RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2024.062

ScienceAsia 50 (3): 2024: ID 2024062: **1–8**

Ginsenoside Rg1 improves spermatogenic dysfunction in cryptorchidism model rats by reducing blood-testis barrier damage

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Received 6 Feb 2023, Accepted 19 Jan 2024
Available online 30 Jun 2024

ABSTRACT: Cryptorchidism is a risk factor for male infertility, for which surgical treatment is the principal clinical treatment, but the sperm quality of cryptorchidism patients may continue to be impaired after cryptorchidism surgery. Studies have found that ginsenoside Rg1 can improve reproductive function damage. Therefore, it is speculated that ginsenoside Rg1 may have the potential to prevent and treat cryptorchidism. This study aims to investigate whether ginsenoside Rg1 plays a protective role in rats with flutamide-induced reproductive function impairment and to explore its effect on blood-testis proteins. Pregnant rats were treated with flutamide and ginsenoside Rg1, and newborn male rat serum and testes were collected for further analysis. ELISA detects the level of testosterone (T), luteinizing hormone (LH), and follicle-stimulating hormone (FSH). Hematoxylin & eosin (H&E) and Periodic acid-Schiff (PAS) staining methods were used to detect the testis tissue. Transmission electron microscope (TEM) was used to observe changes in the blood-testis barrier around testicular tissues. Western blot analysis detects Bax, Bcl-2, cleaved caspase-3, ZO-1, N-cadherin, E-cadherin, and Connexin43 proteins. Ginsenoside Rg1 reversed the decrease in testis weight and serum hormone levels in rats induced by flutamide. Ginsenoside Rg1 can improve the sperm density and sperm motility of rats induced by flutamide, reduce testicular tissue damage and spermatogenic cell apoptosis, and even up-regulate these protein expressions including ZO-1, N-cadherin, E-cadherin, and Connexin43 to affect the blood-testis barrier. Apoptotic germ cells were detected by a terminal-deoxynucleotidyl transferase mediated nick end labeling (TUNEL) assay. The protective effect of ginsenoside Rg1 in spermatogenesis may be related to the regulation of reproductive hormones, reducing tissue damage, and activating spermatogenesis-related proteins.

KEYWORDS: ginsenoside Rg1, testis, hormone, cryptorchid, blood-testis barrier

INTRODUCTION

Cryptorchidism is a common clinical congenital abnormality, and the incidence of full-term newborns is about 4% [1]. Studies have found that cryptorchidism is the risk factor for male infertility [2]. Cryptorchidism can cause a significant increase in the production of free radicals in the testis, a large number of spermatogenic cells apoptosis, and spermatogenesis obstructively [3]. Cryptorchidism not only predisposes the testis to damage and torsion, but more importantly, increases the risk of male congenital infertility, and testicular fixation in childhood does not improve its infertility rate [4]. Surgical treatment of cryptorchidism is a recognized treatment method, but patients with cryptorchidism still have a higher risk of infertility and cancer after surgery [5]. Therefore, it is of great social and economic importance to develop highly effective drugs with clear mechanisms of action and non-toxic side effects for the prevention and treatment of cryptorchidism.

With good clinical efficacy, traditional Chinese medicine has unique advantages in improving semen quality and reproductive function in men and is currently one of the effective methods for treating male infertility [6, 7]. Methanolic extract of moringa oleifera leaves could ameliorate cryptorchidism associated with germ cell loss and oxidative stress [7, 8]. Rosaceae can improve spermatozoa characteristics, antioxidant enzymes, and fertility potential in unilateral cryptorchid rats [9]. Ginsenoside Rg1 is one of the main active ingredients of ginseng (*Panax ginseng C. A. Meyer*) and has a wide and significant pharmacological effect with various protective effects on cardiac muscle cells and kidney tissues as well as vascular regeneration promotion [10–12]. Recent studies have reported that ginsenoside Rg1 can improve reproductive function impairment in mice [13]. In this experiment, we established a rat cryptorchid model by giving anti-androgen flutamide during the embryonic period and observed the protective effect of ginsenoside Rg1 on the spermatogenic function of experimental cryptorchid mice.

MATERIALS AND METHODS

Grouping and administration of experimental animals

Forty female and 40 male SPF SD rats were purchased from the Animal Center of DASHUO [SCXY (Chuan) 2020-034]. They weighed 280–310 g and were raised in the Animal Center of Lanzhou University First Hos-

pital. In this study, all experimental procedures were performed following the guidelines of experimental animals from Lanzhou University, and the number of animals was kept to a minimum. The temperature of the breeding room was 20 °C–25 °C, the relative humidity was 40%–60%, and the animals were free to eat and drink. After a week of adaptive feeding, the female and male rats were caged at a ratio of 1:1. It was recorded as pregnancy 0 day if there were vaginal plugs in the cage on the next day at 8 a.m.

After conception, 40 pregnant rats were evenly allocated to 4 groups by the random number table method. Ten rats in the blank group were fed normally. The skin on the back of the neck was disinfected at 8 a.m. every day from 12 to 20 days of pregnancy, and the same amount of physiological water as the experimental group was injected subcutaneously. In the model group, 10 rats were fed normally, and on 12 to 20 days of pregnancy, 25 mg/kg/d of flutamide (Sigma, USA; catalog no. F9397) was injected subcutaneously every morning at 8 a.m. to induce a cryptorchidism model. Twenty rats in the ginsenoside Rg1 (MCE, USA) treatment group were bred normally. Based on the flutamide-induced cryptorchidism group, ginsenoside Rg1 was injected to spring male rats 1–10 days and 28–30 days after birth (1 mg/kg/d as a low dose and 10 mg/kg/d as a high dose). After completing the experiment, the rats were sacrificed by cervical dislocation, and the rat testicular tissue and blood were collected for subsequent research.

Enzyme-linked immunosorbent assay (ELISA)

One ml of rat fundus venous plexus plasma was centrifuged at 11,200×g at 4 °C for 10 min, and the supernatant was collected for use. ELISA detection kits of T, LH, and FSH were purchased from Abbott Laboratories (USA). Then, the standard was diluted with the diluent, and the absorbance at 450 nm was measured with a microplate reader. The serum T, LH, and FSH expression levels were calculated using the logarithm of the standard concentration as the abscissa and the measured absorbance value as the ordinate.

Histopathologic examination

The rat testes were fixed with paraformaldehyde for 24 h, routinely paraffin-embedded sections were stained with H&E (Mlbio, China), and the histological differences were observed under the microscope. About 15 sub-circular seminiferous tubules were selected from the transverse section of the testis under a 40 × microscope. The diameter and area were measured with the software EIS-elements BR 3.0, and the average value was calculated.

Periodic acid-Schiff stain (PAS)

The testicular tissue was fixed with Carnoy fixative and embedded routinely. Paraffin sections of testicular tissues were routinely deparaffinized and hydrated, reacted with a permanganate alcohol solution for 10 min, washed with 70% alcohol, and treated with a colorless basic magenta solution (Aladdin, China) for 1 h. After washing with running water, counterstaining with hematoxylin for 5 min, and differentiating with 1% hydrochloric acid alcohol, the testicle slice was dehydrated, sealed with a transparent, neutral resin, observed, and analyzed with an optical microscope.

Transmission electron microscopy assay

Rat testicular tissue was fixed in 5% glutaraldehyde for 5 h at 4 °C, washed 3 times with neutral phosphate buffer every 10 min, fixed in 0.1 mol/l osmium acid for 3 h, and washed again in phosphate buffer every 10 min. Every 15 min, gradient dehydration of 50%, 70%, 80%, 90%, 95%, and 100% alcohol was performed. Resin infiltration, embedding, and polymerization were used to create 60–80 nm ultrathin sections, which were subsequently doubly dyed with uranium lead, dried at room temperature, and imaged using TEM.

Terminal-deoxynucleotidyl transferase mediated nick end labeling (TUNEL)

TUNEL (Alexa Fluor 488) was used to detect spermatogenic cell apoptosis. Paraffin sections were routinely deparaffinized, hydrated, and washed with PBS 3 times. The rest of the operations were performed according to the instructions of the TUNEL kit (Progema, USA). Cells with green nuclei under a light microscope are apoptosis-positive cells. Three sections of seminiferous tubules were randomly selected from each rat testicular tissue section, and the average percentage of positive cells was calculated as the apoptosis index.

Western blot analysis

The Radio Immunoprecipitation Assay lysis buffer was used to lyse the testicular tissue, and the total testicular protein was extracted from the lysate with an electric homogenizer. The total protein concentration was determined by the BSA method. Then, a 5 × loading buffer was added to the total protein. The sample was boiled, denatured at 100 °C for 10 min, and stored at −20 °C. The 40 μg total proteins were separated using acrylamide separation gel at 100 V, 90 min. The membrane was transferred with a semi-dry electrotransfer instrument at a constant current for 26 min. The 5% skimmed milk powder solution was blocked for 2 h, and the primary antibody (Bax 1:500, Bcl-2 1:500, cleaved caspase-3 1:500, ZO-1 1:150, N-cadherin 1:200, E-cadherin 1:300, Connexin43 1:500, and β-actin 1:1000) (Abcam, UK) was added and incubated overnight at 4 °C. The membrane was eluted by TBST (Sigma), and the secondary antibody (ImmunoReagents, USA, 1:4000) was added and incubated for 1 h at room temperature. After

TBST washing, the chemiluminescence kit (ECL) was performed. The gray value of the protein band was analyzed with QuantityOne software and the relative expression level with β-actin as the internal reference.

Statistical analysis

The SPSS11.5 software was used to analyze the data of each group. The measurement data was expressed as $\bar{x} \pm s$, and $p < 0.05$ was considered statistically different. One-way analysis of variance (one-way ANOVA) was used for comparison of more than 2 groups, and a T-test was used for expression differences between the 2 groups.

RESULTS

Testicular weight and serum hormone levels

Ginsenoside Rg1 is a tetracyclic triterpenoid derivative with 2 glucose residues (**??**A). Under the induction of flutamide, the weight of rat testis was significantly reduced. Compared with the blank group, the weight of testis in the model group decreased by 453.35 ± 20.35 mg. Ginsenoside Rg1 has a reversal effect on the induction of flutamide. The testis mass of rats in the Ginsenoside Rg1 high-dose treatment group is almost the same as that of the blank group. Compared with the model group, the Ginsenoside Rg1 low-dose treatment group had higher testicular mass ($p < 0.5$) (**??**B). As for serum hormone levels, compared with the blank group, the levels of the hormones T, LH, and FSH in the serum were significantly reduced under the induction of flutamide ($p < 0.5$). Under the induction of ginsenoside Rg1, these hormones tend to be up-regulated, and they are close to the blank group at high doses (**??**C). These results indicate that ginsenoside Rg1 has a regulatory effect on testicular damage repair and hormone levels.

Histochemical detection of rat testis

H&E and PAS staining methods were used to detect the effects of ginsenoside Rg1 on sperm morphology, quantity, spermatocytes, and spermatogenic cells of testis tissue. The results of H&E staining showed that compared with the blank group, the sperm cells and sperms disappeared. Some of the spermatogenic cells degenerated and were necrotic; the nucleus was pyknosis; and the cytoplasmic eosinophilia increased in the model group. However, under the treatment of ginsenoside Rg1, a small number of spermatogenic cells became degenerated and necrotic in the testis, and sperm cells and sperms in some areas were slightly reduced (Fig. 2A). As for PAS staining, compared with the blank group, the epithelium of the spermatogenic tubules was slightly thinner, and the spermatogenic cells were disorganized and unordered in the model group. In contrast, the adverse reactions were significantly reduced under the treatment of ginsenoside Rg1. The spermatogenic tubules were more closely arranged, and the epithelial cells were more neatly arranged (Fig. 2B). Transmission electron microscopy results showed that compared with the blank group, the morphological structure of the blood-testis barrier in the model group was significantly damaged. In the model group, the endothelium was loosely arranged and flexed. The tight junctions between endothelial cells were completely opened in some areas; the capillary basement membrane was disconnected in some areas, and the continuity was disrupted. Moreover, the thickness of connective tissues was uneven; more vesicular structures were seen in the supporting cells. The inter-supporting cell junction complexes disappeared and were completely opened. These symptoms were alleviated after treatment with ginsenoside Rg1 (Fig. 2C). H&E and PAS staining results showed that ginsenoside Rg1 could reduce testicular tissue and promote sperm cell quality by pathological damage.

Effect of ginsenoside Rg1 on cell apoptosis in rat testis

Apoptotic germ cells were detected by a TUNEL assay. Apoptotic cells will be excited to show green fluorescence. In the blank group, there was no green fluorescence. In the model group, there was more green fluorescence as a large number of apoptotic cells. In the low dose of ginsenoside Rg1, the green fluorescence reduced, and apoptotic cells decreased, while there were almost no apoptotic cells in the high dose group (Fig. 3A,B). In addition, compared with the blank group, the expression of apoptosis-related proteins Bax and cleaved caspase-3 increased in the model group, and the expression of the apoptosis-inhibiting protein Bcl-2 decreased. Compared with the model group, the expression of Bax and cleaved caspase-3 decreased, and the expression of Bcl-2 increased after ginsenoside Rg1 treatment (Fig. 3C,D).

Effect of ginsenoside Rg1 on related proteins in testis tissue

The rat testis tissue was taken to detect the effect of ginsenoside Rg1 on ZO-1, N-cadherin, E-cadherin, and Connexin43 proteins. Compared with the blank group, the expression levels of ZO-1, N-cadherin, E-cadherin, and Connexin43 decreased under the induction of flutamide. However, under the treatment of ginsenoside Rg1, these proteins in the low-dose group tended to be up-regulated. Moreover, in the high-dose group, these protein expressions were close to the blank group and significantly higher than those in the model group (Fig. 4A,B).

DISCUSSION

Congenital cryptorchidism is a congenital malformation in which the decline of the testis during embryonic development is affected by heredity, endocrine hormones, and local anatomical factors [14]. The

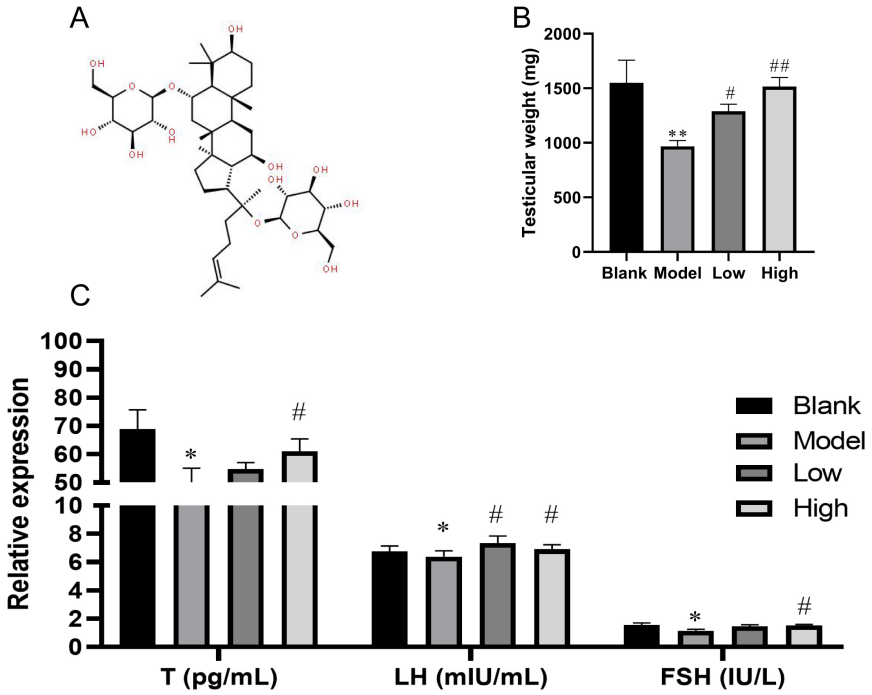

Fig. 1 The effect of ginsenoside Rg1 on testis weight and serum hormone levels in rats. (A) The chemical structure of ginsenoside Rg1. (B) Testicular weight. (C) Serum hormone levels of T, LH, and FSH. * $p < 0.05$ and ** $p < 0.01$, compared with the blank group; $^{\#}$ $p < 0.05$ and $^{\#\#}$ $p < 0.01$, compared with the model group.

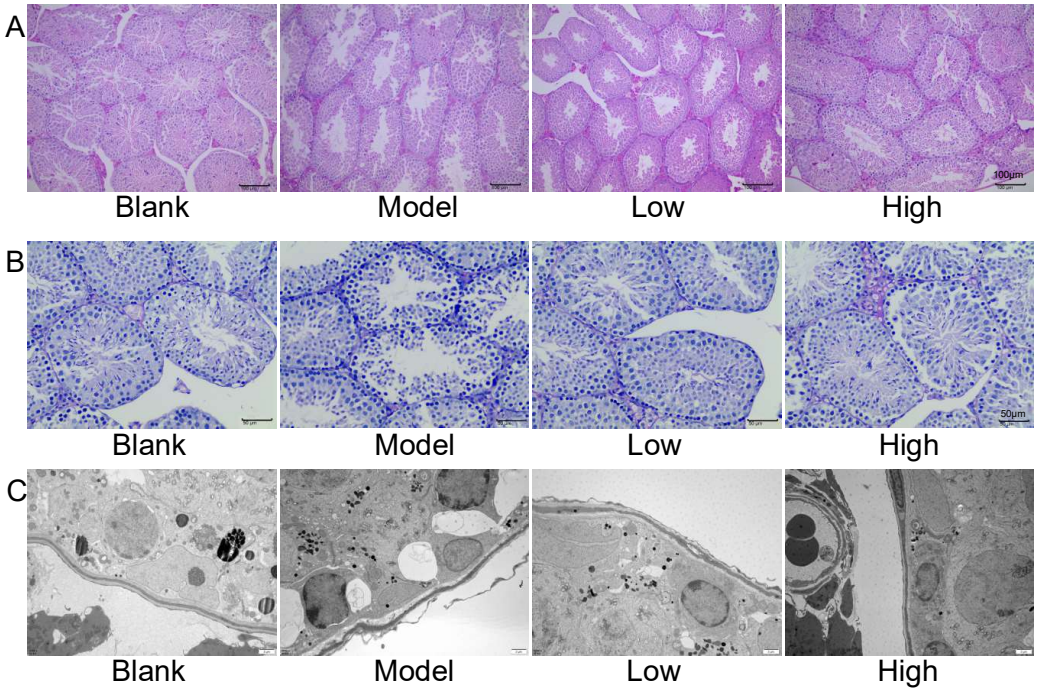

Fig. 2 The effects of ginsenoside Rg1 on histochemistry of rat testis. (A) H&E staining results and (B) PAS staining results of testicular tissue. (C) Testicular tissue examined by transmission electron microscopy.

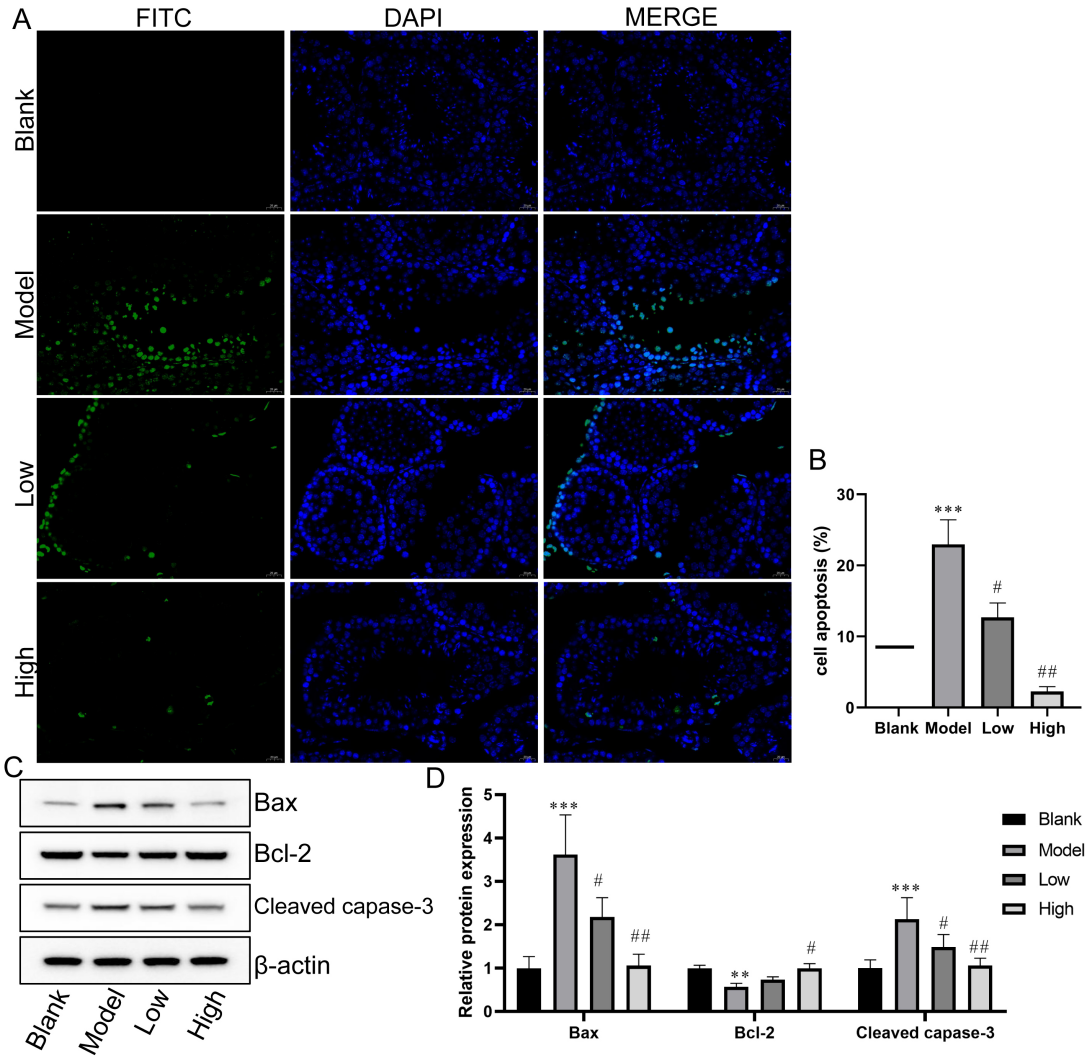

Fig. 3 Testicular tissue apoptosis rescued by Ginsenoside Rg1. (A and B) TUNEL fluorescence detection of the effect of ginsenoside Rg1 on cell apoptosis in rat testis. (C and D) Changes of Bax, Bcl-2, and cleaved caspase-3 proteins in testis tissue. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, compared with the blank group; $^{\#}$ $p < 0.05$ and $^{\#\#}$ $p < 0.01$, compared with the model group.

flutamide-induced cryptorchidism rat model is the main object of research on this disease. Flutamide is a non-steroidal androgen receptor blocker that binds to androgen receptors to prevent testosterone and dihydrotestosterone from exerting their effects [15]. The anti-androgen flutamide given during the embryonic period can block the effect of androgen and affect testicular development [16]. Ginsenoside Rg1 has a variety of biological activities that can improve reproductive function damage [13]. Therefore, this study used ginsenoside Rg1 as an intervention agent to determine whether ginsenoside Rg1 can regulate hormone levels and repair testicular tissue damage.

In this study, the histomorphological alterations of the testes of rats in the model group were obvious. Cryptorchid testes had distinctive pathological changes with increased apoptosis of spermatogenic cells, destruction of the tight structure between supporting cells, and a decrease in sperm count, which may be an important reason for fertility disorders, while ginsenoside Rg1 can restore the spermatogenic environment of cryptorchid testes and inhibit apoptosis of spermatogenic cells.

The hypothalamic-pituitary-testicular axis is the main means to regulate testicular spermatogenic function, and FSH, LH, and T are important components that could affect testicular spermatogenic function [17, 18]. Therefore, the function of the testis can be evaluated by testing the levels of these hormones. Testosterone is mainly synthesized and secreted by

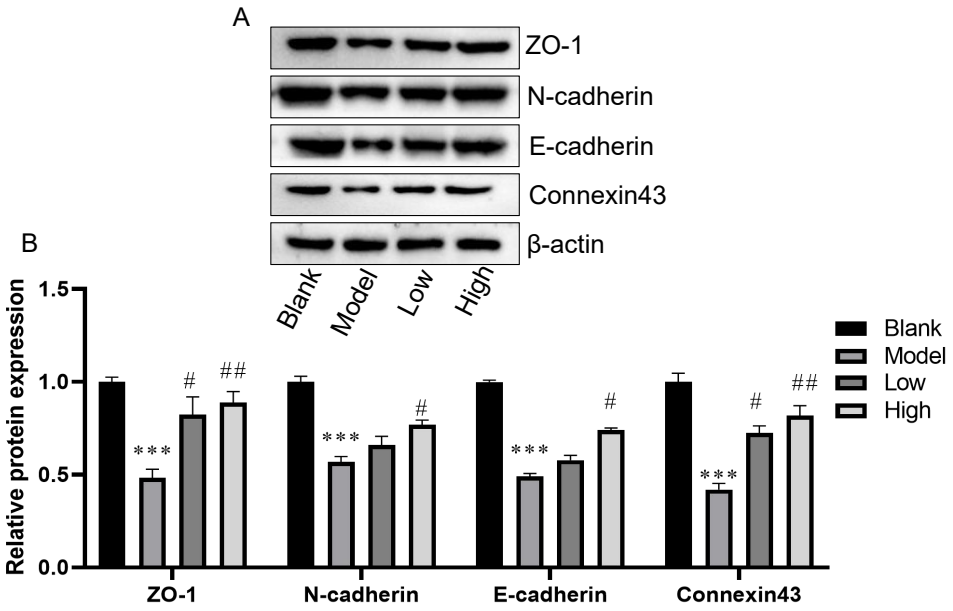

Fig. 4 Effect of ginsenoside Rg1 on blood-testis barrier related proteins. (A and B) Changes of ZO-1, N-cadherin, E-cadherin, and Connexin43 proteins in testis tissue. $^{*}p < 0.05$ and $^{**}p < 0.01$, compared with the blank group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, compared with the model group.

testicular mesenchymal cells (Leydig cells), and its main physiological function is to promote secondary sexual characteristics and the production and maturation of spermatozoa [19]. In contrast, FSH and LH are synthesized and secreted by the anterior pituitary gland [20]. The physiological function of LH in men is to promote the proliferation of testicular mesenchymal cells, synthesize and secrete testosterone, while testosterone negatively regulates the secretion of LH by the pituitary and hypothalamus [21, 22]. FSH can stimulate the development of testicular support cells (Sertoli cells) and promote the production of a protein that binds androgens, through which the developing germ cells receive a steady high concentration of androgens, leading to the production and maturation of sperms [23, 24]. In the present study, flutamide induced a decrease in FSH, LH, and T levels, whereas ginsenoside Rg1 up-regulated these hormones, which also implies that ginsenoside Rg1 can affect the hypothalamic-pituitary-testicular axis.

The blood-testis barrier also plays a significant role in the sperm production process [25]. It is the orderly opening of the blood-testis barrier that ensures the smooth progress of sperm production [26]. The regulation of the blood-testis barrier is a process regulated by many factors such as ZO-1, N-cadherin, E-cadherin, and Connexin43. The p-glycoprotein is involved in regulating the dynamics of the blood-testicular barrier by regulating the phosphorylation state of the occludin/ZO-1 protein complex through interaction with FAK [27]. Connexin43 protein is the most abundant connexin expressed in the testis. It exists between testicular stromal cells and plays an important role in maintaining the integrity of the blood-testis barrier, supporting cell proliferation and differentiation and spermatogenesis [28, 29]. N-cadherin is a type of molecule that calcium-dependently mediates cell adhesion [30]. It plays an important role in maintaining testicular Sertoli cell polarity, spermatogenesis, and intracavitary migration in the human sperm epithelium, and it has been reported to inhibit the formation of tumor cells [31]. E-cadherin is a calcium ion-dependent transmembrane glycoprotein that exists in the epididymal epithelium between the human epithelial cells and promotes the development and maintenance of the blood-epididymal barrier [31]. In the present study, ZO-1, N-cadherin, E-cadherin, and Connexin43 decreased in testis under the induction of flutamide, while all of them were up-regulated by ginsenoside Rg1. The repair effect of ginsenoside Rg1 on testicular injury may be related to the regulatory proteins ZO-1, N-cadherin, E-cadherin, and Connexin43, which further regulate the testicular microenvironment by modulating the blood-testis barrier.

The blood-testis barrier helps maintain the stability of the testicular internal environment and regulates cell apoptosis [32]. Studies have shown that blood-testis barrier damage can lead to increased cell apoptosis, thereby affecting reproductive function [32, 33]. The blood-testis barrier helps reduce cell damage caused by oxidative stress [34, 35], which can lead to cell apoptosis. The blood-testis barrier can

prevent oxidative stress substances from entering the testes, thereby reducing the risk of cell apoptosis [36]. The blood-testis barrier helps prevent immune cells and immune substances from entering the testicles and preventing attacks on their tissues [34, 35]. Once the blood-testis barrier is damaged, immune cells and immune substances can easily enter the testicles, leading to inflammatory reactions and cell apoptosis [37]. In summary, the blood-testis barrier has a protective effect on cell apoptosis and can prevent cell apoptosis caused by factors such as cytotoxic substances, oxidative stress, and immune attack. When the blood-testis barrier is damaged, it could easily lead to increased cell apoptosis, thereby affecting reproductive function. Bax, Bcl-2, and cleaved caspase-3 are apoptosis markers [38, 39], and the role of ginsenoside Rg1 shows that it could inhibit apoptosis proteins and promote the expression of apoptosis-inhibiting proteins. The experimental results of this study showed that ginsenoside Rg1 can repair the blood-testis barrier and rescue germ cell apoptosis.

CONCLUSION

In summary, these findings indicated that ginsenoside Rg1 can improve testicular tissue damage and promote the recovery of testicular weight loss caused by flutamide by regulating the levels of sex hormones T, LH, and FSH. The proteins ZO-1, N-cadherin, E-cadherin, and Connexin43 in the testis may be related to the protective effect of ginsenoside Rg1 on flutamide-induced reproductive function damage in SD rats. Ginsenoside Rg1 can repair the blood-testis barrier and rescue germ cell apoptosis. Ultimately, this research will help the development of natural products and provide efficient and safe drugs for the treatment of cryptorchidism. However, a test to verify its safety is still necessary.

Acknowledgements: Funding: Clinical Basic Research Program of Lanzhou University, ldyyyn2018-46; Gansu Provincial Education Department Innovation Capacity Enhancement Project, 2020B-002; Gansu Provincial Science and Technology Program Grant, 21JR1RA094.

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