

Original Research Article

Fertility-enhancing potential of ethanol extract of *Cuscuta chinensis* seeds in a rat model of unilateral cryptorchidism

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Abstract

Purpose: To investigate the fertility-enhancing potential of the ethanol extract of *Cuscuta chinensis* seeds in a rat model of unilateral cryptorchidism (ULC), and the mechanism(s) of action.

Methods: Healthy male Sprague Dawley rats ($n = 48$; mean weight = 220 ± 10 g) were randomly assigned to 4 groups (12 rats/group): control, ULC, 100 mg extract/kg and 200 mg extract/kg groups. Unilateral cryptorchidism of right testis was induced via standard method using an operating microscope. Rats in the treatment groups received 100 and 200 mg of ethanol extract of *Cuscuta chinensis*/kg orally once a day for 60 days. Sperm count and sperm motility were determined in seminal vesicular fluid (SVF) suspension. Oxidative stress markers and histological changes in rat testis were evaluated. The levels of caspase-3 and caspase-9 in testicular tissue were assessed by enzyme-linked immunosorbent assay (ELISA), while the protein expressions of Nrf2 and heme oxygenase 1 (HO-1) were determined using Western blotting.

Results: Body and reproductive organ weights, sperm count, sperm motility, and activities of glutathione peroxidase (GPx), catalase and superoxide dismutase (SOD) were significantly reduced in ULC group, relative to control group, but these parameters were significantly and dose-dependently increased following extract treatment ($p < 0.05$). Malondialdehyde (MDA), 8-hydroxy-2'-deoxyguanosine (8-OHdG), caspase-3 and caspase-9 levels were significantly higher in ULC group than in control group, but they were reduced significantly and dose-dependently after extract treatment ($p < 0.05$). Moreover, the protein expressions of Nrf2 and HO-1 were significantly downregulated in ULC group, when compared with control group, but they were significantly and dose-dependently upregulated by the extract ($p < 0.05$). Cross sections of testicular tissues of rats in ULC group revealed narrowed and thickened seminiferous tubules (disrupted spermatogonia) characterized by increased apoptotic bodies (increased number of necrotic Sertoli and Leydig cells). However, there were few damaged or necrotic Sertoli and Leydig cells, and complete absence of thickening of seminiferous tubules in testicular tissues of rats treated with the extract.

Conclusion: The ethanol extract of *Cuscuta chinensis* seeds effectively mitigates cryptorchidism in rats via mechanisms involving the regulation of Nrf2/HO-1 signaling pathway, and inhibition of apoptosis and oxidative stress. Thus, the plant extract has potentials for further development for the management of male infertility

Keywords: *Cuscuta chinensis*, Cryptorchidism, Apoptosis, Antioxidant enzymes, Sperm motility, Testis

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INTRODUCTION

Inability to conceive after one year of unprotected sexual intercourse (infertility) results in low birth rate. Infertility is a major global issue which impacts negatively on patients and their family members [1]. In China, the prevalence of infertility among newly married couples ranges from 8 - 13.6 % [2]. Cryptorchidism or undescended testis (a birth defect), genetic disorders (hormonal imbalance/insufficiency), infection, duct obstruction, varicocele, excessive alcohol consumption, and smoking are important factors that contribute to male infertility [3,4]. Surgically-induced cryptorchidism reversibly damages seminiferous epithelium/tubules (germ cells) via induction of oxidative stress and apoptosis, thereby adversely affecting spermatogenesis [5]. Animal model of cryptorchidism remains the best model for assessment of clinical effectiveness of synthetic and natural aphrodisiac drugs [6,7]. Studies have shown that supplementation with antioxidants significantly improve overall sperm quality, thereby enhancing fertility [4,8,9]. *Cuscuta chinensis* Lam. (Chinese Dodder/*Tu-Si-Zi*), a parasitic plant belonging to the *Convolvulaceae* family, is used in Traditional Chinese Medicine (TCM) as an analgesic, and for improving sexual, liver and kidney functions [10]. The plant seeds exhibit a broad spectrum of pharmacological properties such as antioxidant, anti-inflammatory, anti-aging, anti-depressant, and anticancer effects [11-13]. Administration of herbal formulation rich in *Cuscuta chinensis* (KH-204) has been shown to significantly reduce the levels of oxidative stress, heat shock proteins, and germ cell apoptosis, while improving sexual function [14]. Flavones isolated from *Cuscuta chinensis* seeds significantly increased testosterone and protein levels in a mouse model of infertility [15]. Similarly, *Cuscuta chinensis* flavonoids have been reported to significantly improve sperm count and sperm motility in mice [16]. This study investigated the fertility-enhancing effect of ethanol extract of *Cuscuta chinensis* seeds on rat model of ULC.

EXPERIMENTAL

Materials

Glutathione peroxidase (GPx), catalase, SOD and MDA assay kits were products of Sangon Biotechnology (China). Light microscope (E50) was bought from Olympus Co. Ltd. (Japan). Phenol-free DNeasy tissue kit was purchased from Qiagen (USA) and 8-OHgdG ELISA kit was product of Abcam (UK). Caspase-3 and caspase-9 ELISA kits were obtained from Beyotime

Biotech (China). Nuclear/cytosolic fractionation kit was bought from Cell Biolabs Inc. (USA). Primary antibodies for Nrf2, HO-1, histone H3 and β -actin were products of Santa Cruz Biotechnology (USA). Enhanced chemiluminescence (ECL) detection system was purchased from Bio-Rad Laboratories (USA). Horseradish peroxidase (HRP) anti-rabbit IgG antibody was bought from Cell Signaling Technology (USA), while image analyzing software was obtained from Amersham Pharmacia Biotech (UK).

Preparation of ethanol extract of *Cuscuta chinensis* seeds

The seeds of *Cuscuta chinensis* Lam. were obtained from a traditional Chinese drug store in Wuhan, China, and authenticated by Dr. Chang Wang of the Department of Botany, Wuhan University. A herbarium specimen (voucher no. DCWU-12/53/2010) was prepared and deposited in the herbarium of the Department of Botany, Wuhan University. The plant seeds were shade-dried and pulverized using an electric blender. Extraction was done using standard methods [17]. A portion of the powder (500 g) was exhaustively extracted with 1000 mL of 95 % ethanol in a Soxhlet apparatus. The extract was concentrated using a vacuum rotatory evaporator, and the resultant concentrate was freeze-dried by lyophilization. The dried ethanol extract (7.44 %, w/w) was stored at - 20 °C prior to use.

Determination of total phenolic and flavonoid contents of the extract

Total phenolic and flavonoid contents of ethanol extract of *Cuscuta chinensis* seeds were determined using previously described methods [18, 19]. Total phenolic content was expressed as mg gallic acid equivalent (GAE)/g dry extract, while flavonoid content was expressed as mg quercetin equivalent (QE)/g dry extract.

Experimental rats

Healthy male Sprague Dawley rats (n = 48) weighing 210 – 230 g (mean weight = 220 \pm 10 g) were purchased from Wuhan University of Science and Technology. The rats were housed in polycarbonate cages under standard conditions and allowed free access to standard feed (rat chow) and water. They were exposed to 12-h light/12-h dark cycle, and maintained at an average temperature of 22 \pm 1 °C and 40 – 50 % humidity. The rats were acclimatized to the laboratory conditions for 10 days before commencement of study. The study protocol was

approved by the Institutional Animal Ethics Committee of Wuhan University of Science and Technology (approval no. WU-21-AE20). The study procedures were carried out in strict adherence to the Guide for Care and Use of Laboratory Animals, National Academic Press (USA).

Animal grouping and treatments

After the acclimatization period, the rats were randomly assigned to 4 groups (12 rats/group): control, ULC, 100 mg extract/kg and 200 mg extract/kg groups. Unilateral cryptorchidism of right testis was induced via standard method using an operating microscope [20]. The rats were anesthetized with intraperitoneal injection (i.p) of sodium pentobarbital at a single dose of 45 mg/kg after which inguinal incision of 2 cm was made on the right testis of each rat to reveal the spermatic cord. The testis was slowly retracted to the posterior abdominal wall and sutured with 4/0 nylon to the *tunica albuginea* to prevent its movement back to the scrotal sac. Rats in the treatment groups received 100 and 200 mg of ethanol extract of *Cuscuta chinensis*/kg orally once a day for 60 days.

Tissue sample preparation

At the end of the treatment period (on the 61st day), the rats were weighed and euthanized via cervical dislocation under mild ethyl ether anesthesia (20 mg/kg). Both testicle, cauda epididymis, and seminal vesicles were immediately excised, washed with phosphate-buffered saline (PBS) and weighed. A portion of excised testis was homogenized with PBS to prepare 10 % tissue homogenate which was centrifuged at 3000 rpm for 10 min at 4 °C. Biochemical analyses were performed on the supernatant.

Semen analysis

The distal end of caudal epididymis was minced with physiological saline and shaken vigorously on an agitator at 37 °C for 15 min to separate sperm/spermatozoa suspension from SVF. Sperm count and sperm motility were determined using SVF suspension [21]. Sperm count was expressed as millions/mL of SVF suspension, while sperm motility calculated as percentage of motile sperms in total sperm count.

Measurement of oxidative status in rat testis

The activities of GPx, catalase, and SOD, and levels of testicular MDA were determined using their assay kits.

Determination of 8-OHdG levels

Deoxyribonucleic acid (DNA) was extracted from SVF using phenol-free DNeasy tissue kit and then digested. The level of 8-OHdG in the digest was determined with ELISA.

Determination of testicular cell apoptosis

The activities of caspase-3 and caspase-9 in testicular tissue homogenate were assayed using their respective ELISA kits.

Western blotting

Cytosolic and nuclear fractions of testicular tissue homogenate were lysed with ice-cold radio-immunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors (PMSF). The resultant lysate was centrifuged at 15,000 rpm for 15 min at 4 °C, and the protein concentration of the supernatant was determined using bicinchoninic acid (BCA) protein assay kit. A portion of total cell protein (50 µg) from each sample was separated on 12 % sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to a fixed polyvinylidene difluoride membrane at 110 V and 90 °C for 2 h. Subsequently, the membrane was incubated with non-fat milk solution (5 %) in Tris-buffered saline containing 0.2 % Tween-20 (TBS-T), with gentle shaking at 37 °C to block non-specific binding of the blot. Thereafter, the blots were incubated overnight at 4 °C with primary antibodies for Nrf2, HO-1, histone H3 and β-actin, each at a dilution of 1 to 1000. Then, the membrane was washed thrice with TBS-T, and further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 45 min at room temperature. The blot was developed using Enhanced Chemiluminescence (ECL) detection system. Grayscale analysis of the bands was performed using ImageJ Launcher software. The respective protein expression levels were normalized to that of β-actin which served as standard.

Histopathological examination of rat testis

A portion of decapsulated testis (right) was fixed in 10 % formaldehyde. The formaldehyde-fixed testicular tissue was washed in water to remove excess fixative and then dehydrated using graded concentrations of ethyl alcohol. It was then cleared with xylene, and thereafter embedded in paraffin. Tissue sections (5-µm thick) were made using an ultra-microtome. The sections were stained with hematoxylin and eosin (H&E) for 12 h at room temperature according to standard methods, and examined

under a light microscope. Morphological changes in rat testis were recorded and analyzed with ImageJ analysis software.

Statistical analysis

Data are expressed as mean \pm SEM. Statistical analysis was performed with SPSS version 20. Groups were compared using Dunnett's multiple comparison test. Values of $p < 0.05$ were taken as indicative of statistically significant differences.

RESULTS

Total phenolic and flavonoid contents of ethanol extract of *Cuscuta chinensis* seeds

The results of quantitative phytochemical screening showed that total phenolic content of the extract was significantly higher than the total flavonoid content ($p < 0.05$; Table 1).

Effect of extract treatment on body and organ weights

As shown in Table 2, body and reproductive organ (testis, seminal vesicle and epididymis) weights were significantly reduced by ULC, but they were significantly and dose-dependently

Table 1: Phytochemical profile of *Cuscuta chinensis* seeds

Phytochemical	Concentration
Total phenols	48.10 mg GAE/g
Total flavonoids	24.20 mg QE/g

increased by ethanol extract of *Cuscuta chinensis* seeds ($p < 0.05$).

Effect of extract treatment on sperm quality

Sperm count and sperm motility were significantly lower in ULC group than in control group, but these parameters were significantly and dose-dependently increased by extract treatment ($p < 0.05$). These results are shown in Table 3.

Effect of extract treatment on testicular oxidative status

The activities of GPx, catalase and SOD were reduced in ULC group, relative to control group, but they were significantly and dose-dependently increased by extract treatment ($p < 0.05$; Table 4). Malondialdehyde (MDA) and 8-OHdG levels were significantly higher in ULC group than in control group, but they were markedly and dose-dependently reduced by ethanol extract of *Cuscuta chinensis* seeds ($p < 0.05$; Figure 1).

Table 2: Changes in body and reproductive organ weights

Group	Change in body weight (g)	Testis weight(g)	Seminal vesicle weight (g)	Epididymis weight (g)
Control	42.23 \pm 2.60	1.87 \pm 0.10	0.43 \pm 0.03	0.45 \pm 0.04
ULC	36.20 \pm 1.72 ^a	1.34 \pm 0.08 ^a	0.30 \pm 0.02 ^a	0.28 \pm 0.03 ^a
Extract (100 mg/kg)	38.30 \pm 1.80 ^b	1.55 \pm 0.15 ^b	0.35 \pm 0.04 ^b	0.35 \pm 0.04 ^b
Extract (200 mg/kg)	39.70 \pm 2.05 ^b	1.68 \pm 0.10 ^b	0.39 \pm 0.03 ^b	0.41 \pm 0.05 ^b

^a $P < 0.05$ compared with control group; ^b $p < 0.05$ compared with ULC group

Table 3: Changes in sperm quality

Group	Sperm count (million/mL)	Sperm motility (%)
Control	80.92 \pm 7.44	81.56 \pm 9.73
ULC	52.75 \pm 5.82 ^a	56.52 \pm 5.33 ^a
Extract (100 mg/kg)	65.21 \pm 4.03 ^b	70.69 \pm 8.28 ^b
Extract (200 mg/kg)	73.55 \pm 7.35 ^b	75.55 \pm 9.44 ^b

^a $P < 0.05$, compared with control group; ^b $p < 0.05$, compared with ULC group

Table 4: Oxidative status of the treatment groups

Group	GPx (μ g/mg protein)	Catalase (U/mg protein)	SOD (U/mg protein)
Control	8.12 \pm 1.00	14.10 \pm 1.80	6.27 \pm 1.10
ULC	5.08 \pm 0.44 ^a	10.52 \pm 1.63 ^a	4.04 \pm 0.82 ^a
Extract (100 mg/kg)	6.65 \pm 0.82 ^b	12.20 \pm 1.11 ^b	4.97 \pm 0.60 ^b
Extract (200 mg/kg)	7.30 \pm 0.92 ^b	13.09 \pm 1.58 ^b	5.40 \pm 0.75 ^b

^a $P < 0.05$, compared with control group; ^b $p < 0.05$, compared with ULC group. One unit (U) of catalase activity was defined as the amount of enzyme required to decompose 1 μ mol of H₂O₂ per min. One unit of SOD activity was equivalent to the amount of enzyme required for 50 % inhibition of xanthine oxidase

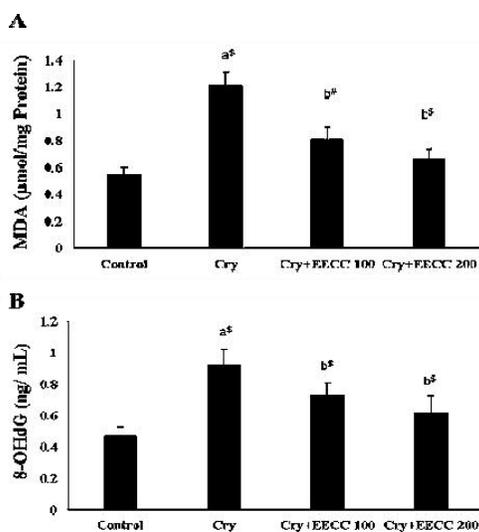


Figure 1: Levels of MDA and 8-OHdG in rats. **(A):** Levels of MDA; **(B):** Levels of 8-OHdG. ^a*P* < 0.05, compared with control group; ^b*p* < 0.05, compared with ULC group

Effect of extract on the protein expressions of apoptosis-related genes

As shown in Figure 2, the levels of caspase-3 and caspase-9 were significantly higher in ULC group than in control group, but they were reduced significantly and dose-dependently by extract treatment (*p* < 0.05).

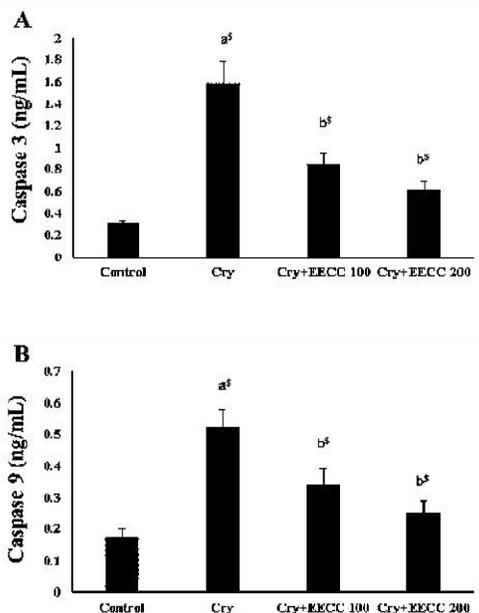


Figure 2: Effect of extract on testicular cell apoptosis. **(A):** Caspase-3 levels; and **(B):** Levels of caspase-9. ^a*P* < 0.05, compared with control group; ^b*p* < 0.05, compared with ULC group

Effect of extract treatment on expression levels of Nrf2 and HO-1

The protein expressions of Nrf2 and HO-1 were significantly downregulated in ULC group, when compared with control group, but they were significantly and dose-dependently upregulated by ethanol extract of *Cuscuta chinensis* seeds (*p* < 0.05; Figure 3).

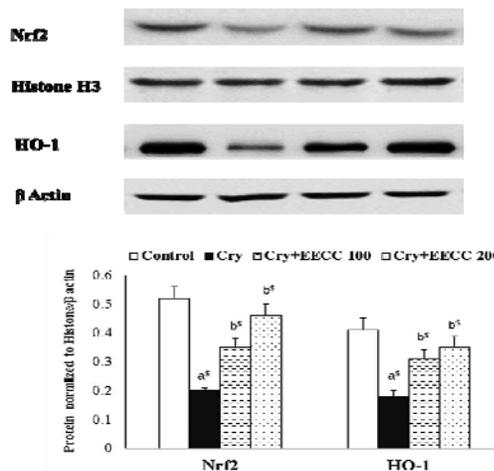


Figure 3: Protein expressions of Nrf2 and HO-1 in rat testis. ^a*P* < 0.05, compared with control group; ^b*p* < 0.05, compared with ULC group

Morphological changes in rat testis

The results of H&E staining showed that rats in control group exhibited normal histology of testicular tissue. Cross section of testicular tissues of rats in ULC group revealed narrowed and thickened seminiferous tubules (disrupted spermatogonia) characterized by increased apoptotic bodies (increased number of necrotic Sertoli and Leydig cells). Testicular tissues of rats treated with 100 mg extract/kg bwt showed less thickening of seminiferous tubules and reduced number of apoptotic bodies. There were few damaged or necrotic Sertoli and Leydig cells and complete absence of thickening of seminiferous tubules in testicular tissues of rats treated with 200 mg extract/kg bwt. These results are shown in Figure 4.

DISCUSSION

Cryptorchidism, a common birth defect in males, is caused by the failure of testis to descend from the abdominal region to the scrotal sac [7]. Unilateral or bilateral cryptorchidism accounts for 4 - 8 % of all known cases of male infertility. A strong correlation exists between the incidence of cryptorchidism and testicular cancer [22].

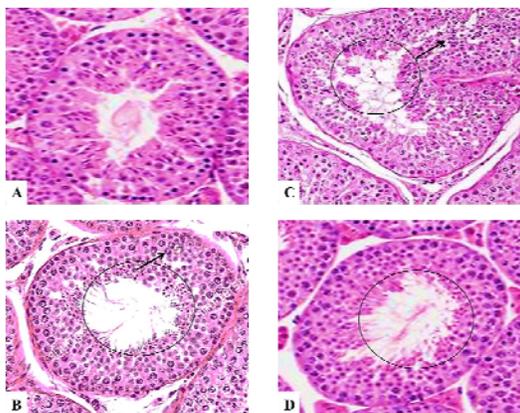


Figure 4: Histopathological features of rat testis. (A): H&E staining of testicular tissues of rats in control group; (B): results of H&E staining of testicular tissues of rats in ULC group; (C): results of histopathological examination of testicular tissues of rats treated with 100 mg extract/kg; and (D): results of histopathological examination of testicular tissues of rats treated with 200 mg extract/kg

At present, there is need for novel and effective anti-cryptorchidism drugs. This study investigated the aphrodisiac effect of ethanol extract of *Cuscuta chinensis* seeds on a rat model of infertility. Two doses were chosen based on results of preliminary dose-response study. Cryptorchidism was induced only in the right testis, thereby allowing the left testis to produce normal levels of sex hormone and spermatozoa.

The results showed that body and reproductive organ weights were markedly reduced in ULC group, relative to the control group. However, extract treatment for 60 days significantly increased the body and reproductive organ weights in a dose-dependent fashion. These results suggest that ethanol extract of *Cuscuta chinensis* seeds may effectively ameliorate ULC-induced testicular damage in rats. The effect of the crude drug may not be unconnected with its antioxidant and anti-apoptotic properties [11,12]. Quantitative phytochemical analysis revealed the presence of high amounts of phenolics and flavonoids in ethanol extract of *Cuscuta chinensis* seeds. Similarly, High Performance Liquid Chromatography (HPLC) revealed the presence of quercetin, hyperoside and kaempferol in the extract (data not shown).

In cryptorchidism rats, sperm count and sperm motility were significantly reduced, but treatment with graded doses of the extract significantly enhanced the sperm quality, as indicated by increased sperm count and sperm motility. Reduction in sperm quality and secretion of sex hormones are thought to be caused by negative

response of the hypothalamus-pituitary axis (HPA). Studies have shown that flavonoids isolated from *Cuscuta chinensis* seeds enhanced the secretion of endocrine hormones (estrogenic effect) via stimulation of HPA [16,23]. In this study, the effect of ethanol extract of *Cuscuta chinensis* seeds on sperm quality, secretion of sex hormones (testosterone and luteinizing hormone) and spermatogenesis could have occurred via stimulation of HPA.

Sperm cell membrane is rich in polyunsaturated fatty acids (PUFAs), phospholipids (PLs) and mitochondria, making it highly susceptible to oxidative stress-induced damage. Oxidative stress results in high levels of lipid peroxidation product (MDA) and DNA-damage product (8-OHdG) [1,3]. Cell apoptosis, reduction in levels of endogenous antioxidants and increased production of lipid peroxidation and DNA damage products contribute to oxidative stress in cryptorchidism rats, and ultimately to infertility [9,20,24].

In this study, the activities of GPx, catalase and SOD in rat testis were significantly decreased in ULC group, relative to control group, while the levels of MDA and 8-OHdG were markedly increased in ULC group, when compared with control group. However, treatment with ethanol extract of *Cuscuta chinensis* seeds significantly and dose-dependently increased the activities of the antioxidant enzymes, but reduced the levels of MDA and 8-OHdG. These results indicate that the extract may possess good antioxidant and anti-apoptotic effects, and are in agreement with those of previous studies [12]. The results of a previous study showed that treatment with a herbal formula rich in *Cuscuta chinensis* (KH-204) significantly enhanced antioxidant levels, while decreasing the level of 8-OHdG in rats with cryptorchidism [14]. The activities of caspase-3 and caspase-9 were significantly higher in ULC group than in control group, but they were significantly reduced by extract treatment.

Cryptorchidism results in over-production of free radicals which trigger oxidative damage in testis via activation of apoptotic cascade. The oxidative stress disrupts mitochondrial membrane, leading to the release of cytochrome c and subsequent activation of the caspase system (especially caspase-3 and caspase-9) [15]. Treatment of ULC rats with graded doses of ethanol extract of *Cuscuta chinensis* seeds significantly decreased the activities of caspase-3 and caspase-9, an indication that the extract may possess anti-apoptotic effect. Studies have shown that polysaccharides isolated from the seeds of *Cuscuta chinensis* significantly decreased the

intracellular Ca^{2+} level and regulated the release of caspases in a rat model of ULC [25].

The Nrf2/HO-1 signaling pathway is a major signaling pathway that regulates redox homeostasis and cell integrity. Under physiological conditions, Nrf2 is localized in the cytosol bound to Keap1 in an inactive form. On stimulation by oxidative stress or pathological conditions, Nrf2 becomes activated (dissociates from Keap1), translocates to the nucleus and becomes bound to antioxidant response element (ARE). While in the nucleus, it regulates the expression of various antioxidant and detoxification enzyme genes such as HO-1, glutathione, SOD, γ -glutamylcysteine synthetase (γ GCS), catalase, and NADPH:quinone oxidoreductase 1 (NQO-1), thereby protecting the cells from damage [26]. The involvement of Nrf2/HO-1 signaling pathway in infertility has been reported [6]. In this study, the protein expressions of Nrf2 and HO-1 were significantly downregulated in ULC group, relative to control group, but they were markedly upregulated after treatment with the extract.

Elevated level of reactive oxygen species (ROS) reduces the antioxidant capacity of cells and negatively regulates Nrf2/HO-1 signaling pathway. The results obtained in this study indicate that the fertility-enhancing effect of ethanol extract of *Cuscuta chinensis* seeds may be positively regulated by Nrf2/HO-1 signaling pathway. The results of a previous study showed that treatment with herbal formula rich in *Cuscuta chinensis* (KH-204) significantly upregulated the protein expressions of Nrf2 and HO-1 in both cell and animal model [26].

The results of histopathological examination provided supportive evidence for the biochemical data. Cross section of testicular tissues of rats in ULC group showed narrowed and thickened seminiferous tubules (disrupted spermatogonia) characterized by increased apoptotic bodies (increased number of necrotic Sertoli and Leydig cells). Testicular tissues of rats treated with 100 mg extract/kg bwt showed less thickening of seminiferous tubules and reduced number of apoptotic bodies. There were few damaged or necrotic Sertoli and Leydig cells and complete absence of thickening of seminiferous tubules in testicular tissues of rats treated with 200 mg extract/kg bwt. These results are in agreement with those reported previously [27]. The aphrodisiac effect was better in 200 mg extract/kg group than in 100 mg/kg group. The major limitation of this study is failure to investigate other necrotic parameters and

expression levels of pro- and anti-apoptotic markers (bax, bcl-2 and bid).

CONCLUSION

The ethanol extract of *Cuscuta chinensis* seeds effectively mitigates cryptorchidism in rats via mechanisms involving the regulation of Nrf2/HO-1 signaling pathway, and inhibition of apoptosis and oxidative stress. Thus, the extract can potentially be developed as anti-infertility therapy.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Jiao Shu, Li Li and Dandan Zhang designed and executed this study. Hui Yu and Dandan Zhang helped in statistical analysis. Hui Yu and Li Li drafted this manuscript. Jiao Shu helped in histological analysis.

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