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Cynomorium Songaricum may protect against spermatogenic damage caused by cyclophosphamide in SD rats

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Abstract

Aim: The aim of the study is to investigate the effect of *Cynomorium songaricum* (CS) on the damage caused by cyclophosphamide (CP) in SD rats. **Methods:** Rats with CP-induced oligoasthenospermia were treated with different concentration of CS. Testicle weight, epididymal sperm count (ESC), sperm motility, and serum testosterone were analyzed, and expression levels of *Thy1*, *Oct4*, *PLZF*, *C-kit*, and *GDNF* were detected in testis tissues. Transmission electron microscopy (TEM) was performed to observe the effect of CS on the spermatogenic damage by CP. **Results:** Compared with the CP group, there were significant differences in testicle weight, ESC, and sperm motility

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($p < 0.05$) observed in all concentrations of CS and CP+VitE groups ($p < 0.05$). There were no significant differences in serum testosterone among the 6 groups ($p > 0.05$). The qPCR results revealed a significant difference in *Thy1*, *Oct4*, *PLZF* and *GDNF* expression between the CP group and CS group ($p < 0.05$), but there was no significant difference in *C-kit* between the two groups ($p > 0.05$). The damage of CP was cured by CS observed under TEM. **Conclusion:** CS can increase sperm counts in the epididymis and improve sperm motility and has a therapeutic effect on the spermatogenic damage caused by CP in SD rats.

Keywords: *Cynomorium Songaricum*, spermatogenic damage, cyclophosphamide, epididymal sperm count, sperm motility

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Introduction

Oligospermia and asthenospermia in men are important factors leading to infertility (1). Thus, improving sperm quality is a critical issue in modern reproductive research.

There are many causes of decreased number of spermatozoa and reduced activity, including reproductive tract infections, semen liquefaction, and endocrine hormone disorders. There may be limited improvement with the use of anti-infective and anti-oxidant treatments. Chinese medicines have several unique effects on this disease. *Cynomorium songaricum* (CS) is a traditional kidney medicine that affects the liver, kidney, and large intestine. CS contains organic acids, flavonoids, steroids, triterpenes, and amino acids. CS has been reported to enhance immune function, scavenge free radicals, and function as an anti-oxidant that impacts aging, and CS has also been shown to reduce hypoxia, stress, and fatigue. CS contains a variety of essential trace elements such as Cu, Mn, and Mi as well as high Fe and Zn content (2). Yang et al. (3) showed that the expression of glial cell line-derived neurotrophic factor (GDNF) mRNA and protein as well as the number of epididymal spermatozoa and the absolute weight of testis are increased after CS administration. The authors also reported a reduction in the number of abnormal sperm and improved sperm motility following CS treat-

ment (4). CS also increases the testicular weight of immature male Wistar rats (5). The aim of this study was to investigate the effects of CS on the number and activity of spermatozoa upon treatment of cyclophosphamide (CP)-related oligospermia in rats. Initial body weight (IBW), final body weight (FBW), testicle weight (TW), epididymal sperm count (ESC), sperm motility (SM), and serum testosterone (ST) were measured, and the expression levels of *Thy1*, *Oct4*, *PLZF*, *C-kit*, and *GDNF* were detected in testis tissue. Transmission electron microscopy (TEM) was performed to observe the effect of CS on the spermatogenic damage caused by cyclophosphamide (CP).

Materials and methods

Animals and Ethics

This study used 36 SPF (Skatepark Formula™) grade Sprague Dawley® Rats (SD) healthy male 8-week-old rats. The rats weighed approximately 215 ± 5 g and the study was provided by the Experimental Animal Center of Xuzhou Medical University (SYXK (Su) 2017-0011).

Drugs

CP was obtained from Jiangsu Hengrui Pharmaceutical Co., Ltd. (Chinese medicine quasi-word H32020856, 0.1 g/support, powder). The CS was produced by Jiangsu Bozhou. Preparation

of CS: Take 100 g dry CS and 1000 ml saline, soak for 60 min, boil for 30 min, and filter with 2 layers of filter cloth. Next, add 500 ml saline, soak for 30 min, boil for 20 min, and filter with 2 layers of filter cloth. Then, add 500 ml saline, soak for 30 min, boil for 10 min, and filter with 2 layers of filter cloth. Combine all 3 filtrates, and filter with 4 layers of filter cloth to remove fried impurities. The filtrate is then added to 80~90°C water to a specific concentration (every 1 ml of liquid CS equivalent to 0.5 g dry CS dose), and the reserve material is stored in the cold.

Grouping and treatment methods

The 36 SD rats were fed for 1 week of adaptation and then treated with drugs via intragastric administration. The rats were divided into 6 groups according to the random concentration table method, including a control group (blank control group) and CP, CP+LCS (low concentrations of CS), CP+MCS (medium concentrations of CS), CP+HCS (high concentrations of CS) groups and a CP+VitE group. All SD rats except the control group were treated with CP (30 mg/kg·day) intraperitoneally for 5 days according to the literature (6). After 4 weeks of normal feeding, the rats in the CP+LCS, CP+MCS and CP+HCS groups were treated with CS at low (0.5 g/kg·day), medium (1 g/kg·day), and high (2 g/kg·day) concentrations, and the CP+VitE group was treated with Vitamin E (50 mg/kg·day) injection. There were no deaths during the intubation model.

Detection of IBW, FBW, TW, ESC, SM, and ST in rats

After 10 weeks, 10% chloral hydrate was used to anesthetize the rats. The testes and epididymis were collected, and the epididymis was placed at 37.5°C in salt solution in an Eppendorf (EP) tube. Smear observation was used to record sperm motility and sperm count in each group, and the testes of each group were weighed and

recorded. A 200-μl sample of venous blood was collected from the abdominal aorta peripheral vein. Siemens ADVIA Centaur CP automatic chemiluminescence immunoassay analyzer and test kit for testosterone of Siemens Medical Diagnostics Co., Ltd were used to measure ST. A blood cell count plate was used to measure the ESC. The direct sperm count, non-linear advance sperm count, and immobile sperm count were measured to evaluate SM. The testicles were observed on 400X microscope with a hematoxylin-eosin (HE) staining.

Real-time PCR

The expression of Thy1, Oct4, PLZF, C-kit and GDNF in testis tissues were compared in the CP and CP+LCS groups. Thy1, Oct4, PLZF, C-kit and GDNF primers were designed using Primer 5.0 software (Table 1). The house-keeping gene β-actin was used as an internal reference. Amplification conditions: 94°C 15 min; 94°C 10 s, 55°C 20 s, 72°C 20 s, 45 cycles; 72°C 5 min. The relative expression of each gene was calculated by fluorescence quantitative analysis using the $2^{-\Delta\Delta C_t}$ method.

Western blot analysis

Western blot was performed to quantify the expression of spermatogonia-related proteins such as Thy1, Oct4, PLZF, C-kit and GDNF. Cells from the CP and CP+LCS groups were used. The BCA approach was used to measure total protein concentration. Proteins (30-50 mg) were resolved on a 10% Bis-Tris gradient SDS-PAGE gel under reducing conditions, transferred onto a polyvinylidene fluoride (PVDF) membrane and blocked with 5% skim milk for 1 h. Membranes were incubated with primary antibodies (Thy1, Oct4, PLZF, C-kit and GDNF) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody at room temperature for 2h. Membranes were washed with TBST 4 times (8 minutes each) and incubated with fluorescein-linked sec-

ondary antibodies at room temperature for 1.5 h. Next, membranes were washed with TBST another 4 times (8 minutes each), and signal detection was carried out using the Pierce™ ECL Western Blotting Substrate Kit (Thermo Fisher Sci. Co, Ltd, America) and X-ray film.

TEM observation

A small amount of little testicular tissue was taken from the CP group and CP+LCS group and fixed in 2.5 ml of 1% glutaraldehyde precooled to 4° after incubation with strong acid. The tissue was dehydrated and embedded with Epon812 resin. Semi-thin sections were cut with an ultramicrotome and stained with toluidine blue and visualized by optical microscopy. Then, the tissue was cut into ultra-thin sections with an ultra-thin slicer and stained with 2% uranyl acetate and lead citrate. After drying, the ultrastructure of the testes was observed and photographed under TEM.

Statistical analysis

Statistical analyses were performed using SAS v8.02 software (SAS Institute Inc., Cary, NC, USA). Continuous data are presented as the mean \pm SD, and categorical data are presented as a percentage. One-way analysis of variance (ANOVA) was performed for the parametric data. Comparisons of numerical data between any 2 groups were performed using the *student's t* test. Categorical data were compared using the chi-square test. A $p < 0.05$ was considered statistically significant.

Results

Effect of CS on rat body weight

The body weights before and after the treatment are shown in Table 1. There were no significant differences among the 6 groups in pre-treatment weight. Compared with the control group, the final body weight of the CP group was significantly reduced.

Effect of CS on TW, ESC, SM and ST in SD rats

The testicle weights in all 6 groups are shown in Table 2. Compared with the control group, the testicle weight, epididymal sperm count, and sperm motility in the CP group were significantly different ($p < 0.05$). Compared with the CP group, there were significant differences in these factors observed in the CP+LCS, CP+MCS, CP+HCS and CP+VitE groups ($p < 0.05$, Figure 1). There were no significant differences in serum testosterone among the 6 groups ($p > 0.05$, Figure 1). Microscopic changes were observed under the microscope (200X, HE staining, Figure 2).

Effect of CS on the expression of Thy1, Oct4, PLZF, C-kit and GDNF in rat testis tissue

The qPCR results revealed a significant difference in Thy1, Oct4, PLZF and GDNF expression between the CP group and CP+LCS group (Figure 3 and Table 3, $p < 0.05$), but there was no significant difference in C-kit between the two

Table 1. Primers for Thy1, Oct4, PLZF, C-kit and GDNF genes

| Gene | Forward primer (5'-3') | Reverse primer (3'-5') | Length (bp) |
|----------------|---------------------------|------------------------------|-------------|
| β -actin | AGAGGGAAATCGTGCGTGAC | GCCGGACTCATCGTACTCCT | 463 |
| Thy-1 | GCAGAGGTCTTGCTTCTCCCG | CACAGTCCAACTTCCCTCATCCATATCT | 369 |
| PLZF | TGGTGCTCCAGCCACATAACCA | GCTCAGCTTGCTTTACAGTCCAGGTT | 395 |
| Oct4 | TGGAGAAGGAGAAGCTGGAGCAAAA | GGCAGATGGTCGTTTGGCTGAATA | 186 |
| c-kit | TTACTCCAACTTGCCAACTGCAACC | TTTACCCCGCCCTTCCCTTCT | 450 |
| GDNF | TCCTGACCAGTTTGATGACG | CTCTGCGACCTTCC | 158 |

Table 2. Effects of CP, CS and VitE on Initial body weight (IBW, g), Final body weight (FBW, g), Testicle weight (TW, g), Epididymal sperm count (ESC, 106/ml), Sperm motility (SM, %), and Serum testosterone (ST, ng/ml)

| Group | Control | CP | CP+LCS | CP+MCS | CP+HCS | CP+VitE |
|-------|-----------|-------------|-------------------------|-------------------------|-------------------------|------------------------|
| IBW | 216.7±4.1 | 216.2±2.4 | 213.7±3.1 | 217.3±6.8 | 214.1±6.7 | 213.6±3.9 |
| FBW | 242.9±9.6 | 174.5±11.0* | 209.3±11.2 [#] | 217.2±11.6 [#] | 214.4±10.6 [#] | 201.0±8.4 [#] |
| TW | 1.9±0.1 | 1.2±0.1* | 1.8±0.1 [#] | 1.8±0.1 [#] | 1.8±0.1 [#] | 1.8±0.1 [#] |
| ESC | 86.3±4.3 | 7.9±3.4* | 79.3±4.0 [#] | 81.5±3.0 [#] | 79.3±3.8 [#] | 74.4±3.0 [#] |
| SM | 49.1±3.1 | 4.8±3.7* | 43.4±3.4 [#] | 48.3±2.8 [#] | 45.9±3.8 [#] | 44.1±4.3 [#] |
| ST | 61.4±5.3 | 59.8±4.5 | 64.0±6.1 | 62.2±2.5 | 58.1±5.3 | 62.0±8.0 |

All the data were expressed as the mean ± SD (n=6). * Significant difference compared with the control group ($p < 0.05$); [#] significant difference compared with the CP group ($p < 0.05$).

groups ($p > 0.05$). The western blot results were consistent with the qPCR data (Figure 4).

CS has a therapeutic effect on the damage caused by CP as observed under TEM

The changes in testicular spermatogonia were observed under TEM. The chromatin of spermatogonial nucleoli in the CP group was fragmented, coagulated and atrophied, mitochondria were enlarged, internal sputa were absent, and a large number of autophagic vacuoles were observed in the cytoplasm (Figure 5A). The spermatogonial cells in the CP+LCS group showed a uniform nucleus, clear nucleoli, and abundant mitochondria (Figure 5B). Therefore, CS has a therapeutic effect on the damage to spermatogonial spermatogenic ability caused by CP.

Discussion

The incidence of infertility has increased annually, and the current worldwide incidence of infertility has reached 15% (7). The proportion of male factors contributing to this problem is approximately 50% (8). Semen analysis indicators are now used to assess male fertility, and the main reasons for infertility are reduced sperm number and decreased sperm viability. Although the etiology of infertility involves genetic, endocrine, immune and environmental factors, there

is still a lack of effective drug treatment to improve sperm count and viability (9).

Traditional Chinese medicine has unique properties for treating oligospermia and asthenospermia. The recent application of modern biological technology has allowed for the evaluation of many herbs in the reproductive system. CS is a traditional kidney medicine with significant positive effects and adequate kidney Qi that can lead to improved semen quality. Other studies have demonstrated that CS increases cell proliferation and neuroblast differentiation in the dentate gyrus of mice by reducing serum corticosterone levels and increasing BDNF levels in this area (10). Wang X et al. demonstrated that compounds from CS exhibit phytoestrogenic and phytoandrogenic activities (11). CS exhibits in vitro and in vivo pharmacological activities and reduces fatigue, hypoxia, oxidation, diabetic symptoms, and viral activity and can modulate the immune system (12). The data from the study by Liu et al. demonstrated important anti-aging effects of CS (13).

Studies of CS both in and outside of China suggest that it plays an important role in the reproductive system. Abdel-Magied EM (14) reported that CS significantly increases the testicular weight of immature male Wistar rats. In immature rats, oral CS has the following effects: increased testicular sperm content and improved

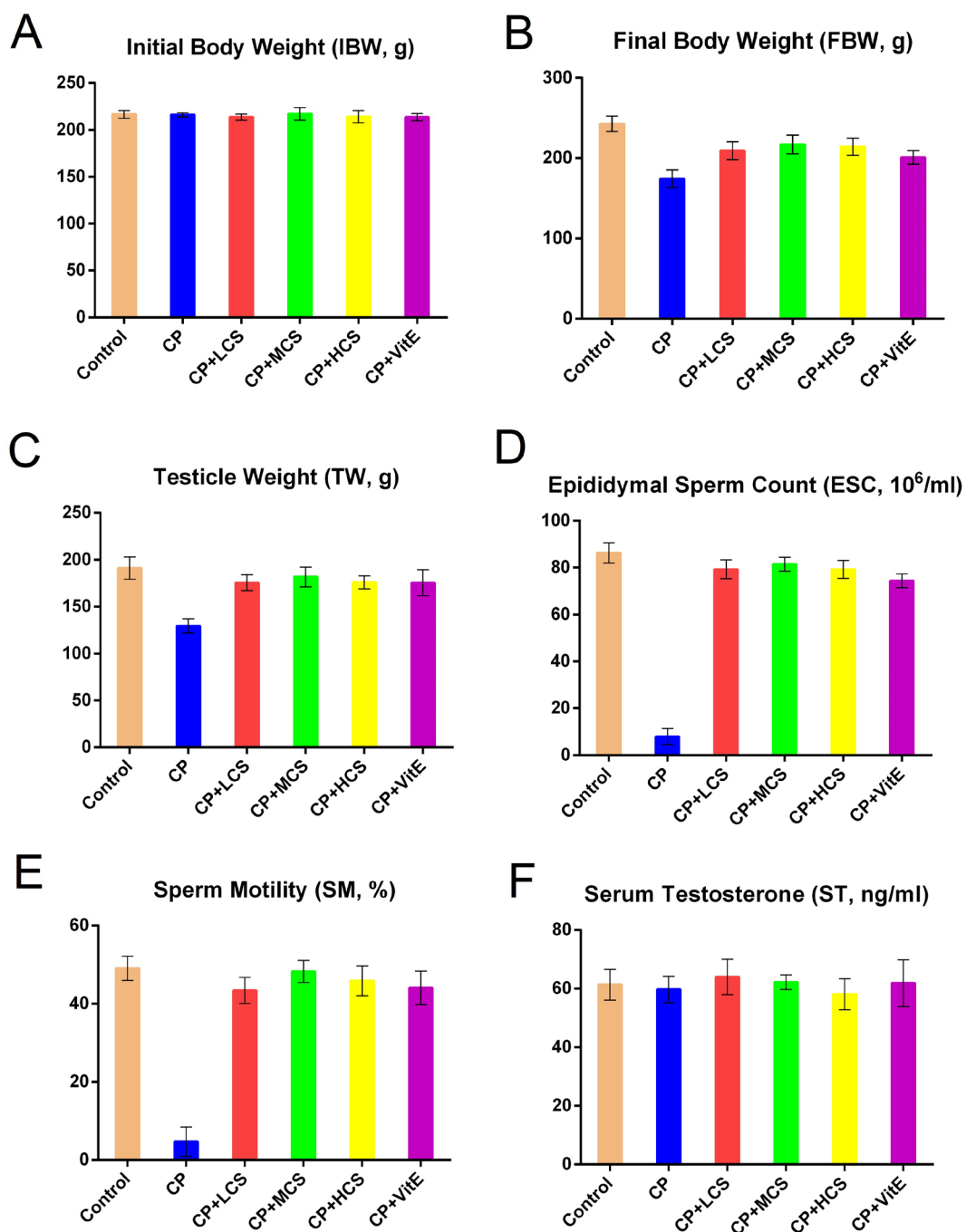


Fig. 1. Effect of CS on body weight, testicle weight, epididymal sperm count, sperm motility and serum testosterone in SD rats

Effects of CP, CS and VitE on Initial body weight (IBW, g), Final body weight (FBW, g), Testicle weight (TW, centigram), Epididymal sperm count (SC, 10⁶/ml), Sperm motility (SM, %), and Serum testosterone (ST, ng/ml). There were significant differences in FBW, TW, ESC and SM between the CP group and control group and between the CP group and the CP+LCS, CP+MCS, CP+HCS, CP+VitE groups ($p < 0.05$, *).

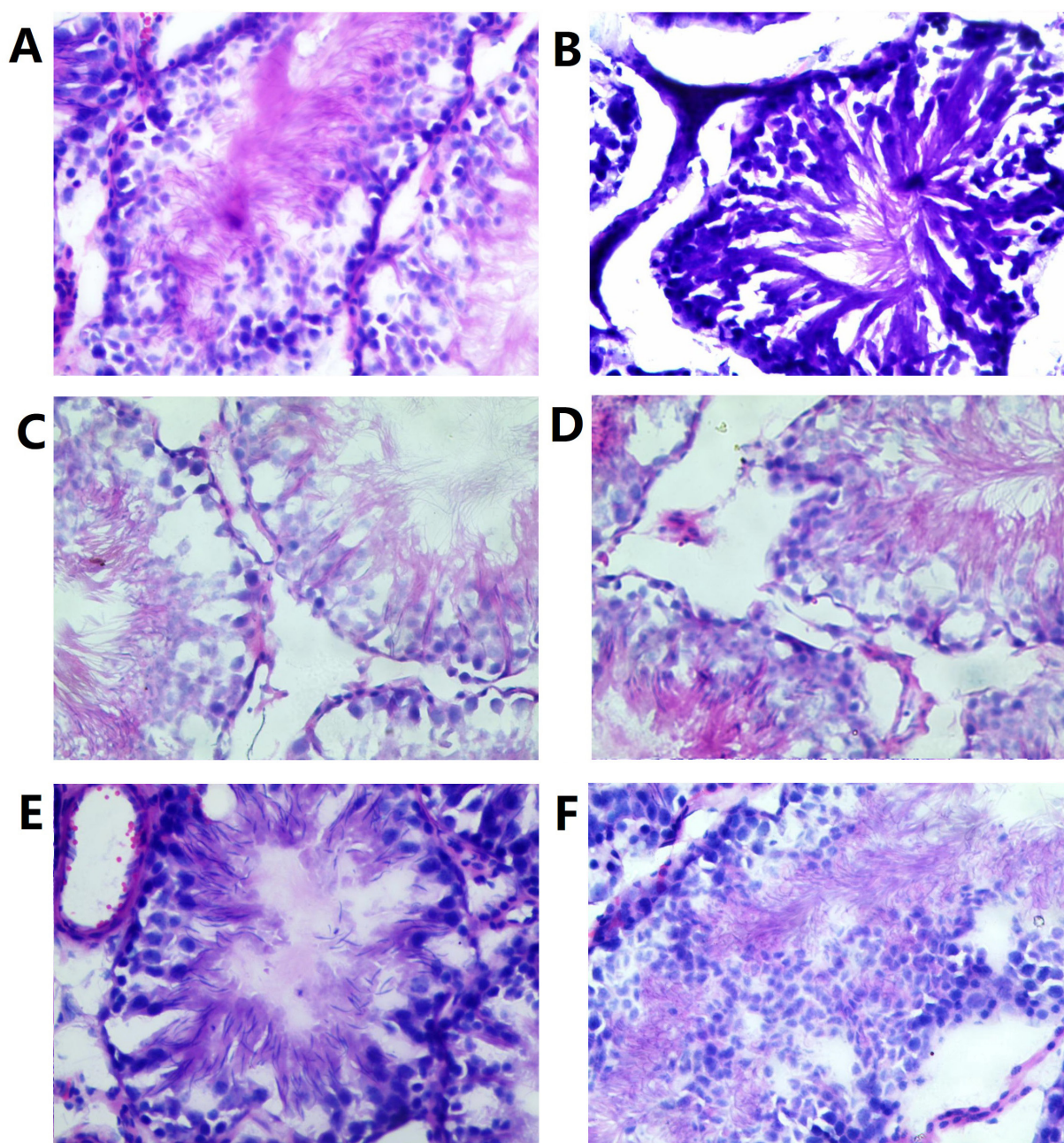


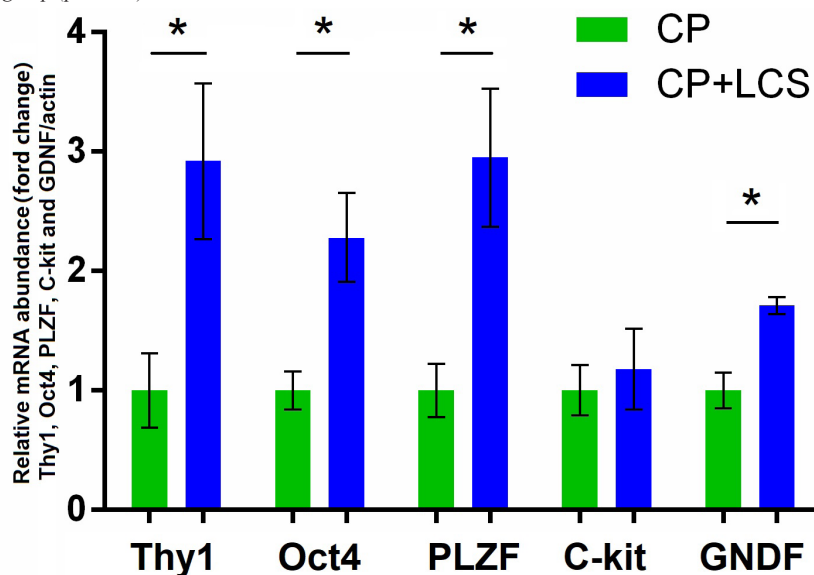
Fig. 2. Microscopic changes of rat testicle

Microscopic changes of rat testicle. A-F: control group, CP group, CP+LCS, CP+MCS, CP+HCS and CP+VitE groups (HE staining, 400X). In the control group, normal seminiferous tubules and spermatogenic cells were observed at all levels. In the CP group, it can be observed that spermatogenic cells of various levels shrink in different degrees and the structure is unclear. The seminiferous tubules and spermatogenic cells at all levels were clearly discernible in the CS or VitE group.

Table 3. qPCR results between the CP and CP+LCS groups

| ΔCt | Group CP | Group CP+LCS | t ratio | p value |
|-------------|-----------|--------------|---------|---------|
| Thy1 | 5.37±1.66 | 15.68±3.49 | 4.62 | 0.0099* |
| Oct4 | 6.65±1.06 | 15.16±2.46 | 5.50 | 0.0053* |
| PLZF | 4.78±1.05 | 14.10±2.77 | 5.44 | 0.0055* |
| C-kit | 8.74±1.84 | 10.31±2.97 | 0.78 | 0.4789 |
| GDNF | 9.76±1.46 | 16.69±0.68 | 7.43 | 0.0018* |

All data were expressed as mean \pm SD (n=3). * Significant difference compared with the control group ($p < 0.05$); # significant difference compared with the CP group ($p < 0.05$).

**Fig. 3. qPCR results of Thy1, Oct4, PLZF, C-Kit and GDNF**

The qPCR results showed significant differences in Thy1, Oct4, PLZF and GDNF ($p < 0.05$, *), but there was no difference in C-Kit expression between the CP group and CP+LCS group ($p > 0.05$).

spermatogenic tubules with sperm, increased epididymal tail sperm count, significantly increased sperm survival rate, increased sperm motility, and reduced abnormal sperm count. The Korean scholar Yang WM also reported similar findings in which oral CS increased glial cell line-derived neurotrophic factor mRNA and protein expression levels (3). The upregulation of GDNF can increase both the number of rat epididymal sperm and absolute testicular weight, reduce the number of abnormal spermatozoa, and increase the percentage of active sperm (4). Chinese scholars have reported that CS can sig-

nificantly improve testosterone levels in male mice. Although several studies have suggested that CS is helpful in the reproductive system, the mechanism of action has not been elucidated.

In this study, we used cyclophosphamide to construct a rat model of oligospermia and asthenospermia (control group). Our data showed that 4 weeks of treatment with different concentrations of CS did not significantly alter the weight of the CS group compared with that of the control group and blank group ($p < 0.05$). These results are inconsistent with previous studies. The differences may be due to separation of rat testicu-

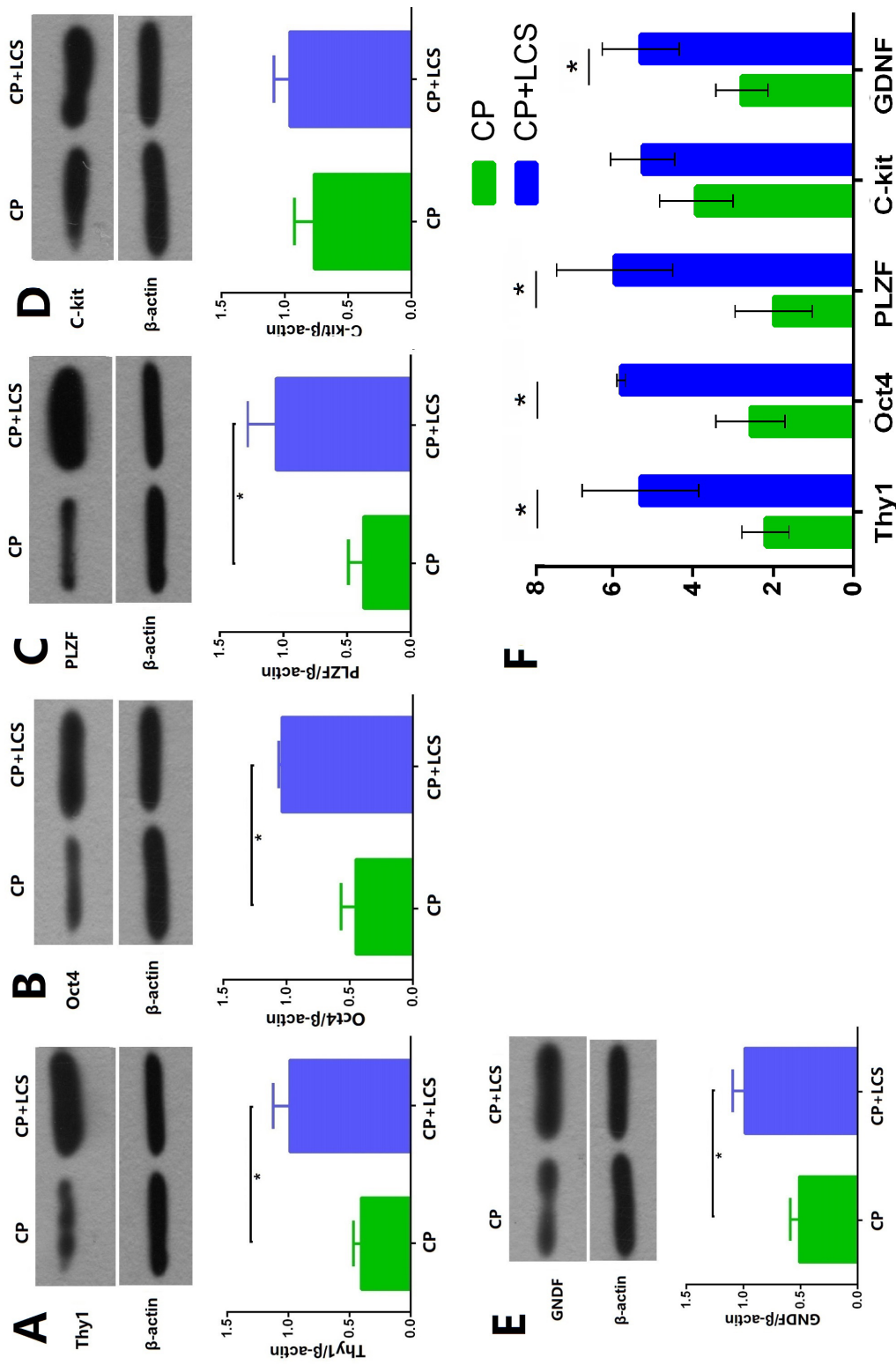


Figure 4 Western blot results to detect the expression of Thy1, Oct4, PLZF, C-Kit, and GDNF

The Western blot results showed significant differences in Thy1, Oct4, PLZF and GDNF ($p < 0.05$), but there was no differences in C-Kit between the CP group and CP+LCS group ($p > 0.05$). The expression of Thy1, Oct4, PLZF and GDNF was increased in the CP+LCS group, but the expression of C-Kit was the same as in the CP group.

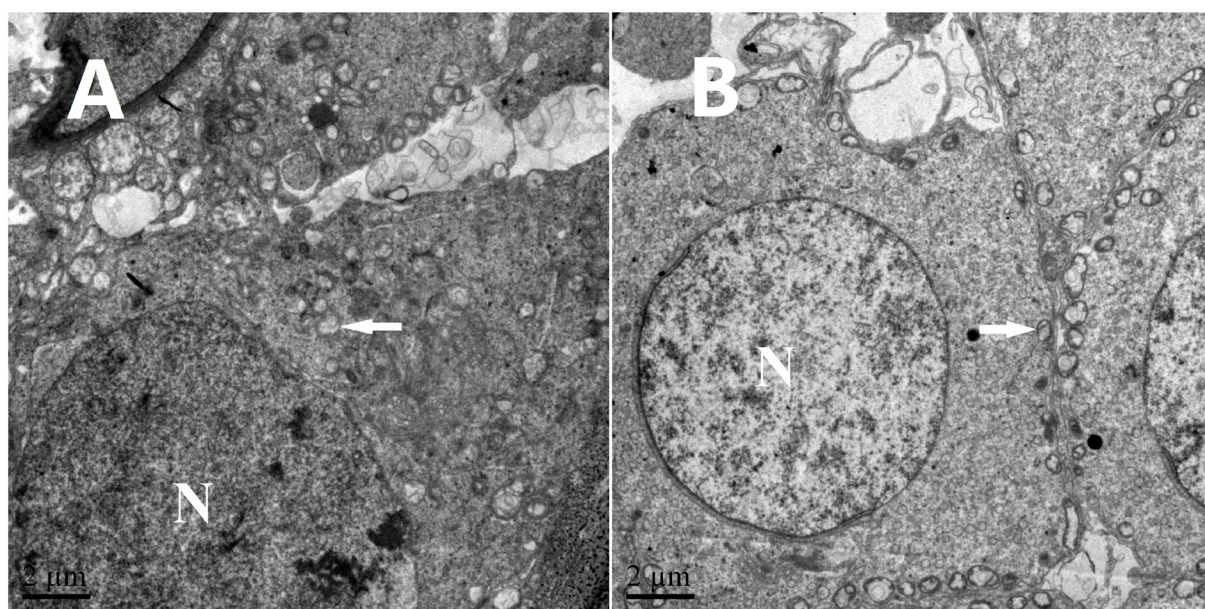


Fig. 5. TEM observations of testicular spermatogonia apoptosis

The changes in testicular spermatogonia were observed under TEM. The chromatin of the spermatogonial nucleus in the CP group was fragmented, coagulated and atrophied (N), mitochondria were enlarged, internal sputum was absent (←), and a large number of autophagic vacuoles were observed in the cytoplasm. The spermatogonial cells in the CP+LCS group showed a uniform nucleus (N), clear nucleoli, and abundant mitochondria (→).

lar tissue in the process. It is also difficult to obtain tissue weight. The number of spermatozoa in the epididymal tail of the CS group increased compared with that in the control group, and the difference was statistically significant ($p < 0.05$). The results of this study are consistent with the results of Yang WM suggested that CS does contribute to sperm content in the testis.

Yang WM also reported that CS may improve spermatogenesis by improving spermatogonia. The three groups of CS experimental animals showed improved spermatogenesis, and there was no obvious concentration effect between the three groups. Therefore, we used real-time PCR to detect the low concentration markers of testicular tissue spermatogonia (Oct4, Thy1, PLZF, and C-kit) (15). The results showed that the mRNA expression levels of Oct4, Thy1, and PLZF in the CS group were higher than those in the control group, and the differences were statistically significant. There was no signifi-

cant change in the expression of C-kit RNA. Studies have shown that Thy1, PLZF, and Oct4 are mainly expressed in undifferentiated spermatogonial stem cells (As, Apr, Aal) (16). Conversely, the C-kit gene is mainly expressed in differentiated A1 to A4 spermatogonia and is not expressed in undifferentiated spermatogonia. These results suggest that CS mainly promoted the proliferation of undifferentiated spermatogonial stem cells and had no significant effect on spermatogonia. During the process of spermatogenesis, spermatogonia receive signals and nutritional support from Sertoli cells, which allow them to proliferate and differentiate. GDNF is indispensable for spermatogonial stem cell self-renewal through its binding to the GFRA1/RET receptor and the subsequent effect on sperm quality (17).

The GDNF gene is expressed in all stages of mouse spermatogenesis and is mainly expressed in Sertoli cells. In this study, we found that the

expression of GDNF mRNA in the CS low concentration group was significantly higher than that in the control group. These findings suggest that CS may promote the expression of undifferentiated spermatogonia by promoting GDNF expression (18). There are many studies on the molecular pathway of GDNF in mice and other model animals. GDNF protein specifically binds to GFR α 1, which is expressed on undifferentiated spermatogonial cell membranes and activates tyrosine phosphorylation of Ret protein. The signal activation causes downstream MAPK-3 activation. This signaling pathway also exists in rats and suggests that CS may play a role that involves the GDNF pathway.

The sperm viability of the three CS groups was significantly higher than that of the control group ($p < 0.05$). Increases in serum testosterone improve the quality and activity of spermatozoa (19). Fatihah et al. (20) found that a CS decoction improved sperm quality by increasing serum testosterone concentration; our results are consistent with these findings. The serum concentration of testosterone in SD rats treated with CS was significantly higher than that in the control group ($p < 0.05$), which suggests that CS may improve serum testosterone level and improve sperm viability.

The complexity of the CS water extract composition may lead to pharmacological diversity. Previous studies have shown that CS is rich in trace elements and has high zinc content (19). Lee et al. suggested that CS extract promotes male fertility by improving spermatogenesis in golden hamsters (21). CS decoction improves rat sperm quality. Recent studies have shown that oxidative stress is an important factor in the manufacture of weak sperm. CS decoction contains a variety of ingredients with potent antioxidant effects, and current research on the anti-oxidant elements of CS has focused on carbohydrates, flavonoids and polyphenols. These compounds are capable of scavenging oxygen free radicals. A

recent study reported that two new water-soluble polysaccharides (22) and songarin A (23) were isolated from CS. Future studies should focus on the research and development of monomer components and expand their use for spermatogonia cell proliferation in vitro.

In conclusion, CS can promote spermatozoa processes in rats with oligoasthenospermia by increasing sperm count in the epididymis and improving sperm motility and has a therapeutic effect on the spermatogenic damage caused by CP in SD rats. However, due to the limitations of Chinese herbal medicine purification methods, the role of individual components of the CS decoction is difficult to obtain, limiting further therapeutic development of CS and other herbs.

Abbreviations

| | |
|-------------|---|
| CS | Cynomorium songaricum |
| CP | Cyclophosphamide |
| IBW | Initial body weight |
| FBW | Final body weight |
| TW | Testicle weight |
| ESC | Epididymal sperm count |
| SM | Sperm motility |
| ST | Serum testosterone |
| TEM | Transmission electron microscopy |
| GDNF | Glial cell line-derived neurotrophic factor |
| SPF | Skatepark Formula™ |
| LCS | low (0.5 g/kg·day) concentrations of CS |
| MCS | Medium (1 g/kg·day) concentrations of CS |
| HCS | High (2 g/kg·day) concentrations of CS |
| HE staining | Hematoxylin-eosin staining |
| EP tube | Eppendorf tube |
| GAPDH | glyceraldehyde-3-phosphate dehydrogenase |
| PVDF | polyvinylidene fluoride |
| SD | Sprague Dawley® Rats |

Authors' contribution

Xiaoxiao Han (Data curation)
 Rongsheng Zhou (Methodology; Software; Supervision)
 Wei Zheng (Investigation)
 Xitao Wang (Data curation)
 Shuyan Mao (Pathology)
 Zhenbei Li (Software)
 Zhiguo Zhang (Data curation)
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 Bo Chen (Data curation)
 Yijuan Cao (Data curation)
 Dianjun Yu (Formal analysis)
 Kun Pang (Writing – original draft; Writing – review & editing)
 Conghui Han (Funding acquisition)

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Conflict of interest statement

The authors declare no conflict of interest in preparing this article

Ethics and consent statements

The study was provided by the Experimental Animal Center of Xuzhou Medical University (SYXK (Su) 2017-0011).

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