A Chinese herbal decoction can increase the intracellular Ca^{2+} concentration and CatSper1 expression in mouse sperm tails

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Abstract. The aim of this study was to investigate the impact of a Chinese herbal decoction on the intracellular calcium (Ca^{2+}) concentration of sperm and the expression of the cation channel 1 of sperm (CatSper1), which is a calcium-channel protein specific to sperm tail, in a murine model of asthenospermia induced with cyclophosphamide. After 34 days of intragastric administration of Chinese herb decoction to the murine model used, routine analyses of the mouse sperm were conducted, the intracellular Ca^{2+} concentration of the sperm tails was measured using flow cytometry, and the expression of CatSper1 protein was detected using reverse transcription-polymerase chain reaction (RT-PCR). Sperm concentration, percentage of grade A and B sperm (i.e., sperm activity) and percentage of grade A, B and C sperm (i.e., overall sperm motility) of the model group mice (MG) were markedly lower compared to the control murine group (CG) (one-way ANOVA, $P<0.05$). Subsequent to treatment, sperm concentration, percentage of sperm activity and overall sperm motility of the large dose of herbal medicine group murine (LG) were markedly increased compared to MG mice ($P<0.05$). Intracellular Ca^{2+} concentration in MG mice was markedly lower compared to CG mice ($P<0.05$). However, following therapy, a significant increase was observed in the intracellular Ca^{2+} concentration in LG mice as compared to MG mice ($P<0.05$). In addition, the expression of CatSper1 in LG mice was significantly higher compared to MG mice ($P<0.05$), while no statistically significant difference was observed for the CG mice. Intraperitoneal injection of cyclophosphamide reduced sperm concentration, percentage of sperm activity and overall sperm motility, intracellular Ca^{2+} concentration and CatSper1 expression. Large doses of this Chinese herbal decoction increased sperm intracellular Ca^{2+} concentration, sperm concentration, and percentages of sperm activity and overall sperm motility by upregulating CatSper1 expression. The findings of this study have demonstrated a therapeutic effect of this decoction on asthenospermia.

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Introduction

Globally, ~8% of reproductive-aged couples are infertile and infertility morbidity ranges from 5 to 35%. In China, the infertility morbidity rate is ~10% and it has been on the increase. The percentage of infertility attributed to males has reached 50% (1). Certain Chinese herbal decoctions have been reported to be efficacious in infertility treatment (2,3). However, the active mechanisms of these remedies remain unknown. This study elucidated the mechanism of one such herbal decoction by examining the sperm-specific intracellular calcium (Ca^{2+}) channel cation channel 1 of sperm (CatSper1), the level of intracellular Ca^{2+} in sperm concentration, and the percentages of grade A and B sperm (i.e., sperm activity), as well as the percentage of grade A, B and C sperm (i.e., overall sperm motility).

Materials and methods

Instruments. Instruments used in this study included GeneAmp® 5700 TaqMAN PCR (Applied Biosystems, Carlsbad, CA, USA); ImageMaster VDS gel image analyzer (Pharmacia Biotech, Inc., Piscataway, NJ, USA); FACSCalibur™ flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA); a high-speed refrigerated centrifuge Allegra 21R centrifuge (Beckman Coulter, Inc., Krefeld, Germany); DU640 ultraviolet spectrophotometer (Beckman Coulter, Inc.); OYY-III 5 electrophoresis system (Liuyi Instrument, Inc., Beijing, China); WL-9000 WeiLi sperm analyzer (Beijing Weili New Century Science and Technology Co., Ltd., Beijing, China) and DR-HW-1 incubator (Medical Device Corporation, Beijing, China).

Reagents. Reagents used in this study included RevertAid™ First Strand cDNA Synthesis kit (Fermentas GmbH, St. Leon-Rot, Germany); Fluo-3/AM (catalog no. 50013; Biotium, Inc., Hayward, CA, USA) and SYBR-Green PCR Master mix: ABI (catalog no. 43049155; Applied Biosystems). The CatSper1 gene

Table I. Components of the Chinese herbal decoction used in this study.

Common name	Chinese name	Latin name	Plant part	Quantity (g)
Adhesive Rehmannia rhizome	Shu Di Huang	*Rehmannia glutinosa* Libosch.	Dried root	20
Epimedium	Yin Yang Huo	*Epimedium*	Dried leaves	20
Ootheca Mantidis	Sang Piao Xiao	*Paratentodera sinensis* Saussure	Praying mantis egg case, dried	20
Rose hips	Jin Ying Zi	*Fructus Rosae Laevigatae*	Dried fruit	20
White mulberry	Sang Ye	*Folium Mori Albae*	Dried leaves	20
Chinese magnolia vine	Wu Wei Zi	*Schisandra henyri*	Dried berries	15
Solomon Seal rhizome	Huang Jing	*Polygonatum odoratum*	Dried root	15
Chinese angelica	Dang Gui	*Angelicae sinensis*	Dried root	12
Fragrant Solomonseal rhizome	Yu Zhu	*Polygonatum odoratum*	Dried root	12
Swordlike rhizome	Cang Zhu	*Atractylodes*	Dried root	12
Peony rubra	Chi Shao	*Paeoniae rubra*	Dried root	9
Safflower (Carthemi flos)	Hong Hua	*Carthamus tinctorius* L.	Dried flowers	9

identification script was obtained from GenBank, while primers were designed using Primer Premier 5.0 and were synthesized by SaiBaiSheng Biotech Co., Ltd. (Beijing, China).

Primer sequences. Primer sequences used in this study were: β-actin F: 5'-GAGACCTTCAACACCCCAGCC-3' and R: 5'-AATGTCACGCACGATTTCCC-3' (the size of the amplified fragment was 263 bp). CatSper1 F: 5'-TGCTCC TTCAGATAAACTCG-3' and R: 5'-ATCTACCAGGACAGC AATCA-3' (the size of the amplified fragment was 444 bp).

Chemical agents. Chemical agents used in this study included BWW low-protein fluid, BWW high-protein capacitation fluid, sodium chloride (NaCl) 554 mg/100 ml, potassium chloride (KCl) 35.6 mg/100 ml, monopotassium phosphate (KH_2PO_4) 16.2 mg/100 ml, bicarbonate of soda ($NaHCO_3$) 210 mg/100 ml, glucose ($C_6H_{12}O_6$) 100 mg/100 ml, sodium pyruvate ($C_3H_3NaO_3$) 3 mg/100 ml, sodium lactate 370 μl/100 ml, gentamicin 125 μl/100 ml and 0.5% phenol red 50 μl/100 ml. BWW low-protein fluid containing 0.3% human serum albumin was used for treatment of sperm. BWW high-protein capacitation fluid containing 0.3% human serum albumin was used for sperm capacitation.

The incubation buffer used in this study was composed of 10 mmol/l HEPES, 140 mmol/l NaCl, 2.5 mmol/l $CaCl_2$, pH 7.4. The phosphate-buffered saline (PBS) buffer used in this study was composed of 8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l $Na_2HPO_4(7H_2O)$, 0.24 g/l $KH_2PO_4(H_2O)$, pH 7.2.

Experimental animals and grouping. Forty male Kunming mice of clean grade with body weight ranging from 18 to 20 g were purchased from the Institute of Laboratory Animal Science (Chinese Academy of Medical Sciences, Beijing, China). Ten mice were randomized into four groups using a digital random method: control (CG), model (MG), small-dose (SG) and large-dose groups (LG). The protocols used in this study were approved by the Institutional Review Board and the Animal Care and Use Committee of the Air Force General Hospital (Beijing, China).

Preparation of Chinese herbal decoction. The components of the herbal medicine used are listed in Table I. The herbal components were placed in a stainless steel pot and soaked for 20 min in 2,000 ml water. They were then boiled for 30 min, and stirred every 5 min. The mixture was transferred to another stainless steel pot and boiled for condensation to a decocted concentration of 1 g/ml. The decoction was refrigerated until use.

Methods. Mice were acclimated for 5 days. Based on the available literature and our pre-experiment data, CG mice were intraperitoneally injected with 60 mg/kg normal saline (NS) while MG, SG and LG mice were intraperitoneally injected with 60 mg/kg cyclophosphamide once daily for 5 consecutive days to induce asthenospermia (4). After 6 days, the Chinese herbal decoction was intragastrically administered to the SG mice at a dose of 3.3 g/kg/day (i.e., the normal human dose equivalent based on human body weight of 60 kg), and to the LG mice at a dose of 16.5 g/kg/day (i.e., 5 times the normal human dose equivalent) for 34 days (treatment days 1-34). The two doses were diluted to the same volume with NS. MG mice underwent gastric lavage with an equivalent volume of NS once daily during the same period. MG mice were intragastrically administered NS of the same volume once daily for 34 days. During the period of intragastric administration, mice were weighed once/week, and the administration volume was adjusted according to their weight. After 34 days of treatment with daily intragastric administration of the Chinese herbal decoction plus NS (LG and SG) or NS alone (MG), the mice were sacrificed, and their testes, epididymides and seminal ducts were removed. During removal, adjunct tissues including adhesive blood vessels, ligaments and adipose tissues were simultaneously dissected.

Collection of mouse sperm. Following dissection, epididymides of the two sides were placed in a clean culture dish. They were cut into two halves using ophthalmic scissors, cutting along the longitudinal axis of the epididymis, and the halves were then cut into six uniform sections by cutting along

the horizontal axis. The epididymal sections were transferred to a tube containing 1 ml PBS buffer at room temperature, while another 1 ml of pre-warmed buffer was used to rinse the culture dish and was transferred to the tube containing the epididymal samples.

Subsequent to removal of the epididymides and testes, a cut was made at the conjunction of the spermatic duct and the tail part of the epididymis, and another at the conjunction of the spermatic duct and the prostate. An injection apparatus containing 1 ml pre-warmed buffer was inserted into the one end of the spermatic duct. The injection forced 1-2 drops of milky fluid containing sperm to drain out the other end of the spermatic duct. This liquid was transferred into the tube containing the epididymal tissue. The remaining pre-warmed buffer in the injection apparatus was used to rinse the epididymal duct and, then, it was transferred to the tube containing the epididymal tissue. The extracted sperm samples were incubated for 30 min at 37˚C.

Routine analysis of sperm samples. Subsequent to incubation, the supernatant was removed and centrifuged at 360 x g for 5 min. Then, 1 ml BWW high-protein capacitation fluid was added to the precipitate, which was placed in the incubator at 37˚C for 2 h. Routine sperm analyses, including sperm concentration, percentage of grade A and B sperm (i.e., sperm activity) and percentage of grade A, B and C sperm (i.e., overall sperm motility) were conducted, using normal human sperm analysis methods.

Extraction of sperm total RNA. Approximately $1x10^6$ mouse sperm was treated with 1 ml TRIzol (Invitrogen, Carlsbad, CA, USA) and placed at room temperature for 5 min for a thorough extraction. Chloroform (200 μl) was added, and the mixture was agitated for 15 sec and placed at room temperature for 15 min. The mixture was then centrifuged at 12,000 x g at 4˚C for 15 min. The supernatant was transferred into a pre-cooled centrifuge tube, 0.5 ml of pre-cooled isopropyl alcohol was added, the mixture was placed at room temperature for 5-10 min after agitation, and then centrifuged at 12,000 x g at 4˚C for 10 min. The supernatant was discarded, and the RNA was precipitated at the bottom of the tube. Then, 1 ml 75% ethanol (pre-cooled) was added, and the tube was gently agitated to suspend the precipitate. The suspension was centrifuged at 7,500 x g at 4˚C for 5 min; and the supernatant was again discarded. The precipitate was left to dry at room temperature for 5-10 min and the precipitated RNA sample was dissolved in 50 μl RNAase-free water at 55-60˚C for 10 min. The A value was measured to determine the RNA concentration.

Detection of RNA concentration. Deionized sterilized water (1.5 ml) was added to a 6-μl RNA sample and then vortexed. The product was transferred to a colorimetric cuvette. The spectrophotometer was calibrated to zero using 1 ml deionized water. Optical density was detected at 260 and 280 nm, and the RNA concentration was calculated as: a 260 x dilution ratio x 40/1,000 and the concentration unit was μg/μl.

Reverse transcription of RNA sample. Reagents and RNA samples were allowed to melt at room temperature, and were placed on ice. Vortexing for homogenization was conducted and the mixture was immediately centrifuged. RNA template (12 μl) was denatured by incubation at 70˚C for 10 min. The reaction mixture was prepared (5X reaction buffer 4.0 μl, 10 mM dNTP mix 2.0 μl, 20 U/μl RNase inhibitor 1.0 μl), and 7.0 μl reaction mixture was added to the RNA template and incubated at 37˚C for 5 min. Then, 1.0 μl M-MuLV reverse transcriptase (200 U/μl) was added. The mixture was incubated at 42˚C for 60 min and again at 70˚C for 10 min. It was stored at -20˚C until use.

Fluorescent quantitative PCR detection. Primers were diluted to 10 pmol/μl. The reaction mix was prepared (2X SYBR-Green PCR Master mix 12.5 μl, forward primer 1 μl, reverse primer 1 μl, nuclease-free H_2O 8.5 μl) and 2.0 μl cDNA template was added. The mixture was homogenized by vortexing and immediately centrifuged. PCR amplification was subsequently performed (95˚C 10 min, 95˚C 25 sec, 55˚C 25 sec, 72˚C 50 sec, for a total of 40 cycles; 72˚C 5 min). Data were analyzed using the PE 5700 for calculation.

Detection of sperm intracellular Ca^{2+} concentration. Sperm samples were incubated at 37˚C for 30 min, and the supernatant was centrifuged twice at 360 x g for 5 min. The precipitate was washed using BWW low-protein fluid and the sperm sample was suspended using BWW capacitation fluid followed by incubation for 4 h. The supernatant was discarded, and the precipitated sperm was suspended in BWW low-protein fluid at a final concentration of $1x10^6$ sperm/ml. A Ca^{2+} fluorescent probe Fluo-3/AM was added to the final concentration of 3 μmol/l and incubated at 37˚C with 5% CO_2 for 45 min under photophobic conditions and was then centrifuged at 360 x g for 5 min. The dissociated Fluo-3/AM was discarded, and the sperm was suspended in BWW low-protein fluid to the final concentration of $1x10^6$ sperm/ml. Fluorescent intensity was detected using flow cytometry. Sperm cell number was ≥10,000.

Statistical analysis. Normally distributed data were analyzed using one-way analysis of variance (ANOVA) with the SPSS11.5 software (SPSS, Inc., Chicago, IL, USA). Results are presented as the mean ± SD (Table II). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Mice under normal conditions. No deaths occurred in the groups of mice prior to sacrifice. Mice exhibited no change in weight after 5 days of daily injection with cyclophosphamide. On the following day (the first day of treatment with herbal decoction), mice gradually began to exhibit symptoms, such as listlessness, less activity, aversion to cold, hair sparseness, lackluster appearance and increased urination. On treatment day 7, statistically significant differences were observed in terms of weight in CG mice compared to MG, SG or LG mice ($P<0.05$). On treatment days 14 and 21, the weight of LG mice remained markedly different compared to CG mice ($P<0.05$). By treatment day 35, no difference of testis and epididymis indices or weight were observed in mice in the groups (Fig. 1).

Table II. Comparison of mouse sperm concentration ($1x10^6$/ml) and motility (mean ± SD) (n=10).

Group	Sperm concentration ($1x10^6$ sperm/ml)	Percentage of sperm activity (%)	Percentage of overall sperm motility (%)
CG	5.20±1.34	14.49±0.30	68.39±15.13
MG	1.73±0.03[a]	6.64±1.88[a]	39.96±4.89[a]
SG	2.08±0.01[a]	11.99±1.01	62.28±4.43[b]
LG	3.31±0.56[a,b]	19.40±3.13[b,c]	73.61±5.05[b]

[a]P<0.05 compared to CG; [b]P<0.05 compared to MG; [c]P<0.05 compared to SG. CG, control group; MG, model group; SG, small-dose group; LG, large-dose group.

Table III. Relative quantification of CatSper1 expression (mean ± SD) (n=10).

Group	CatSper1 expression
CG	0.76±0.05
MG	0.73±0.03
SG	0.75±0.12
LG	0.85±0.04[a,b]

[a]P<0.05 compared to MG; [b]P<0.05 compared to SG. CG, control group; MG, model group; SG, small-dose group; LG, large-dose group.

Table IV. Comparison of mice sperm intracellular Ca^{2+} concentration (mean ± SD) (n=10).

Group	$[Ca^{2+}]i$
CG	133.14±11.86
MG	80.84±6.92[a]
SG	88.05±3.31[a]
LG	99.08±7.17[a,b]

[a]P<0.05 (significant difference) compared to CG; [b]P<0.05 compared to MG. CG, control group; MG, model group; SG, small-dose group; LG, large-dose group.

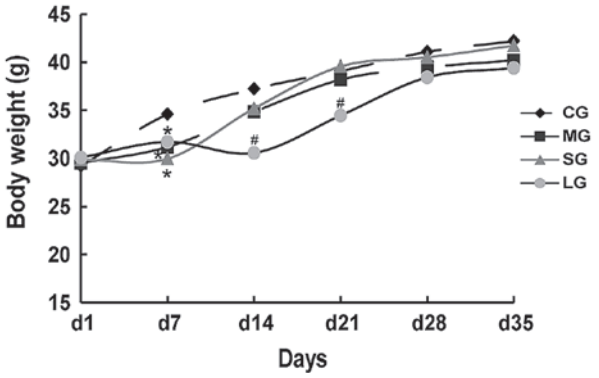

Figure 1. Tendency of mice weight is shown. *The weight of MG, SG and LG mice is significantly lower compared to CG mice (P<0.05); #the weight of LG mice is significantly lower compared to CG mice (P<0.05). CG, control group; MG, model group; SG, small-dose group; LG, large-dose group.

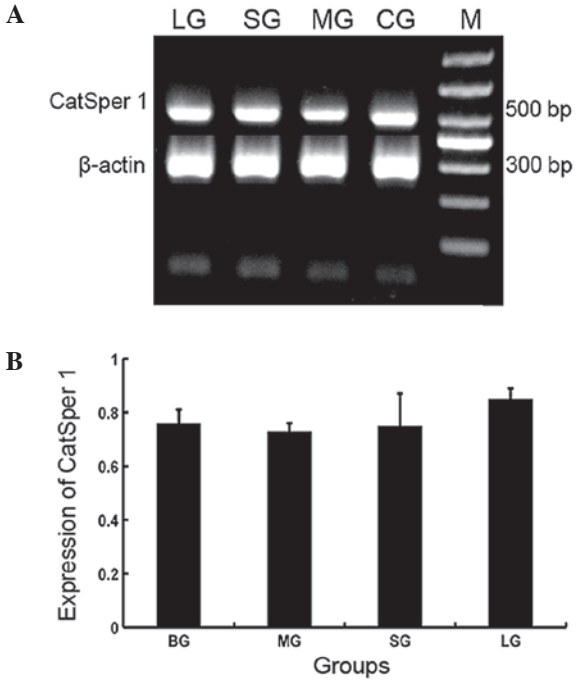

Figure 2. Gel electrophoresis data of CatSper1 is shown. M, DNA marker; CG, control group; MG, model group; SG, small-dose group; LG, large-dose group.

Routine examination of epididymal sperm. Results of this study show that sperm concentration, percentages of sperm activity and overall sperm motility of MG mice were markedly reduced (P<0.05) compared to CG mice (Table II). As a result of treatment with the Chinese herbal decoction , the sperm concentration, percentages of sperm activity and overall sperm motility of LG and SG mice were markedly increased compared to MG mice (P<0.05).

Detection of CatSper1 expression. The targeted relative mRNA expression was calculated based on the ratio of Ct value of *in vivo* reference and target genes. Since the gene expression is inversed to Ct value, for better understanding, we used the reciprocal ratio of the target gene and β-actin, which was the Ct value ratio of the reference gene *in vivo* and the target gene relative to mRNA expression. Our data (Fig. 2

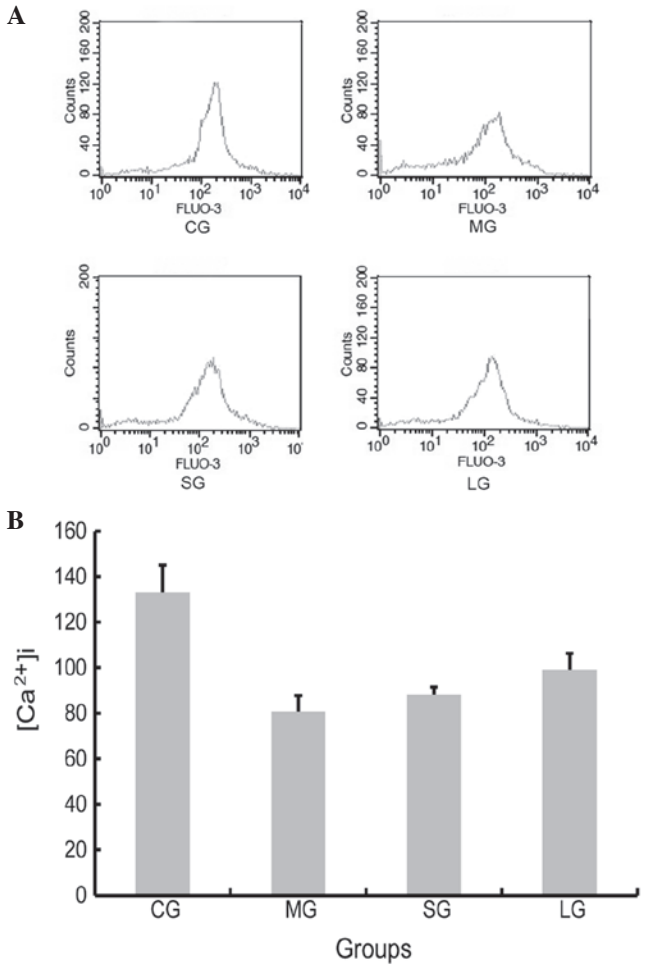

Figure 3. Variation curve of intracellular Ca^{2+} concentration in mouse sperm is shown. CG, control group; MG, model group; SG, small-dose group; LG, large-dose group.

and Table III) demonstrate that following treatment with the Chinese herbal decoction, CatSper1 expression of LG mice was markedly increased compared to MG mice. A statistically significant difference in fluorescent intensity was observed ($P<0.05$), while a statistically non-significant difference was observed compared to CG mice.

Detection of sperm intracellular Ca^{2+} concentration. Calcium concentration is expressed by fluorescent intensity of Fluo-3/AM (Fig. 3 and Table IV). Our results indicate that cyclophosphamide markedly reduced intracellular Ca^{2+} concentration in murine sperm. Following treatment with the Chinese herbal decoction, intracellular Ca^{2+} concentration in LG mice was markedly increased compared to MG mice ($P<0.05$), while no statistically significant difference was observed in CG mice.

Discussion

The alkylating agent cyclophosphamide has been widely used as an antitumor drug. After entering the human body, it is hydrolyzed to phosphomide nitrogen mustard, an active form, by excessive phosphamidase or phosphatase in liver or tumor. This active form cross-links with DNA to suppress DNA synthesis or interfere with RNA function, leading to toxic effects on these tissues (5). After intraperitoneal injection of cyclophosphamide, MG mice exhibited reduced sperm concentration, lower sperm activity and lower overall sperm motility, which are typical symptoms of asthenospermia. We hypothesized that this effect occurred as a result of influencing sperm-specific calcium-channel protein.

Calcium is involved in normal fertilization. During sperm hyperactivation, intracellular Ca^{2+} concentration increases, elevating the fertilization rate by strengthening sperm flagellum activity through the enhancement of its progressive movement and its swerving ability (6,7).

The results of our study have shown that sperm intracellular Ca^{2+} concentration markedly decreases following intraperitoneal injection of cyclophosphamide in mice, while intracellular Ca^{2+} concentration markedly increases in mice following administration of large doses of the Chinese herbal decoction used in the present study. This Chinese herbal decoction reduces asthenospermia symptoms by upregulating intracellular Ca^{2+} concentration in sperm.

This Chinese herbal decoction has been extensively applied to treat asthenospermia, while its reliable effects have been proven in clinical trials (2,3). A previous study has reported that several components of the Chinese herbal decoction improves male genital function. Chinese angelica improves peripheral circulation to trigger cell regenesis and recovery and strengthen the non-specific and specific immune systems. Similar to safflower, it also ameliorates regional microcirculation of the testis and enhances the clearance of reactive oxygen species (ROSs) to fortify spermatogenesis (8). Chinese dodder decoction can markedly increase the mating frequency of *Drosophila melanogaster*, while its water extract is capable of reducing ROS-generated human spermatic membrane injury (9). Cistanche exhibited anti-oxidation and anti-aging effects (10). Rhizome of adhesive *Rehmannia* can increase the activity of monocyte to strengthen its ability against fatigue, aging and free radical clearance (11). White mulberry contains abundant trace elements, such as magnesium, iron and zinc, and its use as an herbal remedy can decrease total cholesterol and glycerin trilaurate levels in serum to improve blood circulation (12). The herb *Epimedium* has been reported to improve the function of the hypothalamus-pituitary-gonadal axis. Additionally, tuber onion seed (Jiu Cai Zi, *Semen allii tuberose*) containing abundant iron, zinc, magnesium and calcium, has an anti-oxidation effect and can increase the weight of the testis, epididymis and vesicular seminalis. Moreover, *Ootheca mantidis* contains eight types of amino acids and phospholipids, and has been demonstrated to decrease fatigue and increase the testicular index (2). These herbal medicines, when combined, synergize to improve male genital function through multiple systems and multiple targets to enhance spermogenesis and improve sperm activity. However, the specific mechanism of this decoction remains to be further elucidated.

Intensive studies have recently demonstrated that CatSper1 is a specific protein that serves as a calcium channel, especially on the sperm plasma membrane located at the sperm tail (6). CatSper1 affects sperm flagellum and enhances sperm activity, capacitation and hyperactivation. CatSper1-deficient sperm lack capacitation, and exhibit impairment of overall activity, tail-swaying ability and rectilinear motion. These

shortcomings prevent sperm from reaching the zona pellucida of the ovum, resulting in fertilization deficiency (13).

We investigated sperm CatSper1 expression in mice treated with large doses of the Chinese herbal decoction for 34 days following intraperitoneal injection of cyclophosphamide for 5 days. Our results suggest that the expression of sperm CatSper1 in LG mice increased markedly compared to that of MG mice, demonstrating that treatment with large doses of the Chinese herbal decoction enhances the expression of CatSper1, thus potentially increasing fertility.

Acknowledgements

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