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Original Article

Determination of the signaling proteins responsible for the antioxidant potential of the Yishenhuoxue formula for treating asthenospermia in rats

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Abstract

Asthenospermia is a predominant cause of male infertility, and antioxidant supplements can be effective in treating asthenospermia. We demonstrate the antioxidant potential of traditional Chinese medicine, the Yishenhuoxue (YSHX) formula, in treating polyglycosides of *Tripterygium wilfordii* (GTW)-induced asthenospermia in rats. Fifty male rats were randomly divided into the normal, model, and treatment groups. HE staining was used to evaluate the improvement of spermatogenic function of rats, and TBA reaction, qRT-PCR, Western Blot and other methods were used to determine the changes of oxidative stress indicators and to evaluate the improvement of antioxidant capacity of rats by YSHX. Comparison with the model group showed significant improvement in pathological damage caused by GTW to seminiferous tubules. MDA and NO content in rat testes decreased, especially in middle- and high-dosage groups. No significant changes were observed in SOD and CAT activity or mRNA expression. GSH-Px activity and GSH mRNA expression were significantly higher in the low-dosage group than in the model group. Compared to the model group, GR activity was significantly lower in the middle and high dosage groups, while the mRNA expression was higher. The PKC-beta level increased, while p-ERK1/2, NF-κB, and the ratio of p-ERK1/2*$(ERK1/2)^{-1}$ decreased significantly in the treatment groups. Therefore, YSHX can alleviate GTW-induced testicular damage, enhance GSH-Px activity, regulate GSH redox cycling, and mitigate oxidative stress injury. Furthermore, YSHX can promote PKC-beta expression and inhibit the phosphorylation of ERK1/2 and NF-κB. Using YSHX may be an effective way to increase sperm motility via the PKC-ERK1/2-NF-κB axis.

Keywords: Asthenospermia, Antioxidant Potential, Yishenhuoxue Formula, Signaling Proteins, *Tripterygium wilfordii*

1. Introduction

Human reproductive health has always been an area of concern as it affects the continuation of human civilization. Globally, infertility affects 8–12% of the reproductive-aged population, with 50 % of the affected population being males [1,2]. Asthenospermia is one of the main causes of male infertility. According to the World Health Organization, it refers to the decrease in motility of sperm [3,4]. The etiology of asthenospermia is diverse and includes infections, genetic factors, endocrine disorders, environmental factors, varicoceles, etc. But these theories cannot perfectly explain the pathogenesis of asthenospermia [5,6]. Treatment of asthenospermia through surgery can only solve some organic lesions, such as varicoceles [7,8]. Drug therapy is still the predominant method for treatment of non-organic disease-induced asthenospermia. Therefore, the search for specific drugs for its treatment still needs to continue.

In recent years, many studies have pointed out that oxidative stress injury to sperms is closely related to asthenospermia. During oxidative stress, the body's antioxidant capacity decreases and excessive reactive oxygen species (ROS) are produced [9]. There is a negative correlation between ROS levels and sperm motility. Excessive ROS can induce a decrease in adenosine triphosphate levels, which leads to a decrease in sperm motility, and sometimes even the loss of sperm motility [10]. Excessive ROS can cause damage to sperm mitochondria. Mitochondria are the main energy-generating organelles in cells and the energy source for sperm movement. It is involved in the process of spermatogenesis, capacitation, swimming and fertilization of spermatozoa [11]. Excessive ROS can affect sperm motility through a variety of molecular mechanisms, such as apoptosis and DNA damage, gene mutation, and so on [12,13]. In relation to the oxidative stress injury to sperms mentioned above, more and more attention has been paid

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to the application of antioxidant therapy for asthenospermia. Several studies have shown that antioxidant supplements, such as coenzyme Q10 and L-carnitine, may help improve sperm quality [14,15]; this provides a new way to treat male infertility.

Research on the application of the antioxidant potential of Chinese herbal medicine (CHM) for treatment of male infertility has also come to light [16-18]. As a complementary and alternative medicine, traditional Chinese medicine (TCM) has accumulated thousands of years of experience in the treatment of male infertility and has the advantages of being cost-effective and having fewer side effects. More importantly, a large number of studies have laid a scientific foundation for understanding the application of TCM prescriptions in male infertility. CHM also has a positive effect on the quality of male sperm: this is increasingly recognized by Western countries [19-21]. According to the TCM theory, the basic pathology of asthenospermia is "dual deficiency of spleen and kidney with blood stasis." [22] With the consensus of several TCM experts, it is believed that invigorating the spleen and kidney, promoting blood circulation, and removing blood stasis are the principles underlying the treatment of asthenospermia. According to the above theory, the Yishenhuoxue (YSHX) formula (alias, Jujing Zhuyu decoction) was proposed and applied to clinical patients with asthenospermia. The clinical effect of YSHX formula in treating male infertility is remarkable in that it can improve the sperm quality of the patients [23,24]. But the exact mechanism of action of the YSHX formula is not clear until now.

The purpose of this study was to investigate the protective effect of YSHX formula on testicular tissue of rats with low sperm motility, induced by the polyglycosides of *Tripterygium wilfordii* (GTW). We also testified the role of YSHX formula in reducing oxidative stress damage and improving the antioxidant capacity of the testicular tissue. We further explored the possible signaling pathway underlying the protective role of YSHX formula on testicular tissue. We were able to detect several proteins (Protein kinase C beta (PKC-beta), extracellular signal-regulated protein kinase 1/2 (ERK1/2), phosphorylated ERK 1/2 (p-ERK1/2), nuclear factor-κB (NF-κB) closely related to sperm motility.

2. Materials and Methods

2.1. Animals

Fifty adult male Sprague Dawley rats (250–300 g, Specific-pathogen free) were purchased from the Laboratory Animal Department of Kunming Medical College (animal license number: SCXK (Yunnan) 2011-0004) for this trial. All animals were kept in the central laboratory animal room of Yunnan Provincial Hospital of Traditional Chinese Medicine (animal license number: SCXK (Yunnan) K2013-0002). The rats were maintained under the standard laboratory conditions (22±1°C, 55 ± 5 % humidity, and a 12 h light/dark cycle) for 1 week of adaptation with *ad libitum* food and water. All the rats were carefully treated according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. All animal experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee of the Yunnan Provincial Hospital of Traditional Chinese Medicine (Kunming, China).

2.2. Preparation of the YSHX Formula

All herb species in the YSHX formula were provided by the Yunnan Provincial Hospital of Traditional Chinese Medicine, in accordance with the standards listed in the National Pharmacopoeia of China. The ingredients in the YSHX formula have been listed in Table 1. The YSHX formula was prepared by boiling the mixture of herbs listed in Table 1 in distilled water at 100℃ for 30 min, three times. The three decoctions were mixed together. The suspended impurities obtained by mixing the three decoctions were removed by filtration (100-mesh sieve) and centrifugation (2000 rpm, 10 min, 25℃). Finally, the solution was concentrated to a concentration of 1.5 g/mL (crude drug) at 60℃ and preserved at 4℃ for further use (Table 1).

2.3. Experimental Design

All rats were fed adaptively for a week, and then randomly divided into 5 groups (10 rats per group): control (C); model (M); and low dosage (L-YSHX), middle dosage (M-YSHX), and high dosage (H-YSHX) YSHX treatment groups. The rats in the 4 groups (except the control group), were induced to develop asthenospermia by administration of polyglycosides of *Tripterygium Wilfordii* (GTW, Hubei Huangshi Feiyun Pharmaceutical Co., Ltd., Huangshi, China) by gavage with 20 mg GTW per kg of rat body weight for 5 weeks; hence, these 4 groups served as established models of asthenospermia. Previous studies have shown that GTW could induce depression in sperm motility in rats [25]. According to the results of previous experiments, the 3 YSHX treatment groups in our established models were given different doses of YSHX (L-YSHX, 5 g/kg; M-YSHX, 10 g/kg; H-YSHX, 15 g/kg) by gavage, twice a day for 5 weeks. The rats in the model group were administered 1 mL of 0.9 % normal saline per 100 g body weight. After the final treatment, all rats were sacrificed with an intraperitoneal injection of sodium pentobarbital (200 mg/kg). The bilateral testes of the sacrificed rats were collected. The left testes were divided into two parts, one part was fixed with 4% paraformaldehyde (Solarbio, Beijing, China). The remaining left testes part and the right testes were stored at −80°C for further assays.

2.4. Histopathological evaluation

The testicular specimens were fixed in 4% paraformaldehyde for 24 h at 4°C. After fixation, the specimens were dehydrated, made transparent, waxed, embedded, sliced (3–5 μm), and stained with hematoxylin-eosin (H&E) for observation by light microscopy. Johnsen's score was used to quantify the changes in spermatogenic function observed during histopathology [26].

2.5. Determination of oxidative stress indicators

2.5.1. Sample pre-processing

The homogenate of testicular tissue was prepared according to the instructions of the kit supplied by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The testicular tissues of rats were accurately weighed and 0.9 % saline was added in the ratio of 1:9 weight (g): volume (mL). A 10 % homogenate was prepared by mechanical homogenization under the conditions of an ice-water bath. The homogenate mixture of testicular tissues was centrifuged at 3000 rpm (4°C) for 10 min. The supernatant was used for subsequent testing.

Table 1. The composition of YSHX formula.

Chinese name	Botanical name	Latin name	Parts used	Amount (g)	Proportion (%)	Source (in China)
Huangqi	*Astragalus membranace*us (Fisch.) Bunge	Radix Astragali Mongolici	Root	30	10.7	Gansu
Zhihuangqi	*Astragalus membranaceus* (Fisch.) Bunge	Radix Astragali Preparata	Root	30	10.7	Gansu
Dihuang	*Rehmannia glutinosa* (Gaertn) Libosch.	Radix Rehmannia	Root	15	5.4	Henan
Shudihuang	*Rehmannia glutinosa* (Gaertn) Libosch.	Radix Rehmannia Preparata	Root	15	5.4	Henan
Danshen	*Salvia miltiorrhiza* Bunge	Radix Salviae Miltiorrhizae	Root	20	7.1	Shandong
Gouqizi	*Lycium barbarum* L.	Fructus Lycii	Fruit	20	7.1	Ningxia
Taizishen	*Pseudostellaria heterophyl*la (Miq.) Pax ex Pax et Hoffm.	Radix Pseudostellariae	Root	30	10.7	Guizhou
Zhiheshouwu	*Polygonum multiflorum* Thunb.	Radix Polygoni Multiflori Preparata	Root	20	7.1	Yunnan
Zhihuangjing	*Polygonatum sibiricum* Red.	Rhizoma Polygonati Sibirici Preparata	Root	15	5.4	Heibei
Tusizi	*Cuscuta chinens*is Lam.	Semen Cuscutae	Seed	20	7.1	Liaoning
Shayuanzi	*Astragalus complanatus* Bunge	Semen Astragali Complanati	Seed	15	5.4	Shanxi
Xuduan	*Dipsacus asperoides* C. Y. Cheng et T. M. Ai	Radix Dipsaci Asperoidis	Root	15	5.4	Yunnan
Yimucao	*Leonurus japonicas* Houtt.	Herba Leonuri Japonici	Entire Plants	15	5.4	Anhui
Jixueteng	*Spatholobus suberectus* Dunn	Caulis Spatholobi	Rattan	20	7.1	Yunnan

2.5.2. Determination of the concentration of malondialdehyde (MDA) and nitric oxide (NO)

The concentration of MDA in rat testis was measured using the thiobarbituric acid (TBA) reaction, with an assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Sample absorbance values were recorded at 532 nm using a microplate reader (Epoch BioTek, USA). MDA levels were expressed as nanomoles per gram protein (nmol/mg protein). NO concentration in rat testis was determined using nitrate reductase with an assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The absorbance was recorded at 540 nm. NO levels were expressed as micromoles per gram of protein (μmol/g protein).

2.5.3. Detection of related antioxidant enzyme activity

The activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and glutathione reductase (GR) in testicular tissue were assayed using commercial standard kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The activity of SOD was determined by measuring nitrite content, formed by the oxidation of hydroxylamine by superoxide anion radicals. The absorbance was recorded at 550 nm. GSH-Px can promote the reaction of hydrogen peroxide (H_2O_2) with reduced glutathione (GSH) to produce H_2O and oxidized glutathione (GSSG). The activity of GSH-Px can be expressed in terms of the rate of its enzymatic reaction. The activity of this enzyme can be determined by measuring the consumption of reduced glutathione in an enzymatic reaction. The absorbance was recorded at 412 nm. Activities of SOD and GSH-Px were reported as units per milligram of protein (U/mg protein). The activity of CAT was determined by measuring the decomposition rate of H_2O_2 at 240 nm. GR activity was determined by detecting the decrease in NADPH absorbance at 340 nm. Activities of CAT and GR were reported as units per gram of protein (U/g protein).

2.6. Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) assay

Total RNA was extracted from testicular tissue using

a total RNA extraction kit (TIANGEN Biotech Co., Ltd., Beijing, China). After RNA extraction, the concentration and purity of the extracted RNA were determined using NanoDrop (Thermo Scientific, Waltham, MA, USA). The reverse transcription reaction was performed according to the manufacturer's procedure of the PrimeScript RT reagent kit (TaKaRa, Dalian, China). Quantitative fluorescence PCR was performed on the Biosystems Veriti Thermal Cycle using the SYBR Premix Ex Taq II (TaKaRa, Dalian, China), and primers (Shanghai Shenggong Bioengineering Co., Ltd., Shanghai, China) mentioned in Table 2. SYBR Green was used as a fluorescent marker and β-actin as an internal reference. Relative gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method.

2.7. Western Blot Analysis

The total protein of the testicular tissue was extracted using the cell lysis buffer for western and IP (Beyotime Institute of Biotechnology, Shanghai, China), and then quantified with the BCA protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China) according to manufacturer's instructions. Proteins were separated by polyacrylamide gel electrophoresis (10% separating glue and 4% concentrated glue) and then transferred to PVDF membranes. The membranes were blocked for 1 h using a 5 % solution of skim milk and incubated with primary antibodies against PKC-beta (Proteintech, Rosemont, IL, USA), ERK1/2, p-ERK1/2, NF-kB (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. Next day, the membrane was washed for 5 min, and then incubated with secondary HRP-conjugated antibody at room temperature for 2 h. The optical density of the strip was visualized using the Odyssey CLx Dual-Color Infrared Laser Imaging system (LICOR Biosciences, Shanghai, China).

2.8. Statistical Analysis

Date analyses were performed using Statistic Package for Social Science (SPSS) 20.0 for Windows (SPSS, IBM, Armonk, NY, USA). All variables were described as mean ± standard deviation (SD). The one-way analysis of variance (ANOVA) and the least significant difference (LSD) tests were used to calculate statistical significance. Differences were classified as statistically significant when $P < 0.05$.

3. Results

3.1. Testicular Histomorphology

The histological structure of testicular tissue is illustrated in Figure 1. In the control group (Figure 1(a)), the seminiferous tubule structure was normal. In the model group (Figure 1(b)), the structure of seminiferous tubule in the testicular tissue was abnormal and showed a cavity-type seminiferous tubule. All types of spermatogenic cells were drastically reduced. Further, the spermatogenic cells were disorderly arranged and loosely structured, had lost their normal layers, and were even falling off into the lumen. Few spermatozoa were found in the lumen of seminiferous tubules. After administration of YSHX formula, the testicular tissue injury in the treatment groups was significantly improved, especially in the middle and high dosage YSHX groups (Figure 1(c),1(d),1(e)). The recovery signs were the reappearance of complete and well-arranged seminiferous tubules. The number of spermatogenic cells at all stages in seminiferous tubules increased significantly, and the number of differentiated spermatozoa in the lumen increased. As shown in Table 3, Johnsen's score of the model group was significantly lower than that of the control group ($P < 0.001$). The Johnsen's score of the L-YSHX group ($P < 0.05$), the M-YSHX group ($P < 0.001$), and the H-YSHX

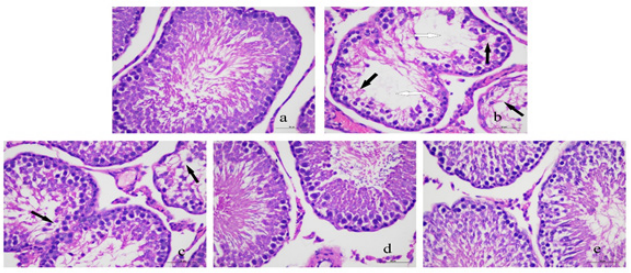

Fig. 1. Effect of YSHX formula on the histomorphology of testicular tissues in control and GTW-treated rats (H&E staining, 400×). (a) Control group; (b) model group; (c) low dosage YSHX group; (d) middle dosage YSHX group; (E) high dosage YSHX group. White arrow: cavity-type seminiferous tubule; Black arrow: spermatogenic cells are disorderly arranged, loosely structured. Bar = 50 μm.

Table 2. Primer name and sequences for qPCR analysis.

Primer name	Forward sequence	Reverse sequence
SOD	GGTTTGCGTCGTAGTCTCCT	GGTCCATTACTTTCCTTCTGCT
CAT	TGCTGAGAAGCCTAAGAATGC	GGTTACACGGATGAACGCTAA
GSH	AGTCGGTGTATGCCTTCTCG	TCGTTCATCTGGGTGTAGTCC
GR	GCTCCTCAACAGCAACAACAG	TCATTCCTTCCAGCACATAGG
β-actin	GGAGATTACTGCCCTGGCTCCTA	GACTCATCGTACTCCTGCTTGCTG

Table 3. Effect of YSHX formula on Johnsen's score of the testicular tissues in rats.

Group	n	Johnsen's score
Control	10	9.78 ± 0.44
Model	10	$6.10 \pm 0.99^{***}$
L-YSHX	10	$6.70 \pm 0.68^{\Delta}$
M-YSHX	10	$9.70 \pm 0.48^{\Delta\Delta\Delta}$
H-YSHX	10	$9.50 \pm 0.53^{\Delta\Delta\Delta}$

All data are presented as mean ± standard deviation. *** $P < 0.001$, versus control group;
Δ $P < 0.05$, versus model group; ΔΔΔ $P < 0.001$, versus model group.

group ($P < 0.001$) increased significantly as compared to the model group.

3.2. Effect of YSHX formula on the concentration of MDA and NO in Rat Testis

As shown in Figure 2, the concentrations of MDA and NO in the testis of the model group were significantly higher than those of the control group ($P < 0.05$). The MDA concentration in the testis of rats treated with different doses of YSHX decreased significantly ($P < 0.05$) as compared to the model group (Figure 2(a)). NO concentration decreased in the testis of rats treated with different dosages of YSHX, especially in the middle and high dosage groups ($P < 0.05$) (Figure 2(b)).

3.3. Effect of YSHX formula on Antioxidant enzyme activity in Rat Testis

As shown in Figure 3, there was no significant difference in the activity of SOD and CAT between the five groups ($P > 0.05$). However, the activity of GSH-Px in the model group decreased significantly ($P < 0.05$), and GR in the model group increased significantly ($P < 0.05$) as compared to the control group. The activity of GSH-Px in the L-YSHX group was significantly higher ($P < 0.05$), while the activity of GR in the M-YSHX and H-YSHX groups was significantly decreased ($P < 0.05$), as compared to the model group.

3.4. Effect of YSHX formula on mRNA expression of related Antioxidant enzymes in Rat Testis

In order to further determine the effect of YSHX formula on the antioxidant enzymes in testicular tissue, we used the qRT-PCR method to detect the mRNA expression

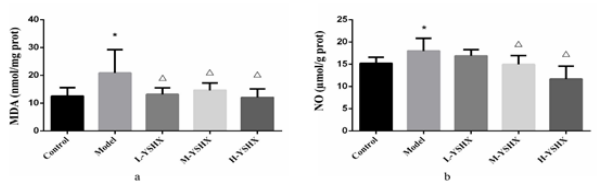

Fig. 2. Effect of YSHX on the concentration of MDA and NO in Rat Testis. (a) MDA concentration; (b) NO concentration. L-YSHX: low dosage YSHX group; M-YSHX: middle dosage YSHX group; H-YSHX: high dosage YSHX group. Data are presented as mean ± standard deviation. ∗ $P < 0.05$, versus control group; Δ $P < 0.05$, versus model group.

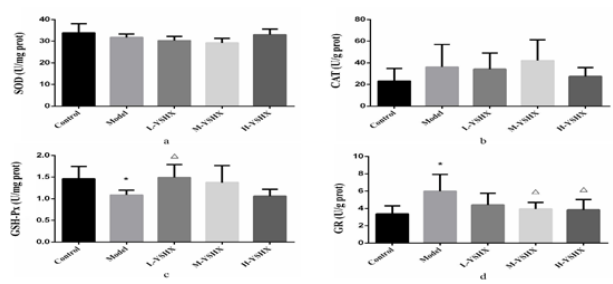

Fig. 3. Effect of YSHX on Antioxidant enzyme activity in Rat Testis. (a) The activity of SOD; (b) the activity of CAT; (c) the activity of GSH-Px; (d) the activity of GR. L-YSHX: low dosage YSHX group; M-YSHX: middle dosage YSHX group; H-YSHX: high dosage YSHX group. Data are presented as mean ± standard deviation. ∗ $P < 0.05$, versus control group; Δ $P < 0.05$, versus model group.

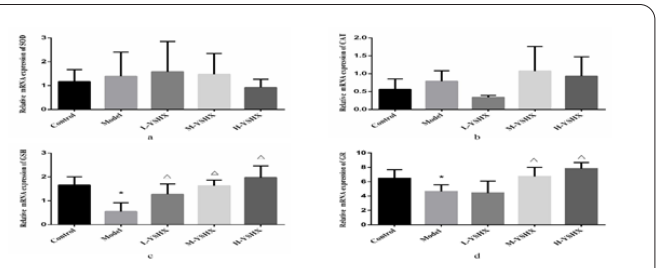

Fig. 4. Effect of YSHX formula on mRNA expression of related Antioxidant enzymes in Rat Testis. (a) Relative mRNA expression of SOD; (b) relative mRNA expression of CAT; (c) relative mRNA expression of GSH; (d) relative mRNA expression of GR. L-YSHX: low dosage YSHX group; M-YSHX: middle dosage YSHX group; H-YSHX: high dosage YSHX group. Data are presented as mean ± standard deviation. ∗ $P < 0.05$, versus control group; Δ $P < 0.05$, versus model group.

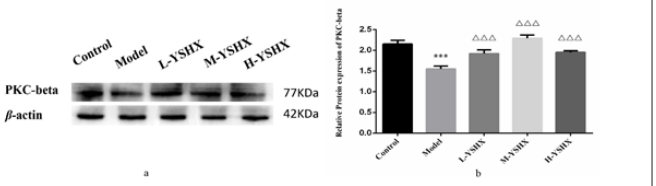

Fig. 5. Effect of YSHX formula on the protein expression of PKC-beta in Rat Testis. (a) Protein electrophoresis map; (b) relative protein expression of PKC-beta. L-YSHX: low dosage YSHX group; M-YSHX: middle dosage YSHX group; H-YSHX: high dosage YSHX group. Data are presented as mean ± standard deviation. ∗∗∗ $P < 0.001$, versus control group; ΔΔΔ $P < 0.001$, versus model group.

levels. As shown in Figure 4, there was no significant difference in the relative mRNA expression of SOD and CAT in the testicular tissue of rats in each group ($P > 0.05$). The relative mRNA expression of GSH and GR in the model group decreased significantly ($P < 0.05$) as compared to the control group. After the administration of the YSHX formula, the relative mRNA expression of GSH and GR in the testicular tissue of rats, especially in the middle and high dosage groups, increased by varying degrees ($P < 0.05$) when compared with the model group.

3.5. Effect of the YSHX formula on the protein levels of PKC-beta, ERK1/2, p-ERK1/2, NF-κB in Rat Testis

To determine the signaling pathway in which YSHX is involved in playing an antioxidant role and in enhancing sperm motility, we analyzed the expression of several proteins closely related to sperm motility. As shown in Figure 5, the relative expression of PKC-beta protein in the model group was significantly lower than that in the control group ($P < 0.001$). The expression of PKC protein significantly increased at different degrees after treatment with different dosages of YSHX ($P < 0.001$) as compared to the model group. As shown in Figure 6(b), (c), the level of ERK1/2 did not change significantly in the model group ($P > 0.05$), while p-ERK1/2 increased significantly ($P < 0.001$) in the model group when compared to the control group. In addition, the ratio of (p-ERK1/2)/(ERK1/2) increased significantly ($P < 0.001$) in the model group as compared to the control group (Figure 6(d)). The expression of ERK1/2 in the high-dosage YSHX group was significantly lower than

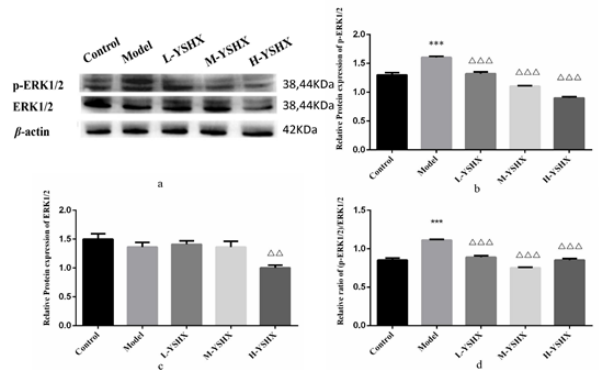

Fig. 6. Effect of YSHX formula on the protein expression of ERK1/2 and p-ERK1/2 in Rat Testis. (a) Protein electrophoresis map; (b) relative protein expression of p-ERK1/2; (c) Relative protein expression of ERK1/2; (d) Ratio of (p-ERK1/2)/(ERK1/2); Data are presented as mean ± standard deviation. *** $P < 0.001$, versus control group; ΔΔΔ $P < 0.001$, versus model group.

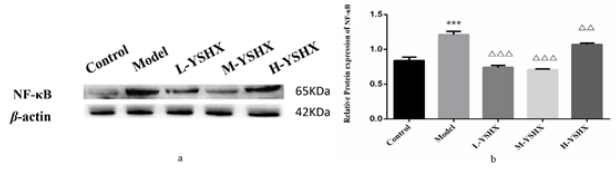

Fig. 7. Effect of YSHX formula on the protein expression of NF-κB in Rat Testis. (a) Protein electrophoresis map; (b) relative protein expression of NF-κB. L-YSHX: low dosage YSHX group; M-YSHX: middle dosage YSHX group; H-YSHX: high dosage YSHX group. Data are presented as mean ± standard deviation. *** $P < 0.001$, versus control group; ΔΔ $P < 0.01$, ΔΔΔ $P < 0.001$, versus model group.

that in the model group ($P < 0.01$). The levels of p-ERK1/2 and the ratio of (p-ERK1/2)/(ERK1/2) in different dosages of YSHX were significantly reduced respectively ($P < 0.001$, Figure 6) when compared to the model group. As shown in Figure 7, the protein expression of NF-κB in the model group increased significantly, compared with the control group ($P < 0.001$). However, the protein expression of NF-κB decreased to different degrees after treatment with different dosages of YSHX ($P < 0.001$), when compared to the model group.

4. Discussion

Spermatogenesis is a complex process, which is influenced by many factors. Testicular cells (such as germ cells, Sertoli cells, Leydig cells, and peritubular cells) play a key regulatory role in this process. The normal structure of seminiferous tubule ensures normal spermatogenesis. All types of spermatogenic cells (spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids) in seminiferous tubule are arranged orderly and tightly. A large number of spermatozoa can be seen in the lumen of seminiferous tubule [27]. Testicular histomorphology showed that there was typical damage to seminal tubules in the model group, which was consistent with previous literature [28,29]. The YSHX formula can reverse the damage caused by GTW in spermatogenic tubules, especially when administered at middle and high dosages. This study provided evidence for the ability of YSHX to improve sperm motility in asthenospermia model rats.

According to the theory of spleen-kidney interaction and sperm-blood interaction in TCM, "dual deficiency of spleen and kidney with blood stasis" should be the basic pathology of asthenospermia-related infertility. "Spleen deficiency", "kidney deficiency," and "blood stasis" are the three key links in the pathogenesis of asthenospermia. The YSHX formula is a clinical prescription created by Guozheng Qin, a famous TCM expert, based on the three key links. In previous animal experiments, we found that YSHX formula could improve sperm motility and sperm viability in rats with asthenospermia [25]. Although the specific mechanism of action of YSHX had been unclear, several ingredients of the formula, such as Astragalus, *Lycium barbarum*, and so on, have been shown to promote spermatogenesis and improve oxidative stress damage in testicular tissues [30,31]. Therefore, we speculated that YSHX formula may protect testicular spermatogenesis by reducing oxidative stress damage.

In order to verify our hypothesis, we first detected the levels of oxidative stress markers, MDA and NO. MDA, an aldehyde product, is a lipid peroxide produced due to oxidative stress and can be used as a biomarker to assess oxidative stress [32]. It has a toxic effect on sperm cells. It damages the mobility and integrity of the sperm membrane to different degrees, increases the permeability of the sperm membrane, and loses the ability of the sperm for intracellular regulation of the concentration of related ions involved in sperm motility regulation, thus resulting in the decrease or even loss of sperm motility [33,34]. The concentration of NO in semen of normal fertile men was significantly lower than that of patients with low sperm motility. There was a significant linear negative correlation between NO concentration and sperm motility. NO can inhibit cell respiration and reduce the production of adenosine triphosphate, thus reducing sperm viability. NO can affect the biosynthesis of testosterone, change the local hormone environment of sperm, and regulate sperm motility [35,36]. In this study, our results showed that the concentrations of MDA and NO in the testicular tissues of rats in the YSHX treatment groups were significantly lower than those in the model group. We also detected the expression of antioxidant enzymes at protein and mRNA levels. Antioxidant enzymes are the most effective defense systems to limit the toxicity of free radicals/reactive oxygen species and play an important role in protecting tissues from the harmful effects of lipid peroxidation. SOD is an important member of the biological antioxidant enzymes. It can transform superoxide anion radicals (O_2^-) produced in the process of biological oxidation into H_2O_2, which is the first defense line of the biological antioxidant system [37]. CAT catalyzes the breakdown of H_2O_2 into oxygen and water [38]. We observed that there was no significant difference in the activity and relative mRNA expression of SOD and CAT in each group. The GSH system is an important defense system to prevent ROS damage. GSH, GSH-Px, and GR are the key components of the glutathione system. GSH-Px specifically promotes the reaction of H_2O_2 with GSH to produce water and GSSG, while GR can reduce GSSG to GSH [39-41]. Some studies have also confirmed that the GSH system is closely associated with male reproduction [42-44]. Our results showed that the activity of GSH-Px and the relative mRNA expression of GSH in the L-YSHX group were higher than those in the model group. This could be because the YSHX formula promoted the activity of GSH-Px and enhanced the reac-

tion between GSH and H_2O_2, which resulted in excessive consumption of GSH. To compensate for the decrease in GSH pool, the body responds by increasing the expression of GSH mRNA. Interestingly, we found that GR activity in L-YSHX and H-YSHX groups was significantly lower than that in the model group, but the relative mRNA expression of GR was significantly higher in the two treatment groups than in the model group. We concluded that this phenomenon may also be the result of YSHX formula promoting GSH-Px activity; as a result, the reaction between GSH and H_2O_2 is strengthened, and excessive GSSG is produced. GSSG can react with GR. GR is also over-consumed, and this results in the increased mRNA expression of GR. To sum up, our study suggests that the YSHX formula enhances sperm motility by enhancing the activity of GSH-Px, mediating GSH-redox circulation, removing excessive ROS, and reducing oxidative stress damage in testicular tissue of rats with GTW-induced asthenospermia.

ROS can be used as a signal molecule similar to a second messenger, to activate many redox-sensitive signaling pathways. In order to further elucidate the molecular mechanism of the YSHX formula in promoting sperm motility, we detected the levels of signal pathway proteins, PKC, ERK1/2, and NF-κB, that are closely related to sperm motility. The activation of PKC can regulate spermatogenesis and improve sperm vitality [45]. We found that the YSHX formula can increase the expression of PKC-beta protein in the testis tissue of GTW-induced rats. Normally, ERK1/2 functions downstream of PKC, and its activation may be positively correlated with spermatogenesis [45]. Zhang et al. showed that the phosphorylation level of ERK1/2 in testicular tissues of rats in the model group increased, and their testicular spermatogenesis was severely impaired. The Wuzi Yanzong pill could reduce the phosphorylation of ERK1/2 induced by GTW [46]. Similar results have been obtained in our study. The YSHX formula could reduce the ratio of p-ERK1/2 to ERK1/2, thus inhibiting the phosphorylation of ERK1/2 in testis tissue of GTW-induced rats and hence, affecting sperm motility. Spermatogenesis is controlled by NF-κB; activated NF-κB causes a defect in spermatogenesis both in human and mice. Activation of the ERK/AKT/NF-κB signaling pathway can lead to reproductive toxicity [47]. A previous study has indicated that the regulation of pro-inflammatory PKC/ERK/NF-κB signaling pathways in the bladder can reduce oxidative stress injury in the bladder [48]. In our study, we found that the expression of NF-κB protein was significantly increased in model group, while its expression was significantly decreased in the YSHX formula-treated groups. Our results suggest that the effect of the YSHX formula on improving sperm motility may be related to the regulation of signaling pathways associated with PKC, ERK1/2, and NF-κB proteins, but the specific mechanism needs to be studied further. Besides, considering the multitarget effects of TCM, the YSHX formula may also affect sperm motility through other pathways, such as p38MAPK, JNK, FAS/FASL, etc. [49,50]. Elucidating the role of these potential pathways in the effectiveness of the YSHX formula in asthenospermia could be the potential future direction for this study.

5. Conclusion

In this study, we found that YSHX formula could alleviate oxidative stress injury, eliminate excessive lipid peroxidation products (MDA, NO), and enhance antioxidant enzyme activity (GSH-Px, GR) in testicular tissue of GTW-induced rats. More importantly, we also found that the YSHX formula may affect spermatogenesis and sperm motility by mediating the PKC/ERK/NF-κB signaling pathways. These findings suggest that the YSHX formula can be effectively used in the treatment of asthenospermia. It also shows the great potential of TCM in the treatment of asthenospermia.

Conflict of Interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

All animal experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee of the Yunnan Provincial Hospital of Traditional Chinese Medicine (Kunming, China).

Informed Consent

The authors declare that no patients were used in this study.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' Contributions

Zhuojun Yuan, Bing Liu and Guozheng Qin designed the study and performed the experiments, Dong Ma, Pengfei Mao and Chunping Wan collected the data, Xiaojun Zhong, Fugang Zhang and Yijian Yang analyzed the data, Zhuojun Yuan, Bing Liu and Guozheng Qin prepared the manuscript. All authors read and approved the final manuscript.

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