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Antagonistic Effect of *Cuscuta chinensis* on a Rat Model with Unilateral Cryptorchidism

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: The aim of this study was to investigate the effect of *Cuscuta chinensis* Lam. on germ cell apoptosis in a rat model of unilateral cryptorchidism.

Material/Methods: Thirty male SD rats were randomly and equally divided into a control group, a model group, and a Cuscuta group (5.0 g/kg/d) (n=10). The rat model of unilateral cryptorchidism in the model and Cuscuta groups was established by removal of the right gubernaculum, while rats in the control group received no treatment. After modeling, rats in the *Cuscuta chinensis* group were intragastrically administered *Cuscuta chinensis* extract (5.0 g/kg/d), while rats in the control group and model group were administered an equal volume of normal saline. After 90 days, all the rats were sacrificed and the testicles were separated and weighed, followed by TUNEL staining to detect germ cell apoptosis, flow cytometry to measure JC-1, ROS, and MDA, and Western blot analysis to evaluate the expression of Bax, Bcl-2, and cleaved caspase3.

Results: Ninety days after the operation, *Cuscuta chinensis* Lam significantly minimized the damage caused by modeling by increasing weight of testis, reducing the germ cell apoptosis, and enhancing the mitochondrial membrane potential of testicles, as shown by levels of JC-1, ROS, and MDA, as well as elevating the level of Bcl-2/Bax and reducing the level of cleaved caspase3 ($P<0.05$).

Conclusions: Treatment with *Cuscuta chinensis* Lam reduced the germ cell apoptosis in rats with unilateral cryptorchidism, which provides new insight for the development of cryptorchidism therapy in the future.

MeSH Keywords: **Apoptosis • Cryptorchidism • Neoplasms, Germ Cell and Embryonal**

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Background

Cryptorchidism is a common congenital disease in the male reproductive system. It is common in full-term male infants, and the incidence rate gradually decreases with age. It is an important factor contributing to male infertility [1,2]. In the occurrence and development of unilateral cryptorchidism, higher temperature in the testis than in the scrotum leads to degenerative changes in sustentacular cells, interstitial cells, and spermatogenic cells in the testis, which affects fertility [3]. In addition to the impact on the homolateral testis, unilateral cryptorchidism can also inhibit the function of the contralateral testis. Some studies have revealed that cryptorchidism can result in hypothalamic-pituitary-gonadal dysfunction, which may also interfere with contralateral testicular function [4]. The balance between production and clearance of reactive oxygen species (ROS) plays an extremely important role in spermatogenesis in testicular spermatogenic cells. The physiological level of ROS can maintain the body's normal physiological functions, whereas excessive ROS can cause apoptosis [5]. The production of ROS in the body and the effect of peroxides maintain the balance of ROS *in vivo*. Lipid oxidation reaction produces malondialdehyde (MDA), which can indirectly reflect the ROS level in cells and tissues [6]. Mitochondria are the main source of adenosine triphosphate, which is essential in energy production in cells, and its dysfunction results in decreased mitochondrial membrane potential and increased ROS production, thereby causing a series of reactions such as DNA damage and apoptosis [7]. Studies have revealed that the increasing apoptosis of spermatogenic cells in cryptorchidism may be closely related to the increased ROS level in cells. Zhen et al. [8] found that the expressions of apoptosis-associated proteins in the testis of mice with cryptorchidism were significantly increased with the rise of ROS. *Cuscuta chinensis* is the dry seed of the Chinese dodder plant, a member of the Convolvulaceae family, the major pharmacological components of which include flavonoids, sterols, polysaccharides, and coumarin [9]. It has been demonstrated by several studies that *Cuscuta chinensis* has anti-oxidation and anti-aging effects, as well as pharmacological effects on the reproductive system and immune regulation [10,11]. Our study aimed to establish a rat model of unilateral cryptorchidism to investigate the effect of *Cuscuta chinensis* on the apoptosis of contralateral testicular spermatogenic cells.

Material and Methods

Animals and grouping

A total of 30 male Sprague-Dawley (SD) rats aged 21 days were purchased from the Laboratory Animal Center of School of Medicine, Wuhan University (laboratory animal production license No.: SCXK2016-0002) and were kept in the specific pathogen-free animal house. Before the experiment, rats were fed adaptively for 1 week at 22±1°C and humidity of 45±5%, with a 12 h/12 h light/dark cycle, and they had free access to food and water. These SD rats were randomly and equally divided into a control group (n=10), a model group (n=10), and a *Cuscuta chinensis* group (n=10). The experimental program was approved by the Laboratory Animal Ethics Review Committee of our hospital. All animal procedures adhered strictly to the regulations in the health guidance of the National Institute about laboratory animal care and use.

Establishment of animal model of unilateral cryptorchidism and drug administration

Model establishment: After rats in each group adapted to the environment under the above feeding conditions for 1 week, rats in the model group and *Cuscuta chinensis* group received the modeling operation according to the method of Loebenstein et al. [12], while rats in the control group received no treatment. The operation was performed as follows: After rats were anesthetized with 10% chloral hydrate, a median abdominal incision was made, the right gubernaculum testis was cut, and the testis was fixed on the posterior abdominal wall using a 7-0 noninvasive suture needle. The left testis was not treated and was placed back into the scrotum, and the abdomen was sutured surgically. Rats in each group were injected with antibiotics to prevent infection.

Drug treatment: Rats in the control group and model group were administrated normal saline (5 mL/kg), while those in the *Cuscuta chinensis* group were administrated *Cuscuta chinensis* (5 mg/kg) through gavage. *Cuscuta chinensis* was purchased from Beijing Tongrentang Co. and was authenticated by the chief pharmacist of traditional Chinese medicine of our hospital. We added 100 g *Cuscuta chinensis* into 1000 mL water, followed by soaking for 2 h, low heat for 1 h, filtering, adding 1000 mL additional water, and continued to be heated on low for 1 h. The liquid was concentrated to 2 g (crude drug)/mL for further use.

Detection of apoptosis via TUNEL staining

After rats in each group were sacrificed, the testis was isolated. The contralateral testis was taken, and the capsule was separated. Then the testis was sliced using a freezing microtome, placed on a glass slide, dried naturally, treated with 3% hydrogen peroxide already prepared for 10 min, and washed 3 times with phosphate-buffered saline (PBS). The following operations were performed according to instructions of the TUNEL staining kit (Beyotime Biotechnology Co.): Proteinase K solution was added dropwise to sections, placed in a wet box for digestion at 37°C for 10 min and washed with PBS for 3 times.

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The mixed solution of 40 µLTdT and DIG-d-UTP was added dropwise to sections, placed in the wet box for labeling at 4°C for 2 h, and washed 3 times with PBS. Then, sections were blocked with 40 µL blocking solution at room temperature for 30 min. Biotinylated anti-digoxin antibody (diluted at 1: 100) was added for incubation in the wet box at 37°C for 30 min and washed 3 times with PBS. The SABC-FITC secondary antibody (diluted at 1: 100) was added for reaction at 37°C for 30 min and washed 3 times with PBS. Then, sections were treated with anti-fluorescence quenching solution, followed by observation and photography under a confocal fluorescence microscope (Nikon, Japan). The ratio of TUNEL-positive cells in the contralateral testis to those in the undescended testis was calculated.

Detection of mitochondrial membrane potential

The contralateral testicular tissues of rats in each group were isolated and the mitochondrial membrane potential in tissues was detected using a mitochondrial membrane potential detection kit (Wuhan Boster Biological Technology Co.). The prepared JC-1 working solution was diluted 5 times, and 900 µL JC-1 diluted working solution was added into 100 µL of purified mitochondria (about 100 µg after purification). The anti-fluorescence quenching sealing solution was added dropwise, and the sections were assessed on a fluorescence microplate reader. The excitation and emission wavelength were 485 nm and 500 nm, respectively. Then, sections were observed and photographed under a laser confocal microscope. Red fluorescence indicated normal mitochondrial membrane potential, while green fluorescence indicated damaged mitochondrial membrane potential.

Detection of ROS level and MDA content

The ROS level in the testis was detected via 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) strictly according to instructions of the ROS detection kit (Beyotime Biotechnology Co.). In brief, testicular tissues of rats in each group were obtained, cut into pieces, and we added normal saline (100 mg: 1 mL), followed by tissue homogenization. After centrifugation at 10 000 g and 4°C for 15 min, the supernatant was taken for subsequent experiments. An appropriate amount of tissue supernatant was taken and added with 10 µM DCFH-DA for treatment in the dark at 37°C for 20 min, followed by washing with PBS and washing solution, and photography under a laser confocal microscope with excitation wavelength of 488 nm and emission wavelength of 525 nm.

The MDA content in tissues was detected strictly according to instructions of the MDA detection kit (Beyotime Biotechnology Co.). An appropriate amount of thiobarbituric acid (TBA) was taken and prepared into 0.37% TBA stock solution. An appropriate amount of standard sample was taken and diluted with distilled water to 1 µM, 2 µM, 5 µM, 10 µM, 20 µM, and 50 µM for making standard curves. Then, a 100 µL sample was placed into a centrifuge tube and we added 200 µL MDA working solution, mixed it evenly, heated it in a water bath at 100°C for 15 min, and cooled it to room temperature, followed by centrifugation at 1000 g for 10 min at room temperature. We added 200 µL supernatant into a 96-well plate, and the absorbance value was measured at 532 nm using a microplate reader and substituted into the standard curve to calculate the MDA content in a sample solution in each group. The MDA content per unit weight in the sample was measured.

Detection of apoptosis-associated proteins

Testicular tissues of rats in each group were obtained and treated with radioimmunoprecipitation assay (RIPA) lysis solution (100 mg: 1 mL), followed by tissue assessment after 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted at 80 V. Then, the protein was transferred onto a polyvinylidene fluoride (PVDF) membrane under constant pressure of 90 V for 100 min. The membrane was blocked using 5% freshly-prepared skim milk. After 2 h, Bax, Bcl-2, cleaved caspase-3, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies (purchased from Cell Signaling Technology, USA, diluted at 1: 1000) were added for incubation at 4°C overnight. It was then washed 3 times (5 min per time) with freshly-prepared Tris-buffered saline with Tween-20 (TBST) incubated with horseradish peroxidase-conjugated secondary antibody (Beyotime Biotechnology Co.) at room temperature for 1 h and washed again 3 times (5 min per time) with TBST. The luminescence solution was prepared for image development and exposure in a dark room. After scanning, the Bcl-2/Bax and cleaved caspase-3/GAPDH ratios were calculated using the analysis.

Statistical analysis

Data in this study are presented as mean ± standard deviation and analyzed using Statistical Product and Service Solutions (SPSS) 19.0 software (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used for statistical processing of data. After homogeneity testing of variance, Bonferroni's test was used in case of homogenous variance. $P<0.05$ suggested that the difference was statistically significant.

Results

Unilateral cryptorchidism-induced changes in the contralateral testis weight

At 90 days after modeling, rats in each group were sacrificed. The contralateral testis to the undescended testis was isolated

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and weighed after the capsule was peeled off. Results showed that at 90 days after modeling, the weight of the contralateral testis of rats in model group was significantly lighter than that in the control group ($P<0.01$), and *Cuscuta chinensis* treatment significantly elevated the weight of the contralateral testis compared to that in the model group ($P<0.05$) (Figure 1).

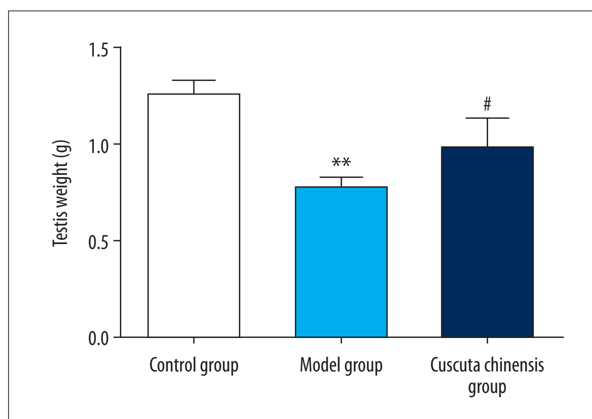

Figure 1. Weight of contralateral testis to the undescended testis in each group of rats. The testis weight of rats in the model group was significantly smaller than that in control group, and it was significantly larger in the *Cuscuta chinensis* group than in the model group (** $P<0.01$ *vs.* control group, # $P<0.05$ *vs. Cuscuta chinensis* group).

Unilateral cryptorchidism-induced changes in apoptosis of spermatogenic cells in the contralateral testis

Unilateral cryptorchidism-induced changes in the apoptosis of contralateral testicular spermatogenic cells of rats in each group were detected via TUNEL staining. Green fluorescence indicates the TUNEL-positive cells, and the number of positive cells refers to the level of cell apoptosis. Results revealed that at 90 days after modeling, the number of TUNEL-positive cells in the contralateral testis of rats in the model group was significantly increased ($P<0.01$), but was significantly decreased after administration of *Cuscuta chinensis* ($P<0.01$), suggesting that the apoptosis level of spermatogenic cells in the testis of rats induced in model group was reversed after treatment with *Cuscuta chinensis* (Figure 2).

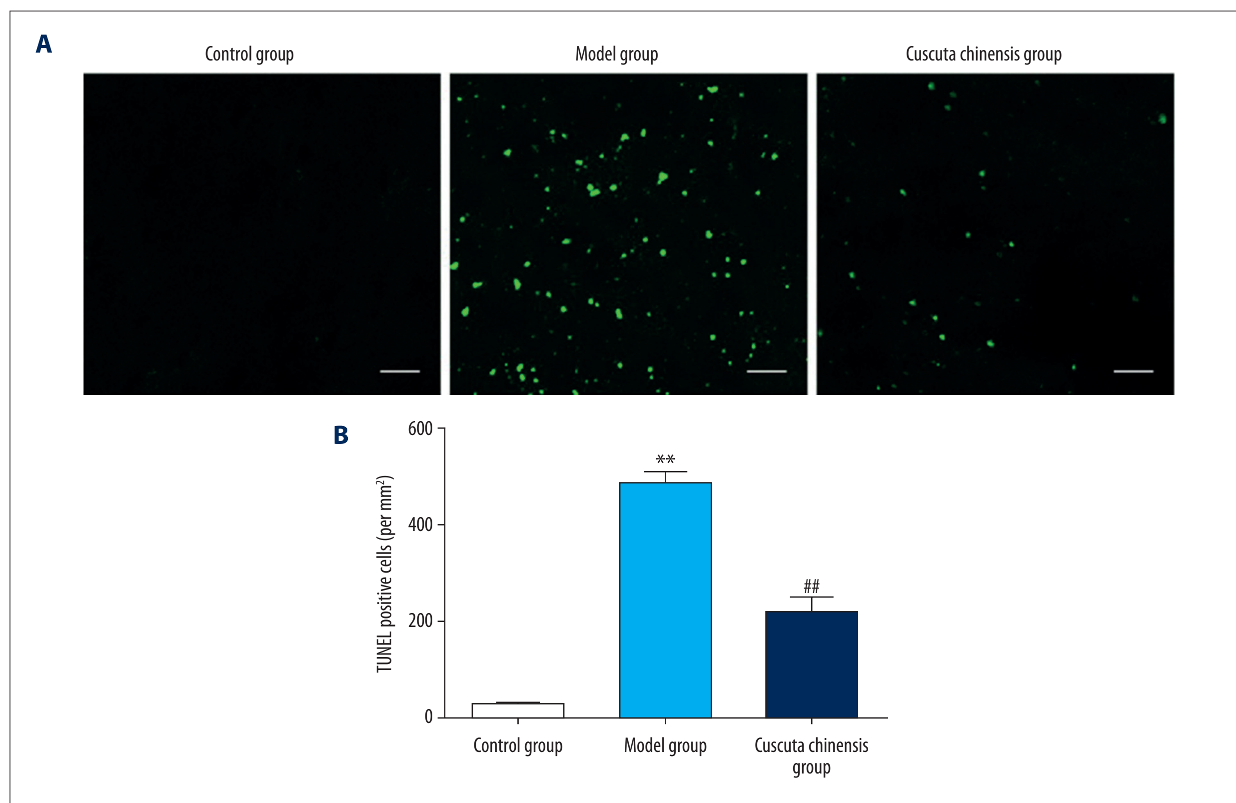

Figure 2. Detection of apoptosis level of contralateral testicular spermatogenic cells of rats in each group via TUNEL staining. (**A**) TUNEL staining micrographs; (**B**) statistical graph. The apoptosis level of testicular cells of rats in the model group was significantly higher than in the control group, and it was significantly lower in the *Cuscuta chinensis* group than in model group (** $P<0.01$ *vs.* control group, ## $P<0.01$ *vs. Cuscuta chinensis* group. Bar=100 μm).

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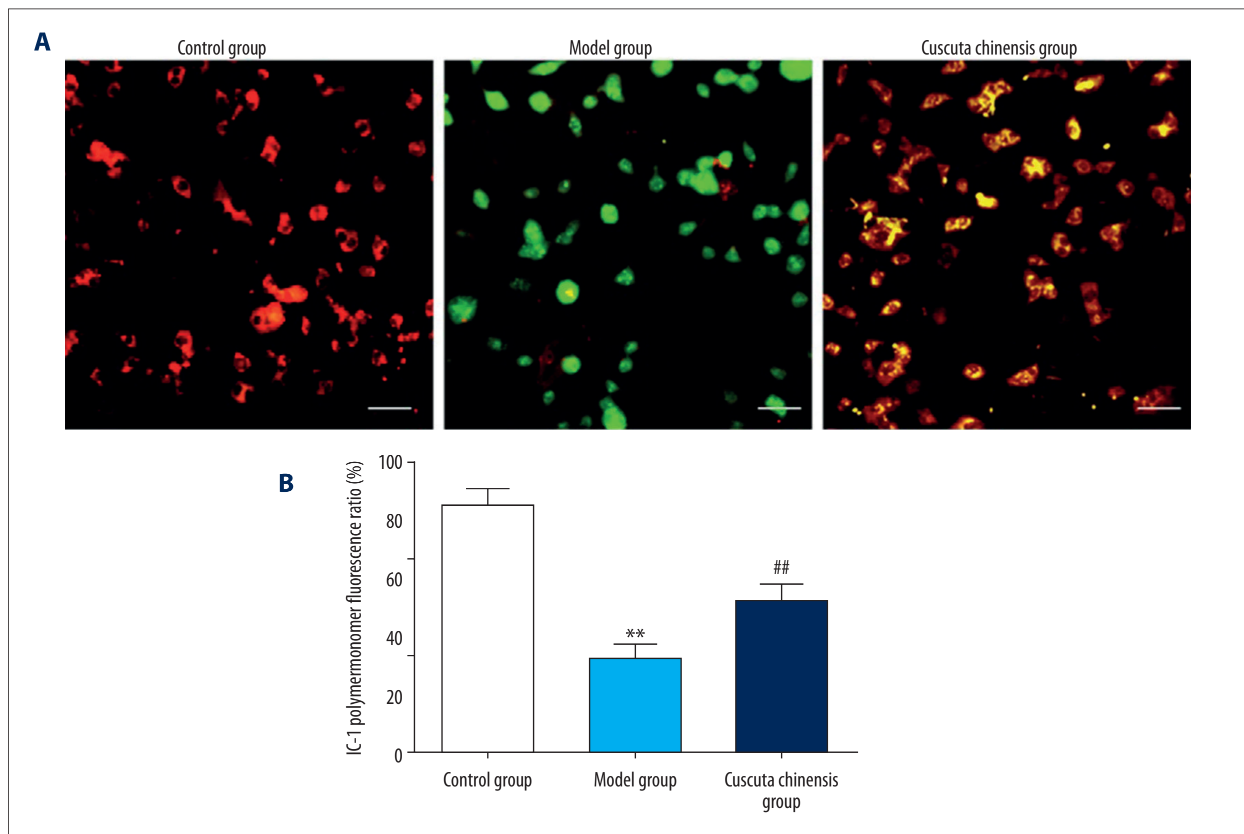

Figure 3. Detection of mitochondrial membrane potential in testicular cells of rats in each group via JC-1. (**A**) JC-1 micrographs; (**B**) statistical results. The mitochondrial membrane potential in the model group was obviously lower than in the control group, and it was obviously higher in the *Cuscuta chinensis* group than in the model group (***P*<0.01 *vs.* control group, ## *P*<0.01 *vs. Cuscuta chinensis* group. Bar=100 μm).

Changes in mitochondrial membrane potential in the contralateral testis of rats in each group

We then evaluated the mitochondrial membrane potential in the contralateral testis. Red fluorescence indicates JC-1-positive cells and suggests normal mitochondrial membrane potential in cells, while green fluorescence refers to the decline of the mitochondrial membrane potential. We found that the number of JC-1-positive cells in the contralateral testis of rats was significantly lower in the model group than that in the control group (*P*<0.01). However, the administration of *Cuscuta chinensis* significantly increased the number of JC-1-positive cells (*P*<0.01), showing that *Cuscuta chinensis* can effectively ameliorate the decrease of the mitochondrial membrane potential in testicular cells (Figure 3).

Changes in the ROS level and MDA content in the contralateral testicular cells of rats in each group

The ROS and MDA levels in testicular cells were detected using the ROS detection kit. Results showed that at 90 days after modeling, the contents of ROS and MDA in testicular cells of rats in the model group was obviously higher than in the control group (*P*<0.01), and this change was significantly retarded after administration of *Cuscuta chinensis* (*P*<0.01) (Figures 4, 5).

Changes in apoptosis-associated proteins in contralateral testicular cells of rats in each group

Expression levels of apoptosis-associated proteins in testicular cells of rats in each group were detected via Western blotting. Results showed that at 90 days after modeling, the Bcl-2/Bax ratio in testicular cells of rats in the model group was significantly lower than in the control group (*P*<0.01), but cleaved caspase-3 was significantly higher than in the control group (*P*<0.01). However, the administration of Cuscuta chinensis significantly increased the Bcl-2/Bax ratio in testicular cells of model rats, with significant down regulation of cleaved caspase-3 (*P*<0.01) (Figure 6).

Discussion

Cryptorchidism, a type of congenital malformation, can directly affect the number and function of spermatogenic cells in the

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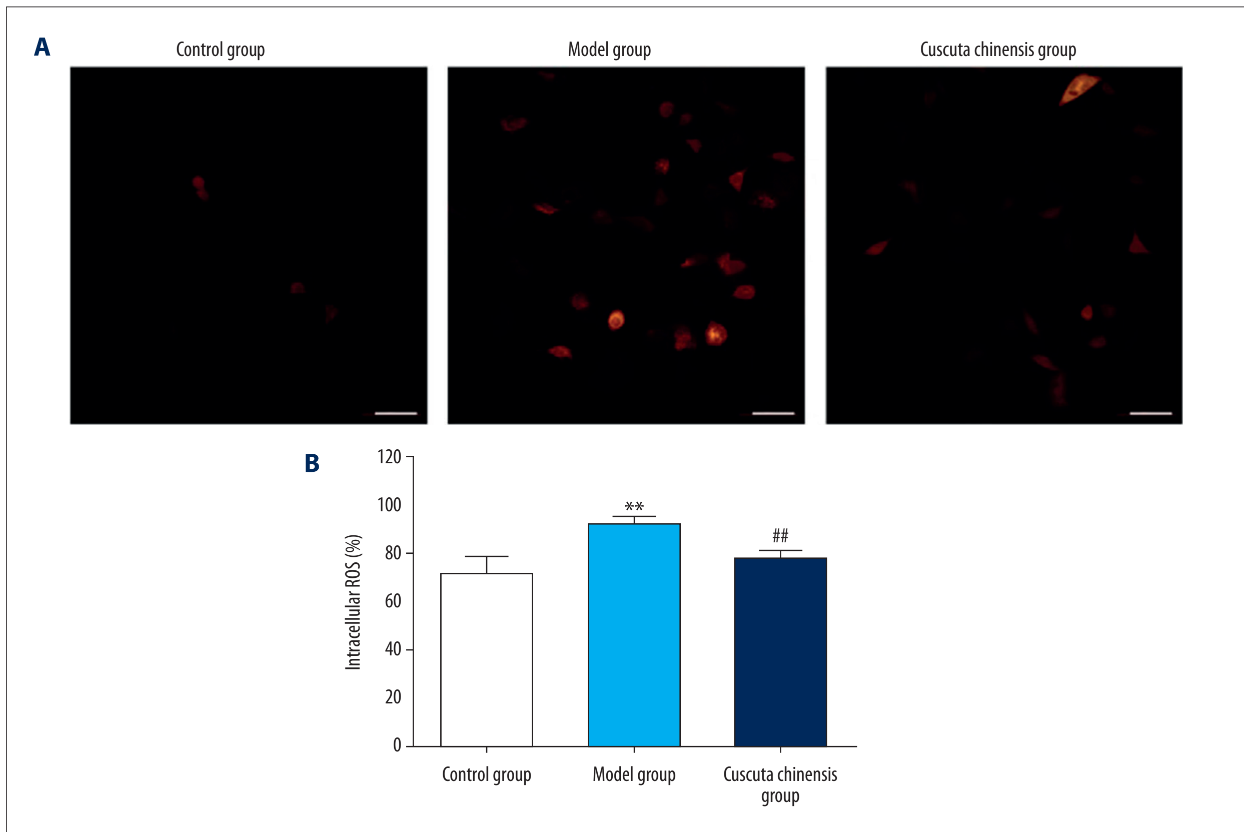

Figure 4. ROS level in testicular cells of rats in each group. (**A**) Micrographs; (**B**) statistical graph. The ROS level in testicular cells of rats in the model group was significantly higher than in the control group, and it was significantly lower in the *Cuscuta chinensis* group than in model the group (***P*<0.01 *vs.* control group, ## *P*<0.01 *vs. Cuscuta chinensis* group. Bar=100 μm).

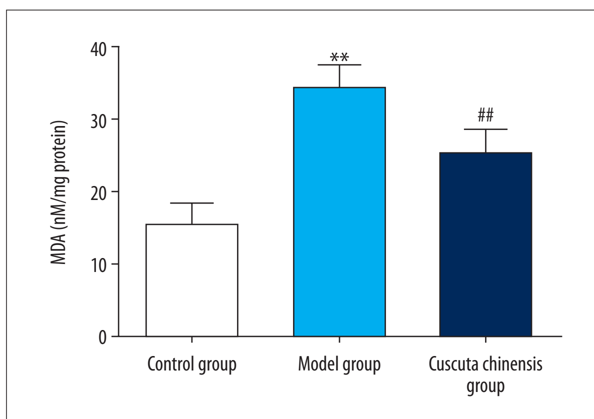

Figure 5. MDA content in testicular cells of rats in each group. Statistical graph. The MDA content in testicular cells of rats in the model group was significantly higher than in the control group, and it was significantly lower in the *Cuscuta chinensis* group than in the model group (***P*<0.01 *vs.* control group, ## *P*<0.01 *vs. Cuscuta chinensis* group).

testis, the onset of which can lead to infertility, with an incidence rate of more than 50% [13]. Hornakova et al. [14] found that unilateral cryptorchidism leads to damage in contralateral testis, with rising apoptosis levels of germ cells and pathological changes in the cell ultrastructure. It also results in the thinning of spermatogenic epithelium, and destroys the normal function of spermatogenic cells. According Aydin et al. [15], the contralateral testicular injury is a kind of internal damage during the progression of unilateral cryptorchidism, which is a disease frequently occurring in neonates. Unilateral cryptorchidism also causes endocrine disorders in the body, affecting the secretion of sex hormone and occurrence of allergic reaction. Apoptosis of spermatogenic cells is an important cause of the subsequent development of cryptorchidism. The abnormal environment in the undescended testis has harmful effects on the genitofemoral nerve, and induces changes in blood circulation and microenvironment in the contralateral testis [16].

In this study, the model of unilateral cryptorchidism was established in rats aged 21 days. At 90 days after modeling, the apoptosis of contralateral testicular spermatogenic cells of rats was evaluated. Results demonstrated that in the model group, the weight of the contralateral testis was significantly

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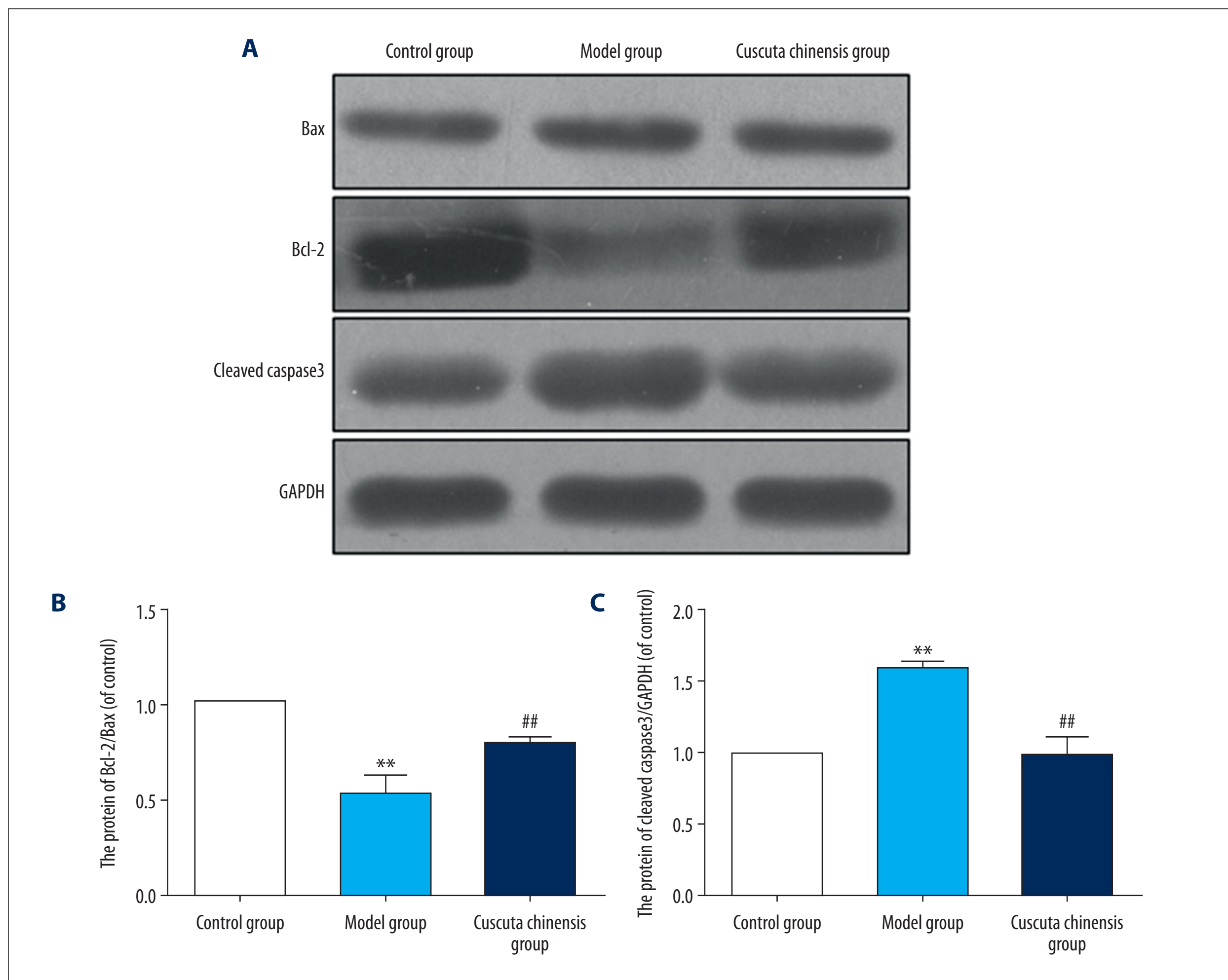

Figure 6. Detection of Bcl-2, Bax, and cleaved caspase-3 protein expression levels in the contralateral testis compared to the undescended testis of rats via Western blotting. The Bcl-2/Bax ratio in testicular cells of rats in the model group was significantly lower than in the control group, but cleaved caspase-3 was significantly higher than that in the control group. The Bcl-2/Bax ratio in testicular cells of rats in the *Cuscuta chinensis* group was significantly higher than in the model group, but cleaved caspase-3 was significantly lower than in the model group (** P<0.01 *vs.* control group, ## P<0.01 *vs. Cuscuta chinensis* group).

reduced and the number of apoptotic cells was significantly increased, indicating that unilateral cryptorchidism impairs the function of spermatogenic cells. Consistent with previous finding that the weights of the reproductive organs, as well as the sperm count and motility, were improved by *Cuscuta chinensis* Lam, we found that the treatment also retarded the damage caused by unilateral cryptorchidism [17]. The most common mechanism of apoptosis in animal tissues and cells is mediated by the mitochondrial pathway. A variety of apoptosis-stimulating factors act on cells and changes the expression of various apoptosis-associated proteins, such as reduction of anti-apoptotic proteins (e.g., Bcl-2 and Bcl-xl) and upregulation of pro-apoptotic proteins (e.g., Bax and Bak) [18]. At the same time, the mitochondrial membrane potential in cells is significantly reduced, leading to dramatic increase of cytochrome C (Cyt C) in the cytoplasm, which was released by mitochondria. Moreover, Cyt C can further interact with apoptotic protease, activating factor 1 (Apaf-1) and caspase-9 to produce apoptotic bodies and promote the expressions of a variety of apoptosis-executing proteins, such as caspase-3, caspase-6, and caspase-7, and causing apoptosis [19]. We also assessed changes in mitochondrial membrane potential, showing that the mitochondrial membrane potential in the contralateral testicular tissues of rats in the model group was significantly reduced, with higher levels of both ROS and MDA. Expressions of apoptosis-associated proteins were detected via Western blotting, showing that in the model group, Blc-2/Bax in the contralateral testis to the undescended testis was significantly decreased, but the expression of cleaved caspase-3 was significantly increased, suggesting that the unilateral cryptorchidism model

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activates the apoptotic pathway and significantly increases the apoptosis level in the contralateral testis. By contrast, our data indicate that *Cuscuta chinensis* counteracted the effect of unilateral cryptorchidism by reducing ROS, MDA, and cleaved caspase-3 and elevating the Blc-2/Bax ratio. These results agree with previous findings in cardiomyocytes that *Cuscuta chinensis* contributes to inhibition of apoptosis by regulating the mitochondrial apoptosis pathway and has hepatoprotective and antioxidant effects by reducing MDA levels [20,21].

Cuscuta chinensis possesses a potent anti-oxidation effect and plays an important role in the reproductive system. In this study, *Cuscuta chinensis* was decocted using the traditional extraction method, and previous studies indicated that *Cuscuta chinensis* treatment mildly promoted the proliferation of MG-63 cells at doses of 500 and 1000 microg/mL in the 24-h culture period, suggesting an important role in osteoblastic bone formation. The ethanol extract of *Cuscuta chinensis*, but not its water extract, was believed to serve as a dietary nutritional supplement to promote human health and prevent oxidation-related diseases, due to its antioxidant properties [22,23]. Our data showed that the administration of *Cuscuta chinensis* increased the weight of the contralateral testis of model rats, reduced apoptosis of spermatogenic cells in the testis, elevated mitochondrial membrane potential, and reduced ROS level, MDA content, and cleaved caspase-3 expression, suggesting that *Cuscuta chinensis* displays a strong antagonistic effect against unilateral cryptorchidism through downregulating unilateral cryptorchidism-induced apoptosis of spermatogenic cells in the contralateral testis, which provides fundamental directions for further clinical therapy. A limitation of this study is that genetic and endocrine factors should also be included in the evaluation of *Cuscuta chinensis* on unilateral cryptorchidism for further clinical use, such as the density and distribution of single-nucleotide polymorphisms (SNPs) anti-Mullerian hormone (AMH) and AMHRII receptors in cryptorchid patients, and determine potential hormone imbalance connected with undescended testes [24,25]. A recent study using a mouse model revealed that Cuscuta species exhibited a protective activity against memory deficit, cholinergic dysfunction, oxidative damage, and neuroinflammation, and combined with the advanced extraction method, it is promising to utilize active ingredients with high quality and purity for the development of its clinical application, for instance, in the treatment of hepatic, reproductive disease and renal failure, in humans [26,27]. Further pharmacokinetic and toxicological research on human subjects is needed to validate its efficacy and safety in further clinical uses.

Conclusions

Cuscuta chinensis exerts an anti-apoptotic effect on the unilateral cryptorchidism-induced apoptosis of spermatogenic cells in the contralateral testis, and also ameliorates the damage of unilateral cryptorchidism by influencing the mitochondrial pathway.

Conflict of interest

None.

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