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Cistanches herba Induces Testis Cytotoxicity in Male Mice

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Abstract We investigated the effects of *Cistanches herba* (CH) on the male reproductive system in mice, assessing CREM gene expression and spermatogenesis. Our results demonstrate that CH treatment lead to a significant decrease in sperm count dose-dependently, 298.3 ± 48.9 vs. 296.6 ± 102.4 (250 mg/kg), 236.7 ± 75.1 (500 mg/kg), 223.0 ± 48.7 × 10^6 (1000 mg/kg), respectively. Additionally, serum testosterone levels decreased following CH treatment to as low as ~57% compared with the vehicle-treated group. CREM gene expression was also down-regulated following CH treatment and histological examination of the testicular seminiferous tubules showed severe damage on CH treatment. These results suggest that CH induces cytotoxicity in the male reproductive system, through the inhibition of spermatogenesis, testicular damage, and limited hormonal function.

Keywords Cistanches herba · CREM · Spermatogenesis · Cytotoxicity

The testis produce mature spermatozoa through a complex process of stem germ cell proliferation and differentiation, by a somatic-germ cell and hormonal interactions in the seminiferous epithelium (Cooke and Saunders 2002). Exposure of the testis to toxicants leads to toxic damage to spermatogenesis, including deficiency and excessive apoptosis of germ cells, hormonal insufficiency, and testicular atrophy (Boekelheide 2005). Additionally, paternal exposure to toxicants results in infertility and adverse progeny outcomes (Codrington et al. 2004). Cyclophosphamide (spermatogonia toxicant), 2,5-hexanedione (Sertoli toxicant), ethylene glycol monomethyl ether (spermatocyte toxicant), and sulfasalazine are known reproductive toxicants that affect the expression of genes related to spermatogenesis (Fairley et al. 1972; Moustafa et al. 2007). Additionally, high levels of ROS, cigarette smoke, and radiation are known effectors of sperm DNA damage (Zini and Libman 2006).

Cistanches herba (CH) is derived from the stems of *Cistanche* species (Orobanchaceae) and the major active component are lignans, phenylethanoid glycosides, and iridoid glycosides, which have activity in regulating immune function, anti-aging, and curing constipation. CH has been shown to exhibit anti-stress, antioxidant, and hepatoprotective activity and to be useful for the treatment of kidney-deficiency-syndrome (Xiong et al. 1996; Qiu et al. 2008). CH is frequently used as an important tonic in traditional Eastern medicine and has been reported to improve impotence and female infertility (Shi et al. 2009). Despite its various effects on biological systems, the cytotoxic effects of CH on the male reproductive system remain uninvestigated.

The aim of this study was to assess the reproductive toxicity of CH in the mouse testis. We performed sperm analyses, hormonal assays to measure serum testosterone levels, histological examinations of the mouse testes, and analyzed CREM gene expression.

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Materials and Methods

C. herba, the stems of *Cistanche deserticola* Y.C.M_A, was purchased from NeiMenggu in China (Sin-Hung pharm co., Korea). Three hundred grams of dried CH was boiled with 6 L of water for 2 h at 100°C, then the suspension was filtered and concentrated under reduced pressure. The filtrate was lyophilized and yielded 31.3 g (10.4%) of powder, which was kept at 4°C. Before each experiment, dried extract was dissolved in distilled deionized water and vortexed for 2 min at room temperature.

Five-week-old male ICR mice were purchased from SLC Inc. (Japan). The animals were housed in a specific pathogen-free environment with a 12/12 h light/dark cycle at the Center for Laboratory Animal Care and Use at Kyung Hee University. Mice had free access to standard rodent pellets (Purina Inc., Korea) and water ad libitum. Animal care and experimental procedures conformed to the "Guide for the Care and Use of Laboratory Animals" (Department of Health, Education, and Welfare, NIH publication # 78-23, 1996).

After 7 days of adaptation to the environment, the mice were randomly divided into four groups; normal group (vehicle treated, n = 10), and several concentrations of *C. herba* (CH) group (250, 500, 1000 mg/kg CH only-treated, n = 8). CH was treated daily for 35 days (250, 500, 1000 mg/kg/day, *p.o.*). The animals were weighed weekly in order to adjust the gavage volume and to monitor their general health. At the end of the treatment period, the mice were anesthetized with urethane (100 mg/kg, *i.p.*). Serum from the whole blood through the cardiac puncture was separated and stored at a deep-freezer for the serum testosterone quantitative analysis. The testes were taken out, cleared of the adhering tissues, and weighed. Epididymis being taken out, they were used for sperm analysis.

Epididymal sperm count and motility were evaluated by the method as described by Connolly et al. (2005), with some modifications. To obtain sperm count, entire epididymis from the mouse were minced in M199 media containing 0.5% BSA and incubated for 5 min at 37°C. The sperm concentration outcomes produced by manual evaluation using the Computer Assisted Semen Analysis system (CASA, Hamilton Thorne, USA). For assessment of sperm motility, sperms were recovered from excised caudal epididymis and allowed to capacitate for 5 min in M199 media containing 0.5% BSA at 37°C. Sperms were scored as motile if any movement was detected and used to analyze the total number of sperm and motility by CASA system. Radioimmunoassay (RIA) for serum testosterone was carried out using Coat-A-Count total testosterone kit according to manufacturer's protocol with minor modifications (Monath et al. 1995). The Coat-A-Count procedure is a solid-phase RIA, based on testosterone-specific antibody immobilized to the wall of a polypropylene tube. ^{125}I-labeled testosterone competes for a fixed time with testosterone in the experimental sample for antibody sites. The tube is then decanted, to separate bound from free, and counted in a gamma counter (Cobra; Hewlett packard, USA). The amount of testosterone present in the experimental sample is determined from a calibration curve. All samples were run in triplicate and two sets of testosterone standard were included in each assay.

For histological studies, the testes were fixed overnight in Bouin's solution, dehydrated in 70, 80, 95, 100% ethanol, xylene and embedded in paraffin. Tissue sections of 5 μm were prepared in order to perform hematoxylin-eosin stain. The sections were deparaffinized and rehydrated in xylene, 100, 95, 80, 70% ethanol. The sections were overstained with hematoxylin, usually 3–5 min and rinsed off excess stain in deionized water. Then they were destained a few seconds in acidic alcohol until sections look red, usually 4–5 dips and rinsed briefly in deionized water to remove the acid. Hematoxylin stained slides from the last tap water were rinsed and placed in 70% ethanol for 3 min. Slides were placed in eosin for 2 min and taken slides through 95, 100% ethanol and xylene. After H&E staining, slides were mounted with Canada balsam and examined by light microscopy.

1 mL of Trizol was added to the testis tissue samples (0.1 g). Then the samples were homogenized and incubated for 5 min at room temperature. 250 μL of chloroform was added and the samples were centrifuged at 12,000 g for 10 min at room temperature. Aqueous phases were transferred to fresh tubes and 400 μL of isopropanol was added. The supernatants were incubated for 10 min at room temperature and centrifuged at 12,000 g for 15 min at 4°C. Then the RNA pellets were washed with 75% ethanol, air dried, and resuspended in DEPC-treated water. Total RNA samples were analyzed by denaturing formaldehyde/agarose/ethidium bromide gel electrophoresis. First strand cDNA synthesis with 5 μg of total RNA was performed using MMLV reverse transcriptase and oligo dT primer for 1 h at 42°C. Subsequently, the PCR-amplification was performed by a modified method originally described by Saiki et al. (1986). Firstly, 5 μL of cDNA was added to 2.5 μL of 10 × PCR buffer, 1 μL of 25 mM $MgCl_2$, 1 μL of 2.5 mM dNTP, 0.5 μL of polymerase (1 U), 1 μL of each primer (4 pmol), and DEPC-H_2O to give a final volume of 25 μL. The mixture was heated at 95°C for 5 min, followed by 35 sequential cycles of primer extension at 72°C for 1 min, heat denature at 95°C for 1 min, and primer annealing at 56°C for 1 min. The sequences of CREM primers were as follows: 5′-GATTGAAGAAGAAAAAT CAGA-3′ as forward primer and 5′-TTGACATATTCTT TCTTCTT-3′ as reverse primer, while for the mouse *β*-actin, 5′-ACCGTGAAAAGATG ACCCAG-3′ and

5′-TACGGATGTCAACGTCACAC-3′ were used (Li et al. 2003). PCR-products were separated on a 1.5% agarose gel, visualized by ethidium bromide using i-MAX gel image analysis system (CoreBioSystem, Korea) and analyzed using Alpha Easy™ FC software (Alpha Innotech, USA). Results from at least three separate experiments were used for statistical analysis.

The protein concentrations were determined by Bradford method. The SDS-PAGE and western blotting were performed as described previously (Florin et al. 2005). Samples for protein extraction were half of the same testes used for RNA extractions. Equivalent amount (50 μg) of protein extracts were separated in 10% Tris–glycine gels by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes using 25 mM Tris and 250 mM glycine containing 20% methanol, pH 8.3. Transfer was performed at a constant voltage of 120 mA for 1 h. After transfer, membranes were blocked in phosphate buffered saline containing 0.05% Tween (PBS-T) with 5% skim milk for 1 h at room temperature and incubated with the primary antibody (1:200) for CREM-1 in PBS-T overnight at 4°C. After incubation, the membranes were rinsed three times with 1 × PBS and incubated with conjugated goat anti-rabbit IgG for 1 h at room temperature followed by three rinses with 1 × PBS.

All quantitative data derived from this study were analyzed statistically. The results are expressed as the mean ± standard deviation (SD.). Differences between groups were assessed by one way ANOVA using the SPSS software package for Windows. Statistical significance at p values <0.05 has been given respective symbols in the tables or figures.

Results and Discussion

Body and testicular weights were measured on the day following full treatment. Mice treated with varying concentrations of CH (250, 500, 1000 mg/kg) displayed little change in body weight compared with the normal group (42.4 ± 2.4 vs. 42.1 ± 2.7, 42.2 ± 2.8, 42.8 ± 1.9 g, respectively). Testes weight in the presence of CH treatment (250, 500, 1000 mg/kg) also did not differ from those of the control group (43.7 ± 2.6 vs. 42.6 ± 2.4, 42.6 ± 2.9, 42.9 ± 1.6 mg, respectively; Table 1).

Figure 1a demonstrates that the number of sperm in the presence of CH (250, 500, 1000 mg/kg) decreased dose-dependently (298.3 ± 48.9 vs. 296.6 ± 102.4, 236.7 ± 75.1, 223.0 ± 48.7 × 10^6, respectively), particularly at a CH concentration of 1000 mg/kg. The sperm motility of the CH treated groups showed a slight reduction compared with the control group, the motility of CH (250, 500, 1000 mg/kg) treated groups being 58.5 ± 15.3 versus 62.0 ± 20.3, 51.5 ± 15.4, and 52.2 ± 13.9%, respectively. However, these differences were not statistically significant (Fig. 1b).

Table 1 Body and testicular weight change after *C. herba* (CH) Treatment

Groups[a]	Normal	CH250	CH500	CH1000
Body weight (g)	42.4[b] ± 2.4	42.1 ± 2.7	42.2 ± 2.8	42.8 ± 1.9
Absolute testis weight (mg)	43.7 ± 2.6	42.6 ± 2.4	42.6 ± 2.9	42.9 ± 1.6

[a] Normal: vehicle treated group. CH group: CH (250, 500, 1000 mg/kg, p.o., 5 weeks) treated group

[b] Values are the mean ± S.D. ($n = 10$ for normal group and $n = 8$ for treatment group)

Solid-phase ^{125}I radioimmunoassay was performed to evaluate the effects of CH on testosterone biosynthesis. Quantitative measurements of testosterone in serum were made using the Coat-A-Count total testosterone kit. The normal serum testosterone levels were 5.61 nmol/L, while the CH-treated group showed decreased testosterone level in a dose dependent manner (5.05, 3.76, and 3.24 nmol/L, respectively; $p < 0.01$; Fig. 1c).

Hematoxylin and eosin staining was performed to observe testicular histological changes. Histological sections of seminiferous tubules were similar to the control group at a CH concentration of 250 mg/kg (Fig. 2, c–d), while slight damage was evident in the 500 mg/kg treated group (Fig. 2, e–f). The 1000 mg/kg CH-treated group showed severe seminiferous tubule damage (Fig. 2, g–h).

To examine the effects of CH on the CREM mRNA levels in mouse testis, RT-PCR was performed. CREM activator transcripts were detected from the pachytene stage to the early round spermatid (Behr et al. 2000). The CREM fragment was detected at 761 bp and β-actin was used as an internal control. The CH treated group displayed an ~80% reduction in CREM mRNA levels compared with those of the control group ($p < 0.05$; Fig. 3a). Consistent with these results, Western blot analysis of the CREM protein levels in mice treated with CH decreased in a dose-dependent manner to ~80% of the control (Fig. 3b).

C. herba is a parasitic plant that is attached underground to the roots of the host dicotyledonous plant *Haloxylon ammodendron*, which grows through the absorption of host nutrients. The parasite is primarily distributed in the desert regions of Northwestern China (Qun et al. 2007). The botanical origin of Cistanis Herba, termed Yuk-jong-yong, is described only as *Cistanche deserticola* Y. C. Ma. As a desert-distributed parasitic plant, CH is widely used in traditional medicine for various therapeutic treatments, including sedative, analgesic

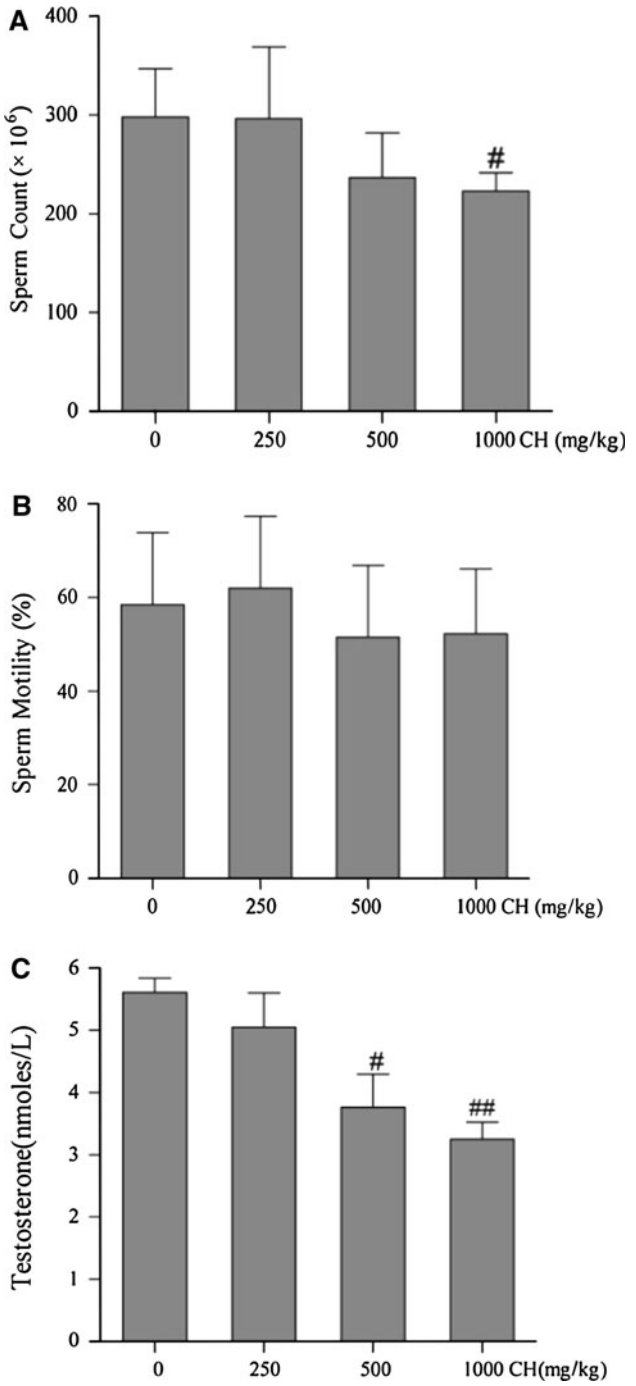

Fig. 1 Effect of *C. herba* (CH) treatment on sperm parameters. **a** Sperm count. Several concentrations of CH were used and analyzed by the CASA system, as described in the Materials and Method. Each column represents the mean ± SD ($n = 10$ for normal group and $n = 8$ for treatment group). # indicates significant differences from the normal value ($p < 0.05$). **b** Effect of CH on sperm motility. Mice were treated with CH and analyzed by the CASA system, as described in the Materials and Method. Each column represents the mean ± SD ($n = 10$ for normal group and n = 8 for treatment group). **c**, effect of CH on serum testosterone levels in mice. Normal is the vehicle-treated group. Each column represents the mean ± SD ($n = 10$ for normal group and n = 8 for treatment group). # indicates that the mean is significantly different from the normal value (##: $p < 0.01$)

and immune-enhancing indications. Additionally, CH exhibits activity for sexual potency, free radical-scavenging, anti-aging, and neuroprotection (Shahat et al. 2005; Gao et al. 2000; Kyriakopoulou et al. 2001; Deng et al. 2004; Geng et al. 2004).

The purpose of this study was to examine the cytotoxic effects of CH on the male reproductive system. Mice treated with CH displayed no significant change in body or testis weight. Standard semen analysis evaluates the major semen parameters of sperm concentration and motility, and a clinical diagnosis of the male partner's fertility relies primarily on the outcome of this analysis (Tomlinson et al. 1999). This is the most basic laboratory investigation of male infertility and measures semen volume, appearance, viscosity, sperm concentration, sperm motility, and morphology. The concentration of motile sperm is the best parameter to predict in vivo conception, because low motile sperm concentrations have the greatest impact on fertility (Larsen et al. 2000).

Testicular function is influenced by endocrine and paracrine factors. The hypothalamic-pituitary–testicular axis plays a central and critical role in the male reproductive system. Testosterone acts as an androgen, playing an important role in the negative feedback control of both gonadotropins, although hypothalamic aromatization to oestradiol also increases the degree of inhibition (Moudgal and Sairam 1998).

CREM belongs to the cAMP response element-binding protein (CREB)-CREM family of transcription factors, which target genes with CRE regulatory regions (Sassone-Corsi 1998). CREM is a master controller gene for spermatogenesis (Nantel et al. 1996) and is involved in gene expression regulation by cAMP (Li et al. 2003). To investigate the cytotoxic effects of CH on CREM expression, RT-PCR and Western blot analyses were performed. The CREM mRNA levels of CH-treated groups were significantly decreased compared with those of the control group. Together, these data demonstrate that the CREM protein levels of mice treated with CH decreased dose-dependently in the presence of CH. These results suggest that CH treatment possesses cytotoxic effects on CREM gene expression and protein biosynthesis in mouse testis.

The results of this study indicate that CH displays cytotoxic effects on the male reproductive system. CH decreased sperm count, and lead to damage of the mouse seminiferous tubules. Additionally, CREM expression decreased significantly following CH treatment. Taken together, we can conclude that CH displays cytotoxic effects, as measured by various sperm parameters, on the male reproductive system, thus it may not appropriate for therapies seeking to improve the function of the male reproductive system.

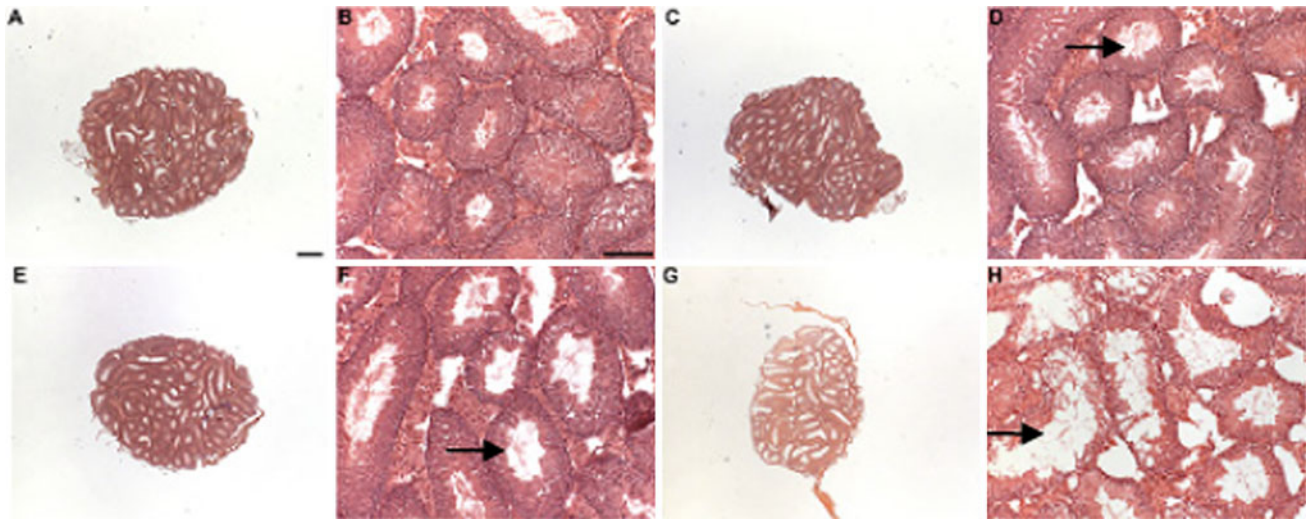

Fig. 2 Examination of histological changes of mouse testes after *C. Herba* treatment with hematoxylin and eosin staining. **a**, **b** control, **c**, **d** 250 mg/kg, **e**, **f** 500 mg/kg, **g**, **h** 1000 mg/kg of CH treated groups. (a), (c), (e), and (g) × 50 magnification. *Scale bar* = 100 μm. (b), (d), (f), and (h) × 200 magnification. *Scale bar* = 200 μm. *Arrows* indicate representative damaged seminefous tubule

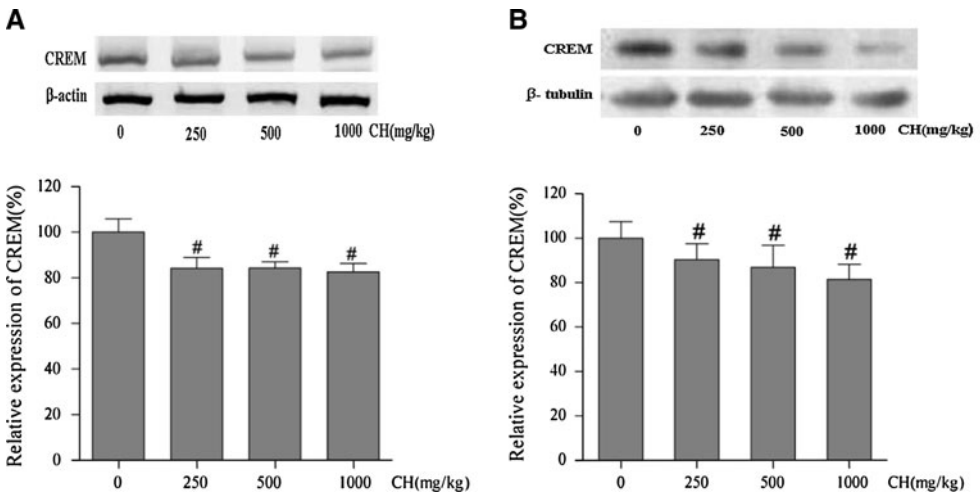

Fig. 3 Effect of *C. Herba* (CH) on CREM gene expression in mouse testes. **a** RT-PCR reaction was performed on isolated RNA using CREM and *β*-actin primers. The levels of CREM mRNA were normalized to the *β*-actin signal. Control indicates the vehicle-treated group. Each column represents the mean ± SD ($n = 3$). # indicates that the mean is significantly different from the normal value (#: $p < 0.05$). (B) Western blot analysis of CREM protein expression in mouse testis lysate (50 μg). Control = vehicle treated group. *β*-Tubulin was used as an internal control. Each column represents the mean ± SD ($n = 3$). # indicates significant differences from the control value (#: $p < 0.05$)

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