 50 male specific pathogen-free SD rats were randomly assigned to five groups (n = 10 per group): the control group, the model group, the low-dose Yishen Tongluo Formula group (YSTL-L), the medium-dose Yishen Tongluo Formula group (YSTL-M), and the high-dose Yishen Tongluo Formula group (YSTL-H). The control group received normal saline throughout the experiment. The other four groups were administered TG tablets to establish the AS model. During modeling, two rats in the model group and two rats in the YSTL-L group died due to gavage-related complications or modeling failure; therefore, eight rats from each group were included in the final analysis. No mid-course dropouts occurred in the remaining groups.

After successful modeling, the model group received normal saline, whereas the YSTL-L, YSTL-M, and YSTL-H groups received Yishen Tongluo Formula at 2.5, 5, or 10 g/kg, respectively, once daily for 5 weeks. One hour after the final administration, all rats were weighed

and anesthetized with isoflurane using a small-animal respiratory anesthesiometer. The rats were euthanized by blood collection from the abdominal aorta under deep anesthesia, followed by cervical dislocation to ensure death. Every effort was made to minimize animal suffering.

The testes and epididymides were collected immediately after euthanasia. The right testis and epididymis were weighed, and the organ index was calculated. The epididymis was transferred to a centrifuge tube containing normal saline, thoroughly mixed, and incubated in a 37°C water bath for 20 min for subsequent sperm analysis. A portion of the testis was fixed in 4% paraformaldehyde for histological and immunohistochemical analyses, and the remaining testicular tissue was cut into blocks and stored at -80°C for Western blotting and quantitative polymerase chain reaction (qPCR).

Analysis of sperm concentration and motility

Sperm viability, progressively motile sperm (PR), non-progressively motile sperm (NP), immotile sperm (IM), and sperm concentration were measured from the epididymal suspension prepared as described above. The measurements were performed using standardized procedures to ensure consistency across samples.

Histopathological analysis

The right testes of three rats from each group were fixed in fixative, embedded in paraffin, and stained with hematoxylin and eosin. Five images of different fields of view were captured for each rat under an optical microscope. CaseViewer software was used to evaluate lesions in the spermatogenic cells of the seminiferous tubules. The diameter of the seminiferous tubules was measured, their area and volume were calculated, and the Johnsen score was assigned.

Quantitative real-time PCR

Total RNA was extracted from approximately 5 mg of testis tissue from each rat in every group, and reverse transcription was performed according to the instructions of the reverse transcription kit. The resulting cDNA was synthesized and subsequently stored at -80°C.

The total reaction volume was 10 µL, including 0.3 µL of each primer (forward and reverse), 1.0 µL of cDNA, 3.4 µL of ddH₂O, and 5 µL of the reaction mix. Quantitative real-time PCR was performed to detect the expression of PINK1, Parkin, p62, LC3, LAMP2, SOD2, Nrf2, and HO-1 genes in the experimental rats of each group.

Western blot method

Fifty mg of testis tissue from each rat was collected, and the protein concentration of the testis protein samples in each group was determined by the bicinchoninic acid method. According to the concentration displayed by the microplate reader, the corresponding samples, ddH₂O, and buffer were added as required, placed in boiling water for denaturation, and then stored at -80°C. PINK1, Parkin, p62, LC3, LAMP2, SOD2, Nrf2, and HO-1 proteins in each group of experimental rats were detected by Western blot. The samples from each group were loaded onto the gel in order according to their concentrations in the electrophoresis solution. First, electrophoresis was performed until bromophenol blue reached the bottom of the gel. The proteins were then transferred to a membrane, blocked with 5% skim milk for 2 hours, incubated with the primary antibody, incubated with the secondary antibody at room temperature, washed, exposed, and imaged. The gray value of each band was analyzed using Image J software.

TEM

A 1 mm² piece of tissue from the left testis was collected and placed in electron microscope fixative at 4°C, then resuspended and fixed for 2-4 h. The sample was pre-embedded in agar, post-fixed, dehydrated at room temperature, embedded in resin, polymerized, sectioned, and stained. The sections were observed under a TEM, and images were acquired and analyzed.

Statistical analysis

All data were analyzed using SPSS 26.0 software and plotted using GraphPad Prism 9 software. Measurement data were expressed as $\bar{X} \pm S$. In the normality test, if the data between groups conformed to a normal distribution, the paired t-test or one-way analysis of variance

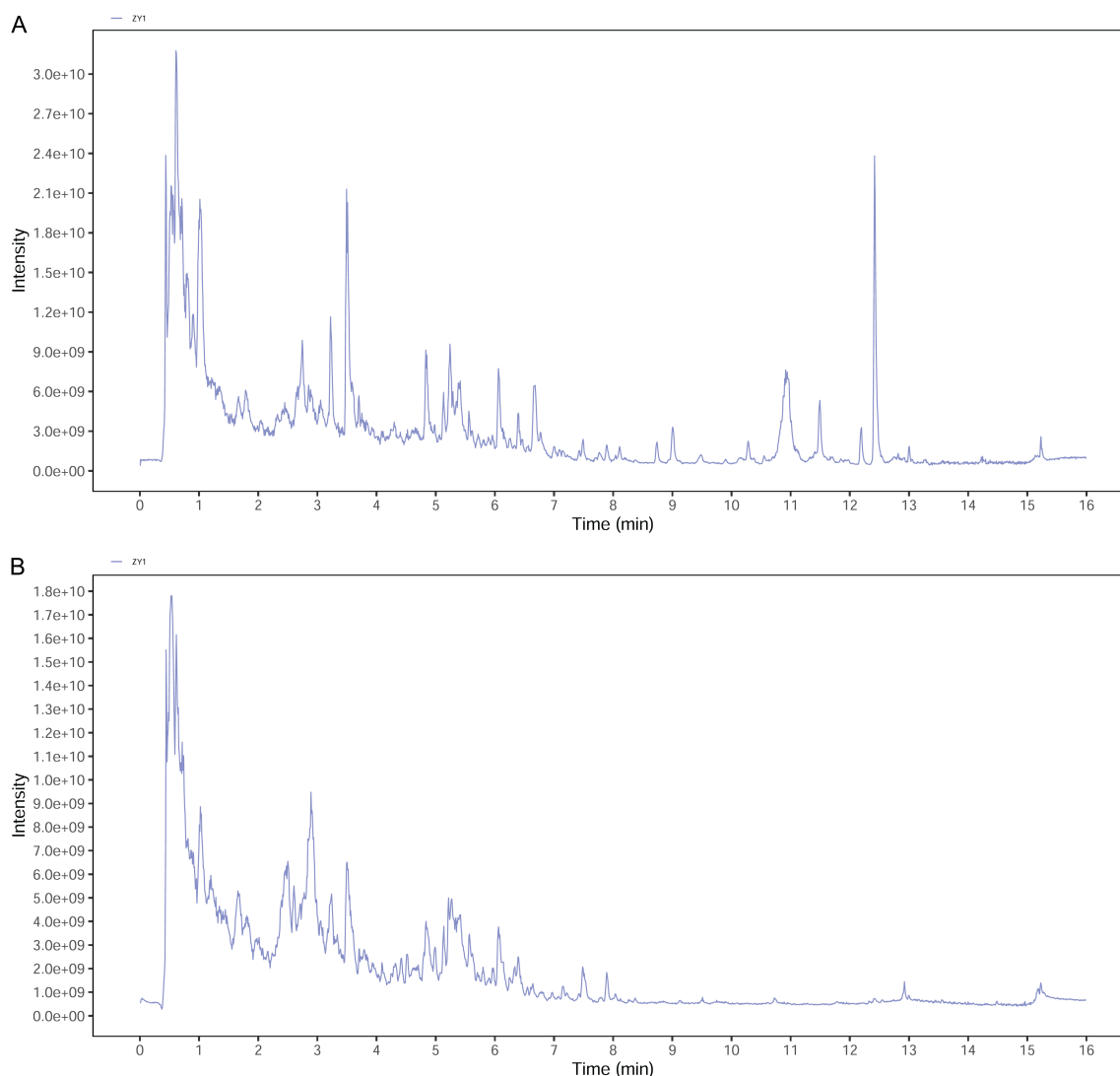


Figure 1. TIC chromatograms of Yishen Tongluo Formula samples. A. Positive-ion mode TIC. B. Negative-ion mode TIC. Note: TIC, total ion current.

was used; if not, the rank-sum test was used for statistical analysis. Comparisons between the YSTL-L, YSTL-M, and YSTL-H and the model group were performed, and $P < 0.05$ was considered statistically significant.

Results

Total ion current chromatogram of Yishen Tongluo Formula

Representative total ion current chromatograms of Yishen Tongluo Formula were obtained in both positive- and negative-ion modes. The chromatograms showed stable signal intensity and abundant chemical information, providing the basis for subsequent identification of blood-entering components (**Figure 1**).

Analysis of blood components of Yishen Tongluo Formula

According to the results of ultra-high-performance liquid chromatography-OE-MS, there are more than 1,200 chemical substances in Yishen Tongluo Formula, including terpenoids, flavonoids, quinones, and other types of substances. Among them, there are 80 active ingredients in blood: terpenoids account for 25, flavonoids for 16, phenylpropanoids for 6, phenols for 3, and alkaloids, isopentenol lipids, and fatty acyls each account for 2. Hydroxyl acids and their derivatives, organic oxygen compounds, quinones, phenylpyrans, aromatic compounds, naphthalenes, cinnamic acids and their derivatives, lignan glycosides, carboxylic acids and their derivatives, linear 1,3-diarylpro-

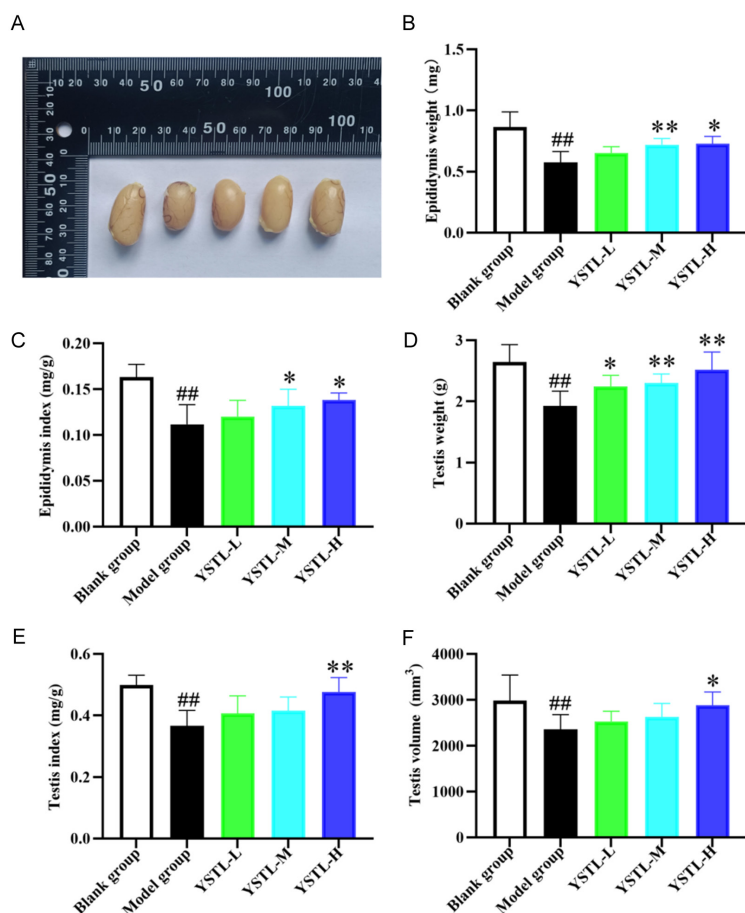


Figure 2. Effects of Yishen Tongluo Formula on gonadal index of TG-induced AS rats. A. Testis appearance size (from left to right: Blank group, Model group, YSTL-L, YSTL-M, YSTL-H). B. Epididymis weight. C. Epididymis index. D. Testis weight. E. Testis index. F. Testis volume ($n = 8$ per group). Statistical significance: Compared with the Blank group, $^{##}P < 0.01$; compared with the Model group, $^{*}P < 0.05$, $^{**}P < 0.01$. Abbreviations: YSTL-L, low-dose Yishen Tongluo Formula; YSTL-M, medium-dose Yishen Tongluo Formula; YSTL-H, high-dose Yishen Tongluo Formula; TG, tripterygium glycoside; AS, asthenospermia.

panols, and esters each accounted for 1 species, and other components accounted for 13 species. Saikosaponin A, formononetin, and lupenone were derived from *Astragalus membranaceus*. Akebia saponin D was derived from *Dipsacus asperoides*. Ginsenoside Re, tanshinone IIA, 20(R)-ginsenoside Rh1, ginsenoside Rg1, salvianolic acid A, and harpagoside were derived from *Salvia miltiorrhiza* and *Panax notoginseng*. Triptolide A, trifolirhizin, Sissotrin, gossypol, Flavone base + 40, 1-Prenyl, 6-methoxy-7-isopentenylcoumarin, Abruquinone B were derived from *Astragali Radix* and *Semen Astragali Complanati*. Withaphysacarpin, (-)-secoisolariciresinol, 1,2-haloperido- α -cyperone, 3,5,5-Trimethyl-4-[3-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxybut-1-enyl] cyc-

lohex-2-en-1-one, Benzenepropanamide, N-[2-(acetyloxy)-1-(phenylmethyl)ethyl]- α -(benzoylamino) were derived from *Lycii Fructus*. Lulutong acid was derived from *Lulutong*, and 4,7-dimethyl-7-(4-methylpent-3-enyl)bicyclo[2,2,1]hept-3-ol, 2-hexyl-5-[2-(4-hydroxy-3-methoxyphenyl)ethyl]furan, demethoxycurcumin were derived from *Curcumae Rhizoma*. Corosolic acid and lindenolide were also identified. Hydroxytanshinone IIA was derived from *Radix Salviae Miltiorrhizae*. ATP was derived from *Rhizoma Dioscoreae*, *Radix Salviae Miltiorrhizae*, *Radix Codonopsis*, *Fructus Lycii*, *Radix Astragali seu Hedysari*, *Radix Notoginseng*, *Radix Polygoni Multiflori*, and *Semen Astragali Complanati*. Furanochromone was derived from *Rhizoma Cyperi* and *Rhizoma Dioscoreae*. Quercetin was derived from *Radix Salviae Miltiorrhizae*, *Radix Astragali seu Hedysari*, *Radix Dipsaci*, and *Semen Cuscutae*. Malic acid was derived from *Radix Salviae Miltiorrhizae* and *Fructus Lycii*. Raffinose was derived from *Radix Morindae Officinalis*, *Radix Astragali seu Hedysari*, and *Semen Astragali Complanati*.

Taxol glucoside and Karakoline were derived from *Radix Polygoni Multiflori*. Due to the complex composition of the prescription, chemical reactions occurred between traditional Chinese medicines during the cooking process, and the remaining 42 components could not be presumed to originate from specific herbs (see [Supplementary Table 2](#)).

Yishen Tongluo Formula improves the gonadal index of TG-induced AS model rats

The right testis and epididymis of rats in each group were weighed to calculate their weight, organ index, and testicular volume. The results are shown in **Figure 2**. Compared with the blank group, the testicular volume, testicular mass, testicular index, epididymal mass, and epididy-

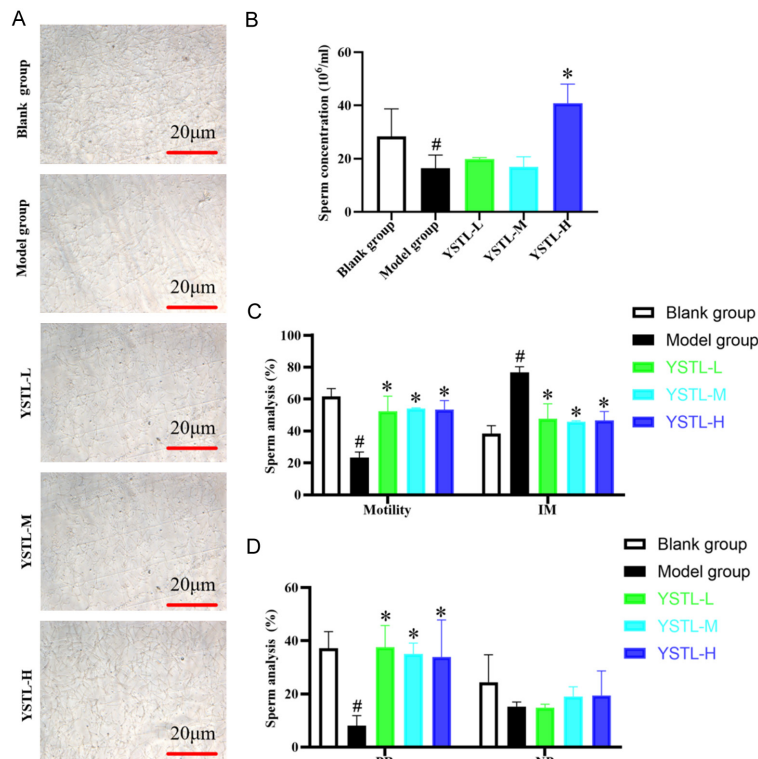


Figure 3. Effects of Yishen Tongluo Formula on sperm quality. A. Representative sperm images observed under a microscope (20× magnification). B. Sperm concentration (n = 8 per group). C. Sperm survival rate and IM proportion. D. Proportion of PR and NP. Statistical significance: Compared with the Blank group, [#] $P < 0.05$; compared with the Model group, ^{*} $P < 0.05$. Abbreviations: YSTL-L, low-dose Yishen Tongluo Formula; YSTL-M, medium-dose Yishen Tongluo Formula; YSTL-H, high-dose Yishen Tongluo Formula; IM, immotile sperm; PR, progressively motile sperm; NP, non-progressively motile sperm.

mal index of rats in the model group decreased to varying degrees ($P < 0.01$). Compared with the model group, the testicular mass (YSTL-L, YSTL-M, YSTL-H), testicular index and volume (YSTL-H), and epididymal mass and index (YSTL-M, YSTL-H) of rats were restored to normal levels to varying degrees after intervention treatment with Yishen Tongluo Formula ($P < 0.05$). The above results suggest that Yishen Tongluo Formula can effectively improve the gonadal index of TG-induced AS rats.

Yishen Tongluo Formula improves sperm quality in TG-induced AS model rats

The right epididymis of the rats was collected to detect and analyze sperm quality. The results are shown in **Figure 3**. Compared with the blank group, PR, sperm survival rate, and sperm concentration of the model group decreased to

varying degrees ($P < 0.05$). IM was significantly increased ($P < 0.05$). NP showed a downward trend, but the difference was not statistically significant. Compared with the model group, sperm concentration (YSTL-H), PR, and sperm survival rate (YSTL-L, YSTL-M, YSTL-H) returned to normal levels to varying degrees after treatment with Yishen Tongluo Formula ($P < 0.05$). IM decreased significantly ($P < 0.05$). NP showed an upward trend, but the difference was not statistically significant. The above results suggest that Yishen Tongluo Formula can effectively improve sperm quality in TG-induced AS rats.

Yishen Tongluo Formula improves testicular lesions in TG-induced AS rats

Rat testicular tissue was routinely embedded, sectioned, and stained with hematoxylin and eosin. Pathological changes in the testicular seminiferous tubules were observed under a light microscope, and the Johnsen score was evaluated.

As shown in **Figure 4A**, in the blank group, the structure of the seminiferous tubules was normal, the seminiferous epithelium was intact, and numerous sperm with normal morphology, orderly arrangement, clear hierarchy, and spermatogenic cells at all levels were observed in the lumen. In the model group, the arrangement of seminiferous tubules was disordered, the spacing between tubules was increased, the lumen was reduced or expanded, the spermatogenic epithelium was thinned, the basement membrane was incomplete, and the spermatogenic cells and mature sperm in the lumen were greatly reduced and disorganized.

Compared with the blank group, the diameter and area of seminiferous tubules and the Johnsen score in the model group showed a decreasing trend ($P < 0.05$). Compared with the model group, after treatment with Yishen

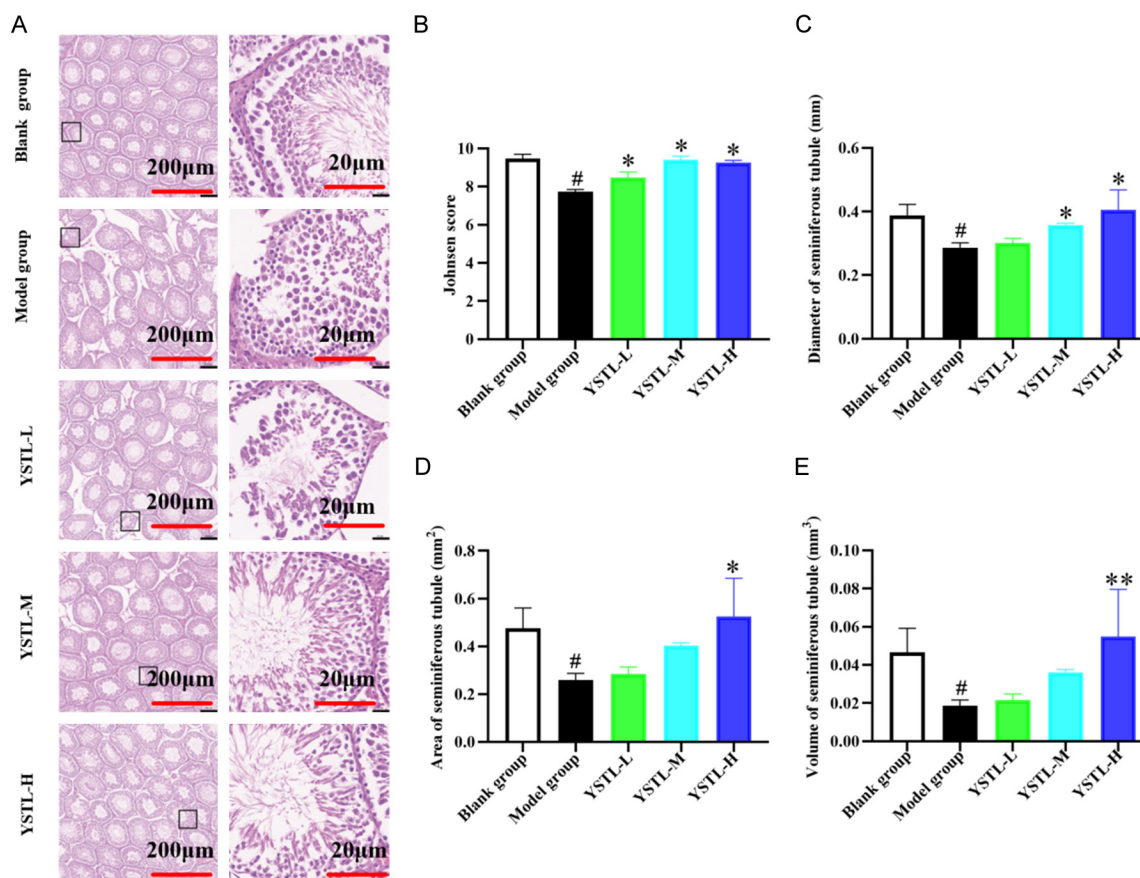


Figure 4. Histological changes in rat testes. A. HE staining of testicular tissue (Scale bar = 200 μ m for 5 $\times$ images, left column; Scale bar = 20 μ m for 40 $\times$ images, right column; from top to bottom: Blank group, Model group, YSTL-L, YSTL-M, YSTL-H). B. Johnsen score. C. Diameter of seminiferous tubules. D. Area of seminiferous tubules. E. Volume of seminiferous tubules (n = 8 per group). Statistical significance: Compared with the Blank group, $^{\#}P<0.05$; compared with the Model group, $^{\ast}P<0.05$, $^{**}P<0.01$. Abbreviations: HE, hematoxylin and eosin; YSTL-L, low-dose Yishen Tongluo Formula; YSTL-M, medium-dose Yishen Tongluo Formula; YSTL-H, high-dose Yishen Tongluo Formula.

Tongluo Formula, the Johnsen score (YSTL-L, YSTL-M, YSTL-H) and the diameter of seminiferous tubules (YSTL-M, YSTL-H) gradually returned to normal levels ($P<0.05$), and both the diameter and area of seminiferous tubules in the high-dose group were significantly increased ($P<0.05$) (Figure 4B-E). The results suggest that Yishen Tongluo Formula can effectively improve testicular seminiferous tubule lesions in TG-induced AS rats.

Yishen Tongluo Formula protects testicular tissue function via oxidative stress reduction

The results of quantitative PCR (qPCR) (Figure 5A-C) showed that, compared with the blank group, the mRNA expression of SOD2, Nrf2, and HO-1 in the model group was significantly decreased ($P<0.05$). Compared with the model

group, the mRNA expression of HO-1 was increased in YSTL-M ($P<0.05$) and significantly increased in YSTL-H ($P<0.01$). The mRNA expression of Nrf2 was increased in YSTL-M and YSTL-H ($P<0.05$). The mRNA expression of SOD2 was increased in YSTL-L ($P<0.05$) and significantly increased in YSTL-M and YSTL-H ($P<0.01$). The results of Western blot (Figure 5D-G) showed that the protein expression level of Nrf2 in the model group was significantly lower than that in the blank group ($P<0.05$). Compared with the model group, the expression levels of HO-1, SOD2, and Nrf2 proteins in YSTL-H of Yishen Tongluo Formula were increased ($P<0.05$). Although the expression levels of proteins in YSTL-L and YSTL-M showed an increasing trend, there was no statistical significance. Notably, a partial discrepancy was observed between mRNA and protein expres-

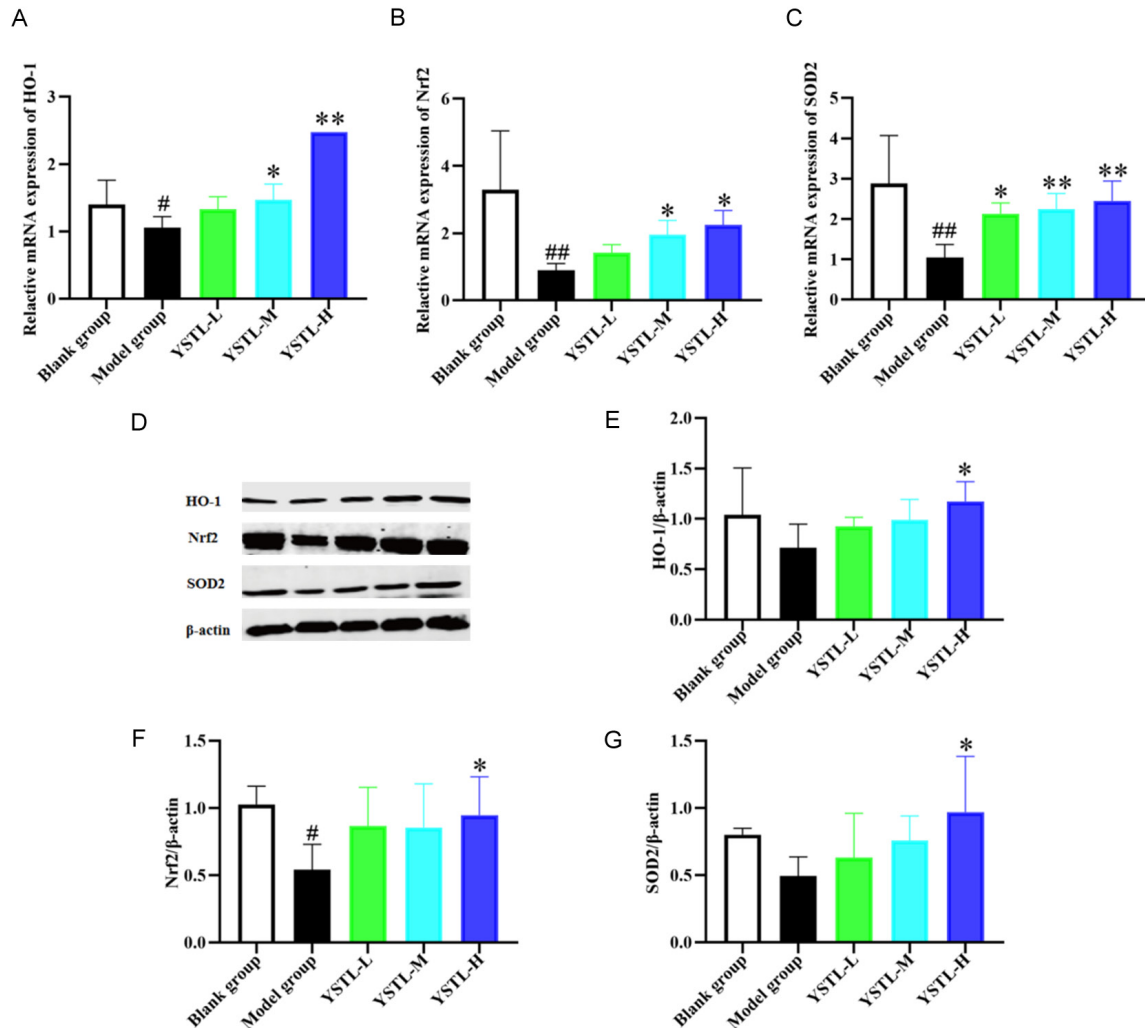


Figure 5. Relative mRNA levels of oxidative stress-related genes and Western blot analysis of protein expression. A. HO-1 transcription level. B. Nrf2 transcription level. C. SOD2 transcription level. D. Western blot analysis of oxidative stress protein gray values. E. HO-1 protein quantitative analysis. F. Nrf2 protein quantitative analysis. G. SOD2 protein quantitative analysis (n = 8 per group). Statistical significance: Compared with the Blank group, [#]*P*<0.05, ^{##}*P*<0.01; compared with the Model group, ^{*}*P*<0.05, ^{**}*P*<0.01. Abbreviations: YSTL-L, low-dose Yishen Tongluo Formula; YSTL-M, medium-dose Yishen Tongluo Formula; YSTL-H, high-dose Yishen Tongluo Formula; HO-1, heme oxygenase-1; Nrf2, nuclear factor erythroid 2-related factor 2; SOD2, superoxide dismutase 2; qPCR, quantitative polymerase chain reaction; β-actin, beta-actin.

sion levels for some markers in certain dose groups. For instance, SOD2 mRNA showed a significant increase in the YSTL-L group (Figure 5C), whereas the corresponding protein increase was not statistically significant (Figure 5G). This phenomenon is not uncommon in biological systems and may be attributed to several factors, including but not limited to: (1) a temporal lag between transcriptional activation and subsequent translation and protein accumulation; (2) post-translational modifications

regulating protein stability or activity without altering abundance detectable by Western blot under the conditions used; and (3) differing sensitivities of qPCR and Western blot techniques. Despite these minor variances at specific doses, the overall concordant upward trend in both mRNA and protein levels of SOD2, Nrf2, and HO-1 across medium- and high-dose groups strongly supports the activation of the Nrf2-mediated antioxidant pathway by Yishen Tongluo Formula. qPCR and Western blot have

different sensitivities and dynamic ranges, which may contribute to discrepancies, especially at lower expression levels.

Yishen Tongluo Formula regulates mitophagy in testicular tissue of TG-induced AS rats

qPCR and Western blot were used to detect LC3, LAMP2, PINK1, Parkin, and p62 in rat testicular tissue. The results of qPCR (**Figure 6A-E**) showed that, compared with the blank group, the mRNA expression of LC3, LAMP2, PINK1, and Parkin in the model group was significantly increased ($P<0.05$) and the mRNA expression of p62 was significantly decreased ($P<0.05$). Compared with the model group, the mRNA expression levels of LAMP2 (YSTL-M, YSTL-H), LC3 (YSTL-L, YSTL-M), and Parkin and PINK1 (YSTL-L, YSTL-M, YSTL-H) were significantly decreased after treatment with Yishen Tongluo Formula ($P<0.05$). The mRNA expression of p62 (YSTL-M, YSTL-H) was significantly increased ($P<0.05$). The Western blot results (**Figure 6F-K**) showed that, compared with the blank group, the protein expression levels of LC3, LAMP2, PINK1, and Parkin in the model group were significantly increased ($P<0.05$), and the protein expression level of p62 was significantly decreased ($P<0.05$). Compared with the model group, the protein expression levels of LAMP2 (YSTL-L, YSTL-M, YSTL-H), LC3 (YSTL-M, YSTL-H), Parkin (YSTL-H), and PINK1 (YSTL-L, YSTL-H) decreased after treatment with Yishen Tongluo Formula ($P<0.05$). The protein expression level of p62 (YSTL-L, YSTL-M, YSTL-H) were increased ($P<0.05$). The above results suggest that Yishen Tongluo Formula can effectively reduce the expression of LC3, LAMP2, PINK1, and Parkin in the testis of TG-induced AS rats and increase the expression of p62, thereby regulating mitophagy and exerting therapeutic effects.

Yishen Tongluo Formula improves mitochondrial morphology and reduces mitophagy in testicular Sertoli cells

As shown in **Figure 7**, TEM observation of Sertoli cells in testicular tissue revealed normal mitochondrial morphology in the blank group, with mitochondria distributed throughout the cytoplasm. These mitochondria had smooth outer and inner membranes, closely arranged cristae, and a granular internal matrix with moderate electron density. Compared with the

blank group, Sertoli-cell mitochondria in the model group showed obvious swelling, increased volume, and unclear cristae. Compared with the model group, the YSTL-L group showed increased mitochondrial electron density and disordered cristae, although some mitochondria with clear cristae were still observed. The YSTL-M group showed clearer mitochondrial cristae, reduced intercrystal matrix density, and visible electron-transparent vacuoles. The YSTL-H group showed near-normal Sertoli-cell mitochondrial morphology, with smooth outer and inner membranes and cristae containing a granular matrix. These results indicate that Yishen Tongluo Formula improves Sertoli-cell mitochondrial morphology and reduces excessive mitophagy.

Discussion

Ancient books of traditional Chinese medicine do not record the name of AS, which is generally classified into the categories of oligospermia and infertility. Most cases are attributed to a lack of congenital endowment or long-term illness. It is recorded in the Dasheng Chapter that “it is thin and weak, such as the seed cannot germinate, so it is difficult to produce”. On the basis of laboratory examinations, modern doctors have a deeper understanding of AS. Ma et al. [4] summarized Professor Wang Qi's view that modern people generally have sufficient food and clothing and that acquired damp-heat toxin, rather than congenital kidney deficiency alone, is an important pathogenic factor; therefore, kidney deficiency with damp-heat stasis toxin was proposed as a key pathogenesis. Lin et al. [5] reported that Jujing Zhuyu Decoction can inhibit apoptosis in asthenozoospermia rats by regulating the mitochondrial apoptosis pathway. In addition, other pathogenesis theories include yin-yang damage and yang deficiency. The treatment focuses on tonifying the kidney and invigorating essence, soothing the liver, activating blood, regulating qi, and resolving phlegm [22]. Western medicine considers AS to be a multifactorial disease related to heredity, environment, endocrine disorders, obesity, smoking, trace elements, infection, drug effects, varicocele, and other factors [23]. Western medical treatment is mainly symptomatic, including lifestyle improvement, anti-infection therapy, hormone therapy, and surgical treatment [24]. Professor Yuan Zhuojun is a

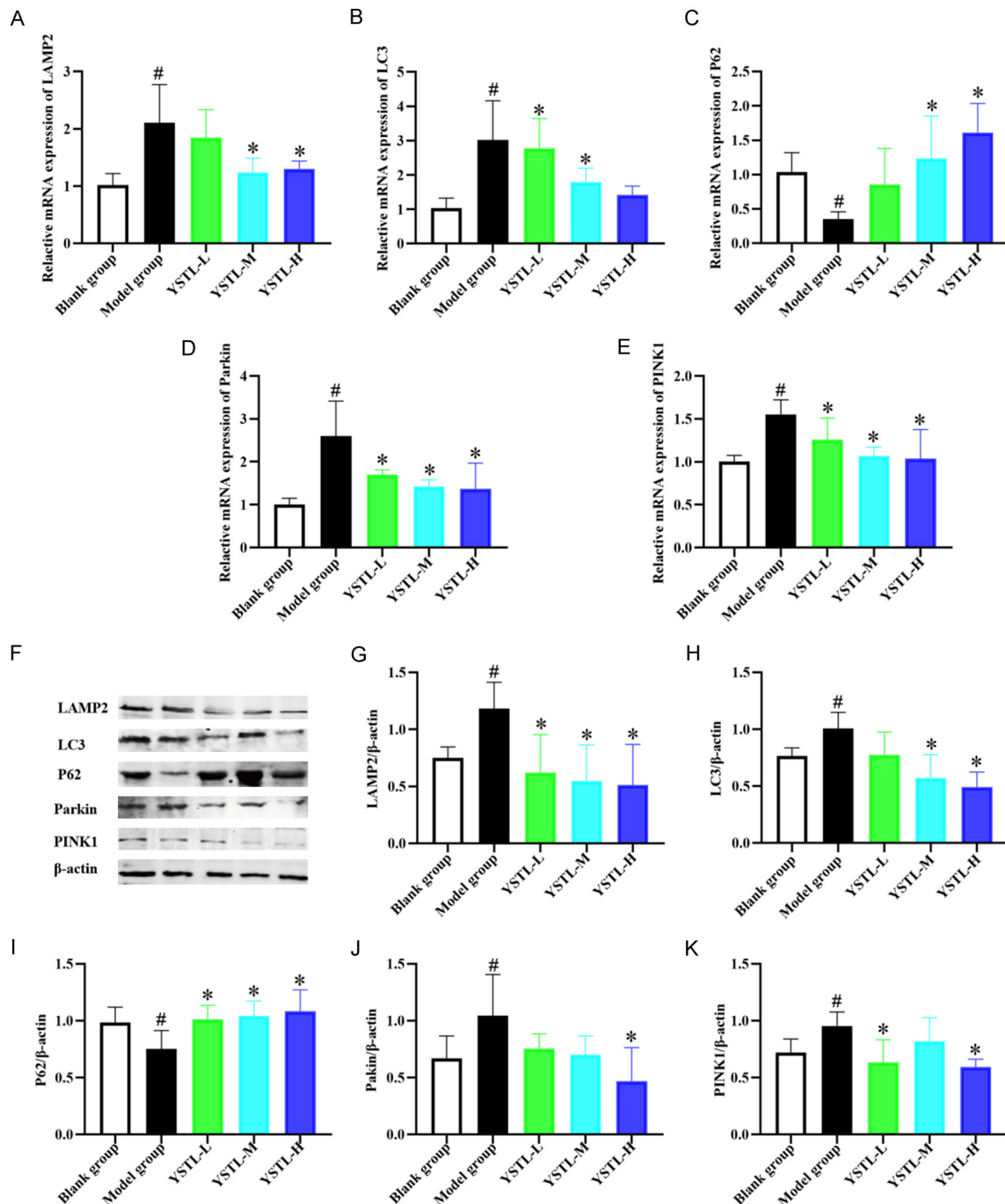


Figure 6. Relative mRNA levels of mitophagy-related genes and Western blot analysis of mitophagy-related protein expression. A. LAMP2 mRNA expression level. B. LC3 mRNA expression level. C. p62 mRNA expression level. D. Parkin mRNA expression level. E. PINK1 mRNA expression level. F. Western blot bands of LAMP2, LC3, p62, Parkin, PINK1, and β -actin. G. Quantitative analysis of LAMP2 protein expression. H. Quantitative analysis of LC3 protein expression. I. Quantitative analysis of p62 protein expression. J. Quantitative analysis of Parkin protein expression. K. Quantitative analysis of PINK1 protein expression (n = 8 per group). Statistical significance: Compared with the Blank group, $^{\#}P < 0.05$; compared with the Model group, $^*P < 0.05$. Abbreviations: LAMP2, lysosomal-associated membrane protein 2; LC3, microtubule-associated protein 1 light chain 3; p62, sequestosome 1; PINK1, PTEN-induced putative kinase 1; β -actin, beta-actin; YSTL-L, low-dose Yishen Tongluo Formula group; YSTL-M, medium-dose Yishen Tongluo Formula group; YSTL-H, high-dose Yishen Tongluo Formula group.

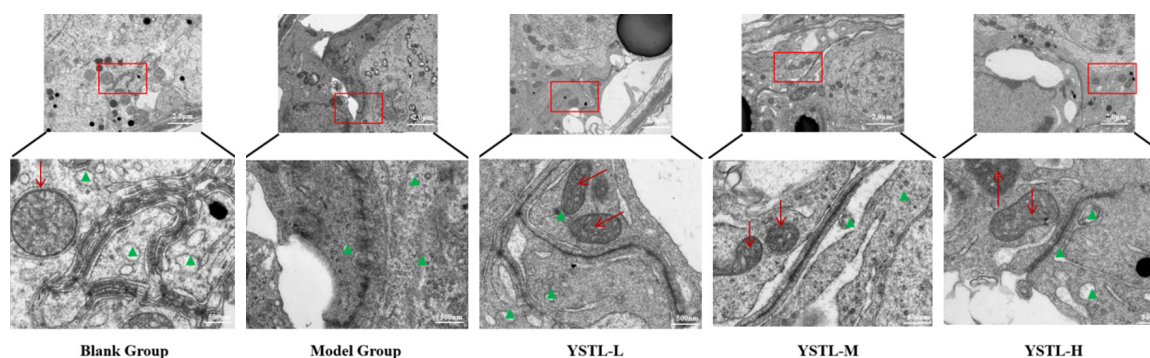


Figure 7. Mitochondrial ultrastructure in testicular Sertoli cells observed by transmission electron microscopy. Representative transmission electron microscopy images show mitochondrial morphology in Sertoli cells. Magnification increased from top to bottom (5,000 $\times$ and 20,000 $\times$); scale bars = 2 μ m and 500 nm, respectively. From left to right: Blank group, Model group, YSTL-L, YSTL-M, and YSTL-H. Red arrows indicate mitochondria; green arrowheads indicate Sertoli cells. Abbreviations: YSTL-L, low-dose Yishen Tongluo Formula group; YSTL-M, medium-dose Yishen Tongluo Formula group; YSTL-H, high-dose Yishen Tongluo Formula group.

third-generation traditional Chinese medicine practitioner in her family. She inherited the academic experience of Professor Wang Qi and Professor Qin Guozheng in the treatment of AS and introduced the theory of “chronic illness entering collaterals” into AS based on her family tradition. It is believed that most patients with AS have no clinical symptoms and are generally identified during examination. Therefore, most patients have been ill for a long time, and the disease evolves from the meridians to collaterals. According to the traditional Chinese medicine theory that “meridians govern Qi and collaterals govern blood”, Qi deficiency without blood circulation is likely to lead to blood stasis; blood stasis damages the kidney, affecting the kidney’s spermatogenic function; Qi and blood are derived from kidney essence, and kidney deficiency leads to Qi and blood deficiency, further aggravating kidney damage. Therefore, Professor Yuan Zhuojun believes that kidney deficiency and blood stasis are reciprocally causative and together lead to the occurrence of AS. On the basis of this pathogenesis, the self-made empirical prescription Yishen Tongluo Formula, which is based on tonifying the kidney and dredging collaterals, has achieved good results in clinical practice.

Analysis of blood-entering components of Yishen Tongluo Formula

In this study, 80 blood-entering components were detected by ultra-high-performance liquid chromatography-OE-MS, and the existing chemical components of 17 traditional Chinese med-

icines in the prescription were searched in the literature. We speculated that these 80 blood-entering components were more likely to pass through the intestinal barrier, although they were not necessarily the most abundant components in the prescription. Among the 80 compounds, terpenoids accounted for 25 species, flavonoids accounted for 16 species, phenylpropanoids accounted for 6 species, phenols accounted for 3 species, alkaloids, isopentenol lipids, and fatty acyls each accounted for 2 species, hydroxyl acids and their derivatives, organic oxygen compounds, quinones, phenylpyrans, aromatic compounds, naphthalenes, cinnamic acids and their derivatives, lignan glycosides, carboxylic acids and their derivatives, linear 1,3-diarylpropanols, and esters each accounted for 1 species, and other components accounted for 13 species. Among them, terpenoids accounted for the largest proportion. Studies have shown that terpenoid compounds have anti-inflammatory [25], anti-tumor [26], neurotrophic, and other activities [27]. Flavonoids also accounted for a large proportion. Studies have shown that flavonoids have antioxidation [28], anti-inflammatory [29], and blood viscosity-reducing effects [30]. Therefore, Yishen Tongluo Formula may achieve the effect of treating AS through mechanisms such as antioxidation, anti-inflammation, and improvement of local blood circulation.

Preparation of TG-induced AS model rats

TG is a commonly used drug in rheumatology and is the root extract of *Tripterygium wilfordii*. Studies have shown that long-term use of

Tripterygium wilfordii can lead to reversible male infertility, including reduced sperm count, decreased sperm motility, and decreased sperm survival rate. Therefore, its reproductive toxicity can be used to prepare a rat model of AS. Most scholars have given GT at 20 mg/(kg·d) to rats for 4 weeks, and the results show that an AS rat model can be successfully prepared [31]. Therefore, this experiment was based on the previous model. The results of this study showed that the gonadal index of rats in the model group was significantly lower than that in the blank group after modeling with TG, indicating that TG could affect the reproductive development of rats, thus affecting their sperm motility. After treatment with Yishen Tongluo Formula, the gonadal index was improved to varying degrees. This shows that Yishen Tongluo Formula can improve the development of the reproductive system after TG modeling, significantly improve the sperm motility of rats with AS, and provide experimental data support for the clinical treatment of AS with Yishen Tongluo Formula.

Effect of Yishen Tongluo Formula on oxidative stress-related indicators

Among the many causes of AS, most mechanisms are related to oxidative stress. ROS are by-products of aerobic respiration and metabolism. At the physiological level, ROS play an important role in the processes of spermatogenesis, development, maturation, and capacitation. Under the action of various physical and chemical factors, when the concentration of ROS exceeds the physiological level, OS occurs in the body and destroys the antioxidant defense system of sperm and seminal plasma, which has a negative impact on the ultrastructure, morphology, and function of sperm. Semen contains a variety of antioxidant components, such as SOD, glutathione peroxidase, and HO-1, which are classified as enzymatic antioxidants. SOD plays an important role in the elimination of O_2^- free radicals. It can resist the spermatogenic toxicity caused by ROS and maintain the oxidation-antioxidation system in germ cells in a balanced state. The concentration of SOD was positively correlated with the level of spermatogenesis. There are three kinds of SOD isoenzymes in the human body [32]: soluble SOD, mitochondrial SOD (SOD2), and extracellular SOD, among which SOD2 is more

highly expressed in human sperm. SOD2 has the effects of scavenging ROS and antagonizing oxidative stress, thereby protecting cells. It is mainly distributed in mitochondria and is expressed in the acrosome region and neck of sperm [33]. Reduced glutathione can reduce H_2O_2 to H_2O , thereby removing H_2O_2 and protecting the structure and function of the cell membrane. Nrf2, as an important intracellular oxidative stress-related transcription factor, can maintain the internal oxidative balance of cells, so that the body can effectively resist endogenous and external oxidative stress damage. Under hypoxia, various stresses, heat shock, and other conditions, Nrf2 can regulate the antioxidant protein SOD2 in sperm and activate the expression and activity of downstream HO-1 protein. The former is mainly located in mitochondria and is expressed in the acrosome region and neck of sperm. As one of the most important endogenous antioxidant protective factors in the body, the latter has a strong cytoprotective effect and mediates an antioxidant response. The qPCR results showed that after TG modeling, the mRNA expression levels of SOD2, Nrf2, and HO-1 in AS rats were significantly decreased. Western blot results showed that Nrf2 protein expression was significantly decreased, whereas HO-1 and SOD2 protein expression showed a decreasing trend. This indicates a compromised endogenous antioxidant defense system. Intervention with Yishen Tongluo Formula, particularly at medium and high doses, effectively reversed this trend by upregulating the expression of these factors. Specifically, our data (**Figure 5**) demonstrated that Yishen Tongluo Formula increased the mRNA and protein levels of the central regulator Nrf2 and its downstream effectors SOD2 and HO-1, mainly in the medium- and high-dose groups. This coordinated upregulation suggests that the prescription activated the Nrf2-mediated antioxidant signaling pathway. Consequently, the increased expression of these antioxidant enzymes (SOD2 and HO-1) may enhance antioxidant defense, thereby protecting testicular tissue function from oxidative stress. Therefore, Yishen Tongluo Formula can protect testicular tissue function by reducing oxidative stress, as evidenced by the overall upregulation trend of the Nrf2 pathway components despite minor methodological discrepancies at specific data points. The 5-week inter-

vention may allow for transcriptional activation without full translational accumulation in low-dose groups, whereas high-dose treatment may sustain signaling long enough to achieve detectable protein increases. Notably, while SOD2 mRNA was significantly elevated in the YSTL-L group, the corresponding protein increase did not reach statistical significance. This discrepancy may reflect that the 5-week intervention period was sufficient for transcriptional activation but not for sustained protein accumulation, possibly due to translational regulation or post-transcriptional feedback. In contrast, the high-dose group showed coordinated upregulation of both mRNA and protein, indicating that a threshold dose may be required for robust pathway activation. These findings are consistent with the known biology of Nrf2-mediated antioxidant responses, which involve both transcriptional and post-translational control mechanisms.

Abnormal activation of the PINK1/Parkin signaling pathway is an important mechanism in the development of AS

The PINK1/Parkin signaling pathway is an important pathway in mitophagy. PINK1 is one of the initial signals that triggers mitophagy. Under normal conditions, PINK1 is continuously imported into healthy mitochondria through the translocase of the outer membrane/translocase of the inner membrane complexes in a mitochondrial membrane potential-dependent manner, cleaved by mitochondrial proteases, and subsequently degraded by the proteasome, resulting in very low basal PINK1 levels on the mitochondrial outer membrane. When mitochondria are damaged, the mitochondrial membrane potential is reduced, and PINK1 transport is blocked. PINK1 accumulates and stabilizes on the outer mitochondrial membrane and recruits and activates Parkin from the cytoplasm to the outer membrane through phosphorylation. Parkin can amplify the mitophagy signal [34]. On the one hand, it provides more phosphorylated substrates for PINK1 and promotes PINK1 recruitment and activation of more Parkin. On the other hand, it promotes ubiquitination of proteins on the mitochondrial membrane [35], and ubiquitinated proteins on mitochondria interact with p62. p62 binds to LC3 to form p62-LC3, and LAMP2 participates in the fusion of autophagosomes with lyso-

somes to form autolysosomes, which are finally degraded. With the increase in autophagy, LC3 and LAMP2 showed an upward trend, while p62 was continuously degraded with the increase in mitophagy activity, showing a downward trend [36]. In this study, we provided direct experimental evidence linking the therapeutic effect of Yishen Tongluo Formula to the modulation of the PINK1/Parkin-mediated mitophagy pathway in TG-induced AS rats. Our qPCR and Western blot results (**Figure 6**) revealed that the TG model group exhibited a marked increase in the expression of mitophagy initiators and markers - PINK1, Parkin, LC3, and LAMP2 - accompanied by a significant decrease in the selective autophagy receptor p62. This expression profile was indicative of excessive mitophagy activation under pathological conditions. Crucially, treatment with Yishen Tongluo Formula reversed these alterations: it significantly downregulated the mRNA and protein levels of PINK1, Parkin, LC3, and LAMP2, while upregulating p62 expression. The downregulation of LC3 and LAMP2, coupled with the accumulation of p62, suggests that Yishen Tongluo Formula may suppress excessive mitophagy activation. This pharmacological modulation of the PINK1/Parkin pathway by Yishen Tongluo Formula was consistent with the observed improvement in mitochondrial ultrastructure (**Figure 7**), where damaged mitochondria with swollen morphology and unclear cristae in the model group were partially restored toward a normal appearance after treatment. Therefore, our data collectively indicated that Yishen Tongluo Formula ameliorated AS, at least in part, by inhibiting injury-induced overactivation of the PINK1/Parkin mitophagy pathway, thereby preserving mitochondrial integrity and function in Sertoli cells.

Effect of Yishen Tongluo Formula on mitochondrial ultrastructure of Sertoli cells

Spermatogenesis and maturation are essential for normal sperm concentration and motility, and Sertoli cells provide structural and metabolic support for germ-cell development within the seminiferous epithelium. Because Sertoli-cell mitochondrial function is closely related to the seminiferous epithelial microenvironment, we observed mitochondrial ultrastructural changes in testicular Sertoli cells by TEM. Compared with the blank group, Sertoli-cell mito-

chondria in the model group were swollen and enlarged, and the cristae were unclear. Compared with the model group, the low-dose group showed increased mitochondrial electron density and disordered cristae, although some mitochondria with clear cristae were still observed. In the medium-dose group, the mitochondrial cristae were clearer, although the intercrystal matrix density was reduced and electron-transparent vacuoles were visible. In the high-dose group, Sertoli-cell mitochondria showed near-normal morphology. These findings indicate that different doses of Yishen Tongluo Formula can gradually improve TG-induced Sertoli-cell mitochondrial injury and protect mitochondrial ultrastructure.

Conclusion

The results of serum pharmacochimistry showed that there were more than 1,200 components in Yishen Tongluo Formula and 80 blood-entering active components. These 80 components were likely to be absorbed through the intestinal barrier, although they were not necessarily the most abundant components. Among these components, terpenoids and flavonoids accounted for relatively large proportions. Terpenoids have anti-inflammatory, anti-tumor, neurotrophic, and other activities, and flavonoids have antioxidant, anti-inflammatory, blood viscosity-reducing, and other effects; therefore, Yishen Tongluo Formula may treat AS through mechanisms such as antioxidation, anti-inflammation, and improvement of local blood circulation.

Yishen Tongluo Formula can improve sperm motility in AS model rats, alleviate mitochondrial functional damage in testicular tissue, and protect the mitochondrial ultrastructure of Sertoli cells. It may increase the expression of SOD2, Nrf2, and HO-1 in AS model rats. The expression of PINK1, Parkin, LC3, and LAMP2 was downregulated, while the expression of p62 was upregulated, suggesting that Yishen Tongluo Formula may alleviate excessive mitophagy and thereby improve sperm motility. The therapeutic mechanism may involve regulation of the Nrf2-mediated antioxidant defense system and inhibition of the overactivated PINK1/Parkin-mediated mitophagy pathway, as evidenced by alterations in the expression of pathway-related molecules (SOD2, Nrf2, HO-1,

PINK1, Parkin, LC3, LAMP2, and p62) and improvement in mitochondrial morphology.

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Disclosure of conflict of interest

None.

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Yishen Tongluo Formula improves AS via mitophagy and oxidative stress pathways

Supplementary Table 1. The composition of Yishen Tongluo Formula

Chinese name	Botanical name	Latin name	Amount (g)	Efficiency
Zhihuangqi	<i>Astragalus membranaceus</i> (Fisch.) Bunge	Radix Astragali Preparata	30	Invigorating spleen and tonifying qi
Dangshen	<i>Codonopsis pilosula</i> (Franch.) Nannf.	Codonopsis Radix	30	
Huangjing	<i>Polygonatum sibiricum</i> Red.	Rhizoma Polygonati Sibirici Preparata	15	Tonifying qi and nourishing yin, spleen kidney tonifying
Zhishouwuwu	<i>Polygonum multiflorum</i> Thunb.	Radix Polygoni Multiflori Preparata	10	Tonifying kidney and nourishing liver, nourishing yin and filling marrow
Danshen	<i>Salvia miltiorrhiza</i> Bunge	Radix Salviae Miltiorrhizae	20	Activating blood to promote menstruation
Tusizi	<i>Cuscuta chinensis</i> Lam.	Semen Cuscutae	15	Nourishing liver and kidney, nourishing kidney yin
Gouqizi	<i>Lycium barbarum</i> L.	Fructus Lycii	15	
Shayuanzi	<i>Astragalus complanatus</i> Bunge	Semen Astragali Complanati	15	Supplementing the kidney to control the nocturnal
Bajitian	<i>Morinda officinalis</i> How	Morindae Officinalis Radix	15	Reinforcing kidney to strengthen yang
Cuxiangfu	<i>Cyperus rotundus</i> L.	Cyperus Rhizoma	15	Tonifying kidney, tonifying qi and blood, activating
Xudian	<i>Dipsacus asperoides</i> C. Y. Cheng et T. M. Ai	Radix Dipsaci Asperoidis	15	blood and dredging collaterals
Shanyao	<i>Dioscorea opposita</i> Thunb.	Dioscoreae Rhizoma	20	
Shengsanqi	<i>Panax notoginseng</i> (Burk.) F. H. Chen	Notoginseng Radix Et Rhizoma	9	
Sanleng	<i>Sparganium stoloniferum</i> Buch.-Ham.	Sparganii Rhizoma	10	
Ezhu	<i>Curcuma phaeocaulis</i> Val.	Curcumae Rhizoma	10	
Wangbuliuxing	<i>Vaccaria segetalis</i> (Neck.) Garcke	Vaccariae Semen	10	
Lulutong	<i>Liquidambar formosana</i> Hance	Liquidambaris Fructus	10	
Yubiaojiao	/	/	15	Tonifying liver and kidney

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Supplementary Table 2. Analysis of blood components of Yishen Tongluo formula

No.	Name	Formula	mzmed	ms2Adduct	herbSet
1	Saikosaponina	C42H68O13	825.4639099	[M+FA]-	Radix Astragali Preparata
2	Lupenone	C30H48O	425.3776319	[M+H]+	
3	Asperosaponin VI	C47H76O18	973.4987374	[M+HCOO]-	Radix Dipsaci Asperoidis
4	Withaphysacarpin	C28H40O7	489.2795108	[M+H]+	Fructus Lycii
5	Liquidambaric acid	C30H46O3	455.3512436	[M+H]+	Liquidambaris Fructus
6	1,7-Dimethyl-7-(4-methyl-3-penten-1-yl)bicyclo[2.2.1]heptan-2-ol	C15H26O	205.1949578	[M-H ₂ O+H]+	Curcumae Rhizoma
7	Corosolic acid	C30H48O4	473.3624571	[M+H]+	Radix Salviae Miltiorrhizae
8	Ginsenoside Re	C48H82O18	945.5394442	[M-H]-	Radix Salviae Miltiorrhizae, Notoginseng Radix Et Rhizoma
9	Tanshinone IIA	C19H18O3	295.132935	[M+H]+	Radix Salviae Miltiorrhizae
10	(20R)-Ginsenoside Rh1	C36H62O9	683.4370132	[M+FA]-	Radix Salviae Miltiorrhizae/Notoginseng Radix Et Rhizoma
11	Ginsenoside Rg1	C42H72O14	845.4858381	[M+FA]-	
12	Wilforlide A	C30H46O3	455.3511663	[M+H]+	Radix Astragali Preparata/Semen Astragali Complanati
13	methyl2-[(4aR,5R,6R,7S,8aR)-8a-ethenyl-5,7-dihydroxy-4-methylidene-3-oxo-1,4a,5,6,7,8-hexahydroisochroman-6-yl]prop-2-enoate	C16H20O6	331.1169582	[M+Na]+	-
14	Alisol B 23-acetate	C32H50O5	515.3702172	[M+H]+	-
15	Alisol C 23-acetate	C32H48O6	529.3502035	[M+H]+	-
16	Oleanonic acid	C30H46O3	455.3512677	[M+H]+	-
17	Polyporusterone B	C28H44O6	477.3186535	[M+H]+	-
18	Betulin	C30H50O2	443.3880252	[M+H]+	-
19	Dehydroandrographolidesuccinate	C28H36O10	531.2223041	[M-H]-	-
20	Dihydroartemisinin	C15H24O5	283.1552173	[M-H]-	-
21	Epoxyganoderiol A/Ganoderiol A	C30H48O4	473.3624003	[M+H]+	-
22	cynaropicrin	C19H22O6	347.1484568	[M+H]+	-
23	Karakoline	C22H35NO4	378.2613596	[M+H]+	Radix Polygoni Multiflori Preparata
24	Ginkgolide K	C20H22O9	407.1336799	[M+H]+	-
25	gamma-Eudesmolrhamnoside	C21H36O5	369.2629506	[M+H]+	-
26	Trifolirhizin	C22H22O10	491.1190304	[M+FA]-	Radix Astragali Preparata/
27	GOSSYPIN	C21H20O13	481.0972199	[M+H]+	Semen Astragali
28	Flavonebase+40,1Prenyl	C20H18O6	355.1144516	[M+H]+	Complanati
29	Sissotrin	C22H22O10	491.118837	[M+FA]-	

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30	Ononin	C22H22O9	431.133204	[M+H] ⁺	Radix Astragali Preparata
31	Khellin	C14H12O5	283.0598938	[M+Na] ⁺	Cyper Rhizoma, Dioscoreae Rhizoma
32	Quercitrin	C21H20O11	487.0638142	[M+H] ⁺	Radix Salviae Miltiorrhizae, Radix Astragali Preparata, Radix Dipsaci Asperoidis, Semen Cuscutae
33	Isoschaftoside	C26H28O14	563.1410996	[M-H] ⁻	-
34	cirsimarín	C23H24O11	475.1247205	[M-H] ⁻	-
35	Isosakuranin	C22H24O10	447.1287928	[M-H] ⁻	-
36	Croton(chalcone)	C20H20O5	341.1382602	[M+H] ⁺	-
37	Estragonoside	C22H22O12	479.1185684	[M+H] ⁺	-
38	Flavonolbase+4O,0-Hex,1MeO	C22H22O13	493.0990306	[M-H] ⁻	-
39	Isorhamnetin3-O-[4-Hydroxy-E-cinnamoyl-(→6)-b[5-D-glucopyranoyl-(1→2)-a-[6-L-rhamnopyranoside]	C37H38O18	771.2099348	[M+H] ⁺	-
40	Naringenin4'-O-glucuronide	C21H20O11	449.1079206	[M+H] ⁺	-
41	Hesperetin3'-O-glucuronide	C22H22O12	477.1388188	[M+H] ⁺	-
42	Tiliroside	C30H26O13	593.1295988	[M-H] ⁻	Radix Salviae Miltiorrhizae
43	2,3-bis[(4-hydroxy-3-methoxyphenyl)methyl]butane-1,4-diol	C20H26O6	361.1656646	[M-H] ⁻	Fructus Lycii
44	Salvianolic acid A	C26H22O10	493.1144457	[M-H] ⁻	Radix Salviae Miltiorrhizae/Notoginseng Radix Et Rhizoma
45	Harpagoside	C24H30O11	495.1853293	[M+H] ⁺	-
46	6-methoxy-7-(3-methylbut-2-enoxy)chromen-2-one	C15H16O4	299.067059	[M+K] ⁺	Radix Astragali Preparata/Semen Astragali Complanati
47	(-)-Podophyllotoxin	C22H22O8	415.1365928	[M+H] ⁺	-
48	Astringin	C20H22O9	405.1194	[M-H] ⁻	Radix Polygoni Multiflori Preparata
49	Benzoic acid	C7H6O2	123.0439127	[M+H] ⁺	Notoginseng Radix Et Rhizoma
50	3'-Deoxyoleacein	C17H20O5	305.1358754	[M+H] ⁺	-
51	Adenosine	C10H13N5O4	268.1038782	[M+H] ⁺	Dioscoreae Rhizoma, Radix Salviae Miltiorrhizae, Codonopsis Radix, Fructus Lycii, Radix Astragali Preparata, Semen Astragali Complanati, Notoginseng Radix Et Rhizoma, Radix Polygoni Multiflori Preparata
52	Meperidine	C15H21NO2	248.164121	[M+H] ⁺	-
53	3beta-Acetoxy-12-oxo-28,13beta-oleananolid	C32H48O5	513.3555618	[M+H] ⁺	-
54	1,3,5,11-Bisabolatetraen-10-one	C15H20O	217.1587454	[M+H] ⁺	-
55	Pantoyllactone glucoside	C12H20O8	293.1221854	[M+H] ⁺	-
56	Dioscoretine	C13H23NO3	242.1749404	[M+H] ⁺	-
57	Malic acid	C4H6O5	133.0142264	[M-H] ⁻	Radix Salviae Miltiorrhizae, Fructus Lycii

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58	Raffinose	C18H32O16	549.1666027	[M+FA]-	Morindae Officinalis Radix/Radix Astragali Preparata/Semen Astragali Complanati
59	AbuquinoneB	C20H22O8	413.1205197	[M+Na]+	Radix Astragali Preparata/Semen Astragali Complanati
60	1,5,8-Trihydroxy-3-methyl-2-prenylxanthone	C19H18O6	343.1165081	[M+H]+	-
61	OudemansinA	C17H22O4	291.1566022	[M+H]+	-
62	2-Hydroxy-4-(4-methoxyphenyl)-1H-phenalen-1-one	C20H14O3	303.0989314	[M+H]+	-
63	1,5-Bis(4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one	C19H18O5	327.1203393	[M+H]+	-
64	L-N-(1H-Indol-3-ylacetyl)glutamic acid	C15H16N2O5	305.1145442	[M+H]+	-
65	Prupaside	C27H36O12	553.2259295	[M+H]+	-
66	Olivin	C17H16O6	317.1017088	[M+H]+	-
67	Acanthospermolide	C20H26O6	380.2045621	[M+NH4]+	-
68	2-2-Hexyl-5-[2-(4-hydroxy-3-methoxyphenyl)3-ethyl]furan	C19H26O3	303.195067	[M+H]+	Curcumae Rhizoma
69	Demethoxycurcumin	C20H18O5	339.1203557	[M+H]+	-
70	1,2-Dehydro-alpha-cyperone	C15H20O	217.1587371	[M+H]+	Fructus Lycii
71	Benzenepropanamide,N-[2-(acetyloxy)-1-(phenylmethyl)ethyl]-alpha-(benzoylamino)-	C27H28N2O4	445.2114733	[M+H]+	Fructus Lycii
72	3,5,5-Trimethyl-4-[3-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxybut-1-enyl]cyclohex-2-en-1-one	C19H30O7	415.1978826	[M+HCOO]-	Fructus Lycii
73	Hydroxytanshinone	C19H18O4	311.1275395	[M+H]+	Radix Salviae
74	2-Octenoicacid	C8H14O2	141.0921776	[M-H]-	Miltiorrhizae
75	Pelargonidin-3-O-glucoside	C21H21O10	431.098593	[M-2H]-	-
76	7-methyl-1-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-1,4a,5,6,7,7a-hexahydrocyclopenta[c]pyran-4-carboxylic acid	C16H24O9	359.134451	[M-H]-	-
77	(1R,2E,7R,10E,12S,13S,15R)-12,15-dihydroxy-7-methyl-8-oxabicyclo[11.3.0]hexadeca-2,10-dien-9-one	C16H24O4	319.129965	[M+K]+	-
78	Egonol	C19H18O5	327.1219506	[M+H]+	-
79	Tetrasaccharides (Hex-Hex-Hex-Hex)	C24H42O21	711.2182848	[M+HCOO]-	-
80	Demecolcine	C21H25NO5	372.1804391	[M+H]+	-