

Down-regulation of CatSper1 channel in epididymal spermatozoa contributes to the pathogenesis of asthenozoospermia, whereas up-regulation of the channel by Sheng-Jing-San treatment improves the sperm motility of asthenozoospermia in rats

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Objective: To determine the expression of CatSper1 channel in epididymal spermatozoa in a rat model of asthenozoospermia, induced by cyclophosphamide (CP), and further examine the effects of soluble granules of Sheng-Jing-San (SJS), a traditional Chinese medicine recipe, on CatSper1 expression and sperm motility in the CP-induced asthenozoospermic rats.

Design: Placebo-controlled, randomized trial.

Setting: Neuroscience Research Institute, Peking University, China.

Animal(s): Sexually mature male Sprague-Dawley rats (n = 60).

Intervention(s): In the CP group, CP at the dose of 35 mg/kg intraperitoneally injected into rats once a day for 7 days; in the normal saline (NS) group, 0.9% saline solution was injected as control.

Main Outcome Measure(s): Sperm motility and count were evaluated by computer-assisted sperm assay (CASA); protein and mRNA expression of CatSper1 channel in epididymal spermatozoa was determined by Western blotting and quantitative real-time RT-PCR, respectively.

Result(s): The rats were randomly divided into five groups with 12 rats in each group: CP, normal saline (NS), CP + SJS, CP + NS, and treatment naïve. In the CP + SJS group, after the last injection of CP, SJS at a dose of 30 mg/kg was intragastrically administered to rats once a day for 14 days; in CP + NS group, saline solution instead of SJS was administered as control. In the treatment naïve group, rats were normally fed for 21 days as controls. We found a statistically significant reduction of the CatSper1 channel, which is associated with an impairment of sperm motility in the epididymal spermatozoa of CP-induced asthenozoospermic rats. Soluble granules of SJS could dramatically restore the CP-induced down-regulation of CatSper1 in epididymal spermatozoa, which greatly improved the sperm motility in the asthenozoospermic rats.

Conclusion(s): Down-regulation of the CatSper1 channel in epididymal spermatozoa likely contributes to the pathogenesis of asthenozoospermia, whereas up-regulation of the channel by SJS improves sperm motility and thus can be used as an effective therapeutic strategy for the treatment of male infertility diagnosed with asthenozoospermia. (Fertil Steril® 2012; ■ : ■ - ■ . ©2012 by American Society for Reproductive Medicine.)

Key Words: Asthenozoospermia, male infertility, CatSper1 channel, cyclophosphamide, Sheng-Jing-San recipe

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Received August 17, 2012; revised October 11, 2012; accepted October 17, 2012.

Y.-N.W. has nothing to disclose. B.W. has nothing to disclose. M.L. has nothing to disclose. C.-Y.H. has nothing to disclose. B.Z. has nothing to disclose. J.C. has nothing to disclose. W.S. has nothing to disclose. G.-G.X. has nothing to disclose.

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Supported by grants from the National Natural Science Foundation of China (31171063, 81072951) and the Beijing Natural Science Foundation (7112079).

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Fertility and Sterility® Vol. ■, No. ■, ■ 2012 0015-0282/\$36.00

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<http://dx.doi.org/10.1016/j.fertnstert.2012.10.030>

Asthenozoospermia, one of the most common findings present in infertile men, is characterized by reduced or absent sperm motility, but its etiology remains unknown in most cases. It is well established that Ca^{2+} signaling is of particular significance in sperm cells, where it is a central regulator in many key activities including capacitation, hyperactivation, chemotaxis, and acrosome reaction (1). Impairment of Ca^{2+} signalling in sperm is associated with male subfertility (2, 3). Recently, a family of sperm-specific CatSper (cation channel of sperm) Ca^{2+} channels have been clearly shown to be required for sperm-hyperactivated motility, egg coat penetration, and male fertility (4, 5). Entry by Ca^{2+} through the principal piece-localized CatSper channels has been implicated in the activation of hyperactivated motility and male fertility (5, 6).

Among the four members of the CatSper channels family (CatSper1–4) that are exclusively expressed in the testis (7–9), CatSper1 and CatSper2 have been identified as essential for mouse sperm motility and male fertility (6, 10–12). Male mice deficient in either the Catsper1 or the Catsper2 gene are completely infertile, and neither Catsper1-null nor Catsper2-null sperm display hyperactivation in capacitating conditions (6, 9, 11, 13). A report identifying mutations in the Catsper1 channel protein that cause human male infertility has suggested that Catsper1 is essential for normal male fertility in humans (14). Numerous studies have now demonstrated that CatSper1 is required for depolarization-evoked Ca^{2+} entry and for hyperactivated movement, a key flagellar function of sperm (6, 15). We therefore hypothesized that asthenozoospermia is likely associated with the down-regulation of CatSper1 in the spermatozoa, and hence pharmacologic augmentation of the CatSper1 channel in spermatozoa may have a therapeutic effect on asthenozoospermia.

Accumulating evidence from clinical observations suggests that soluble granules of Sheng-Jing-San (SJS), a traditional Chinese medicine recipe, can effectively improve oligozoospermia and asthenozoospermia (16–19). The treatment effect of SJS on spermatogenesis has been documented in murine models of oligozoospermia (20–22), but the mechanism underlying the ameliorative effect of SJS in asthenozoospermia remains largely unknown. In a previous study, we found that SJS contained a large number of trace elements, including selenium (Se), zinc (Zn), manganese (Mn), iron (Fe), copper (Cu), antimony (Ti), and calcium (Ca) (23), all of which are required for spermatogenesis and the maturation and quality of spermatogenic cells (24–28). It has been reported that the CatSper gene family can be up-regulated by trace element such as selenium (29). Given that the down-regulation of the CatSper1 channel underlies the pathogenesis of asthenozoospermia, we speculated that the soluble granules of SJS might impose their therapeutic effect on asthenozoospermia by up-regulating the CatSper1 channel in spermatozoa.

In our present study, we validated our hypotheses in a rat model of asthenozoospermia induced by intraperitoneal injection of cyclophosphamide (CP). We show that both CatSper1 protein and messenger RNA (mRNA) expression statistically significantly decreased in epididymal spermatozoa in

CP-induced asthenozoospermic rats, while soluble granules of SJS dramatically ameliorated asthenozoospermia by restoring the CP-induced down-regulation of CatSper1 in asthenozoospermic rats.

MATERIALS AND METHODS

Chemicals, Antibodies, and Animals

Cyclophosphamide (CP) was purchased from local medical stores (Hengrui Medical Co., Ltd.) and was dissolved in sterile 0.9% saline solution to the desired concentrations just before our experiments. Rabbit anti-CatSper1, mouse anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase), and horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-rabbit or mouse) were purchased from Santa Cruz Biotechnology. All other chemicals or reagents were obtained from Sigma, Invitrogen, or Pierce/Thermo Scientific except when otherwise mentioned in the text.

Hualu Pharmaceutical Co., Ltd. (Shandong, China) produced soluble granules of Sheng-Jing-San (SJS) (Table 1), which contained the following (in grams): Shu Di Huang (*Radix Rehmanniae Preparata*), 20 g; Lu Rong (*Cornu Cervi Pantotrichum*), 3 g; Gou Qi Zi (*Fructus lycii*), 15 g; Che Qian Zi (*Plantago asiatica* L.), 12 g; Wu Wei Zi (*Fructus Schisandrae*), 12 g; Jiucaizi (*Semen Allii Tuberosi*) 15 g; Buguzhi (*Fructus Psoraleae*) 12 g; Shan Zha (*Crataegus pinnatifida*), 9 g; Sha Ren (*Fructus Amomi*), 15 g; Yin Yang Huo (*Herba Epimedii*), 30 g; San Qi (*Panax notoginseng*), 6 g; Fu Ling (*Poria cocos* (Schw.) Wolf), 12 g; and He Tao Ren (*Juglandis Semen*), 20 g. The soluble granules of SJS were dissolved in sterile 0.9% saline solution to the desired concentrations just before the experiments.

Sexually mature male Sprague-Dawley rats weighing 300 to 320 g at the beginning of the experiment were provided by the Department of Experimental Animal Sciences of the Peking University Health Science Center. The rats were housed in separated cages with free access to food and water. The room temperature was kept at $24 \pm 1^\circ\text{C}$ under a natural light/dark cycle. All animal experimental procedures were approved by the Animal Care and Use Committee of Peking University.

Rat Model of Asthenozoospermia

A rat model of asthenozoospermia was accomplished by intraperitoneal injection of cyclophosphamide (CP), according to the method previously described elsewhere with some modifications (30). Briefly, CP at a dose of 35 mg/kg body weight was intraperitoneally injected into adult male rats once a day for a period of 5 days. The control rats received a normal saline solution throughout the experiment. At 14 days after the last injection, the animals were killed by decollation, and the epididymides were quickly removed for further examination. Development of asthenozoospermia in the rats was determined by assessment of the sperm motility and count.

Study Design and SJS Treatment

Sixty rats were randomly divided into five groups with 12 rats per group: CP, NS (normal saline solution), CP + SJS, CP +

TABLE 1

Composition and origins of Sheng-Jing San (SJS).

Chinese name	Alternative name	Pharmaceutical latin	Type of compound	Dosage (g)
Shu Di Huang	Shu Di	Radix Rehmanniae Preparata	Prepared Rehmannia root	20
Lu Rong	Ban Long Zhu	Cornu Cervi Pantotrichum	Hairy antler	3
Gou Qi Zi	Chinese wolfberry; Barbary wolfberry fruit	<i>Fructus lycii</i>	Fruit of Barbary wolfberry	15
Che Qian Zi	Plantain seed	<i>Plantago asiatica</i> L.	Plantago seeds	12
Wu Wei Zi	Fructus Schisandrae Chinensis	<i>Schisandra chinensis</i> (Turcz.) Baill.	Schisandra fruit	12
Jiucazi	Leek seeds	Semen Allii Tuberosi	Seeds of <i>Allium tuberosum</i> Rottler ex Spreng	15
Buguzhi	Fructus Psoraleae; Malaytea scurfpea fruit	<i>Psoralea corylifolia</i> L.	Fruit of Malaytea scurfpea	12
Shan Zha	Fructus crataegi	<i>Crataegus pinnatifida</i>	Hawthorn fruit	9
Sha Ren	Bastard cardamom	Fructus Amomi	Amomum fruit	15
Yin Yang Huo	Horny goat weed	Herba Epimedii	Epimedium leaves	30
San Qi	Tian Qi	<i>Panax notoginseng</i> (Burk.); Radix Notoginseng	Root of <i>Panax notoginseng</i> (Burk.)	6
Fu Ling	Poria; Indian bread; Tuckahoe; <i>Poris cocos</i>	<i>Wolfiporia cocos</i>	Fungi; <i>Poria cocos</i> (Schw.); Wolf of Polyporaceae	12
He Tao Ren	Hu Tao Ren; Hu Tao Rou; Juglandis semen; English walnut seed	Semen Juglandis Regiae; <i>Juglans regia</i> L. (Juglandaceae)	Seeds of <i>Juglans regia</i> L.	20

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NS, and treatment naïve. In the CP group, CP at the dose of 35 mg/kg was intraperitoneally injected into rats once a day for a period of 7 days; the rats were then normally fed for 14 days. In NS group, 0.9% saline solution instead of CP was injected into the rats as a control. In the CP + SJS group, CP was first injected at the same manner as described in CP group, then soluble granules of Sheng-Jing-San (SJS) at the dose of 30 mg/kg (in 1-mL 0.9% saline solution) was intragastrically administered to the rats once a day for 14 days. In the CP + NS group, after the last injection of CP, 1 mL of saline solution instead of SJS was administered as a control. In treatment naïve group, rats were fed normally for 21 days as a naïve control. On day 21, all animals were killed by decollation, and the epididymides were quickly removed for further examination.

Sperm Motility and Count

To assess the sperm motility, corpus epididymal sperm cells were collected and prepared as described elsewhere (30). In brief, one caudal epididymis was placed in modified HEPES medium containing 120 mM NaCl, 2 mM KCl, 1.2 mM MgSO₄·7H₂O, 0.36 mM NaH₂PO₄, 25 mM NaHCO₃, 10 mM HEPES, 5.6 mM glucose, and 1.1 mM sodium pyruvate as well as penicillin (100 IU/mL) and streptomycin (100 µg/mL), adjusted to pH 7.4 with NaOH. The cauda was cut into two to three pieces and incubated at 37°C for 10 minutes in a CO₂ incubator. The sperm was gently filtered through nylon gauze, centrifuged, and resuspended in 1-mL fresh M199 medium (Sigma Chemical), from which 10 µL of the sperm suspension was used for assessment of sperm motility and count by computer-assisted sperm assay (CASA) with a sperm motility analyzer (CASA-QH-III, Qinhua Tongfang, China). The following parameters of sperm motility were evaluated: progressive motility (grade A + B) (%), straight-line velocity (VSL, µm/s), curve-line velocity (VCL, µm/s), average path velocity (VAP, µm/s), beat cross frequency (BCF, Hz), amplitude of lateral

head displacement (ALH, µm), linearity (LIN, %), and straightness (STR, %). Sperm concentration, expressed as ×10⁶/mL, was determined by use of the hemocytometer method on two separate preparations of the semen sample.

Sperm viability was visualized by eosin and nigrosin staining (31). Briefly, one drop of sperm suspension was mixed with two drops of 1% eosin Y. After 30 seconds, three drops of 10% nigrosin were added and mixed well. A smear was made by placing a drop of the mixture on a clean glass slide, which was allowed to air dry. The prepared slide was examined. Pink-stained dead spermatozoa and unstained live spermatozoa were counted under the light microscope. The viability of spermatozoa was expressed as the percentage of viable spermatozoa.

Western Blot

Rats were deeply anesthetized with 10% chloral hydrate (0.3 g/kg IP), and the epididymides were removed and immediately homogenized in ice-chilled lysis buffer containing 50 mM Tris (pH 7.4), 250 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA, pH 8.0), 0.5% NP40 (Sigma), 1 mM phenylmethanesulfonyl fluoride (PMSF; Sigma), and 4 mM NaF. The homogenates were centrifuged at 1,200 × g for 10 minutes at 4°C, and the supernatant was analyzed. The concentration of protein was measured with a bicinchoninic acid (BCA) assay kit (Pierce/Thermo Scientific), and an equal amount of protein samples (20 µg) were denatured and then separated through sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% running gels and transferred to a polycyridene difluoride filters (PVDF) membrane (Bio-Rad Laboratories). After blocking with 5% nonfat milk in TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) for 60 minutes at room temperature, the membranes were incubated with primary antibody, rabbit anti-CatSper1 antibody (1:100, Santa Cruz Biotechnology), or mouse anti-GAPDH (1:1,000, Santa Cruz Biotechnology)

at 4°C overnight. The blots were washed in TBST and then incubated in HRP-conjugated goat anti-rabbit IgG (1:300, Santa Cruz Biotechnology). Protein bands were visualized using an enhanced chemiluminescence detection kit (ECL; Santa Cruz Biotechnology) followed by autoradiography using Hyperfilm MP (Santa Cruz Biotechnology). The standardized ratio of CatSper1 to GAPDH band density was used to calculate the alteration in CatSper1 expression.

Real-Time RT-PCR Assay

For the RNA extraction, the total RNA from the liquid-nitrogen-frozen epididymides were extracted with Trizol Reagent (Invitrogen) according to the manufacturer's instructions. The purity of total RNA was checked spectrophotometrically at 260 and 280 nm. A minimum optical density (OD 260/280) ratio 1.80 was required for the following real-time reverse-transcriptase polymerase chain reaction (RT-PCR).

For the RT-PCR, the total RNA (1.0 µg) was reverse transcribed to complementary DNA by use of oligo dT primers and MMLV reverse transcriptase (Promega). The complementary DNAs were further amplified by PCR using the following primers for CatSper1: forward 5'-TTT ACC TGC CTC TTC CTC TTC T-3' and reverse 5'-ACC AGG TTG AGG AAG ATG AAG T-3'.

The quantitative real-time RT-PCR was performed on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems) with SYBR Premix Ex Taq II (TaKaRa) according to the protocol previously described elsewhere (32). In brief, a 20-µL PCR reaction was used that included 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 200 µM dNTPs, 1.5 IU TaKaRa TaqTM DNA polymerase, 0.4 × SYBR Green 1 (Invitrogen), and 0.15 µM of each primer. We used β-actin in parallel for each run as an internal control. A four-step experimental run protocol was used, and the amplification conditions were as follows: 95°C for 10 minutes (initial denaturation), 35 cycles of 25 seconds at 95°C (denaturation), 30 seconds at annealing temperature, 30 seconds at 72°C (elongation), and 8 seconds at fluorescence measurement temperature (60°C). A melting curve was generated at the end of every run to ensure product uniformity. Relative quantification of the expression of genes was calculated using the 2^{-ΔΔC_t} method (33), where C_t is the cycle threshold. The ΔC_t was the difference between the PCR product and its internal control (β-actin). The ΔΔC_t was the difference between the naïve group and the other two groups. Data are expressed as 2^{-ΔΔC_t}, that is, the fold change in CatSper1 mRNA.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism 5 for Windows (GraphPad Software). All data were expressed as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test was used for multiple comparison. *P* < .05 was considered statistically significant.

RESULTS

Effects of CP and SJS on Sperm Motility

Computer-assisted sperm assay (CASA) revealed that except for linearity (LIN), all parameters of sperm motility statisti-

cally significantly decreased in the CP-treated rats as compared with naïve or saline solution (NS) control rats (*P* < .05-.001) (Fig. 1). A statistically significant decrease in the proportion of progressive motility (including grade A, rapid progressive, and grade B, slow progressive) was found in the CP-treated rats (23.3% ± 1.4%) in contrast to the naïve (45.6% ± 1.8%) or NS (44.4% ± 2.0%) control rats (*P* < .001) (see Fig. 1A). A decrease of 15% to 35% was observed for the velocity parameters of straight-line velocity (VSL) (see Fig. 1B), curve-line velocity (VCL) (see Fig. 1C), and average path velocity (VAP) (see Fig. 1D). The lower velocity of the CP-treated spermatozoa could be due to a reduction of the beat cross frequency (BCF) (~31%) (see Fig. 1E) and the amplitude of lateral head displacement (ALH) (~28%) (see Fig. 1F) in the CP-treated spermatozoa in comparison with the naïve or NS-treated spermatozoa.

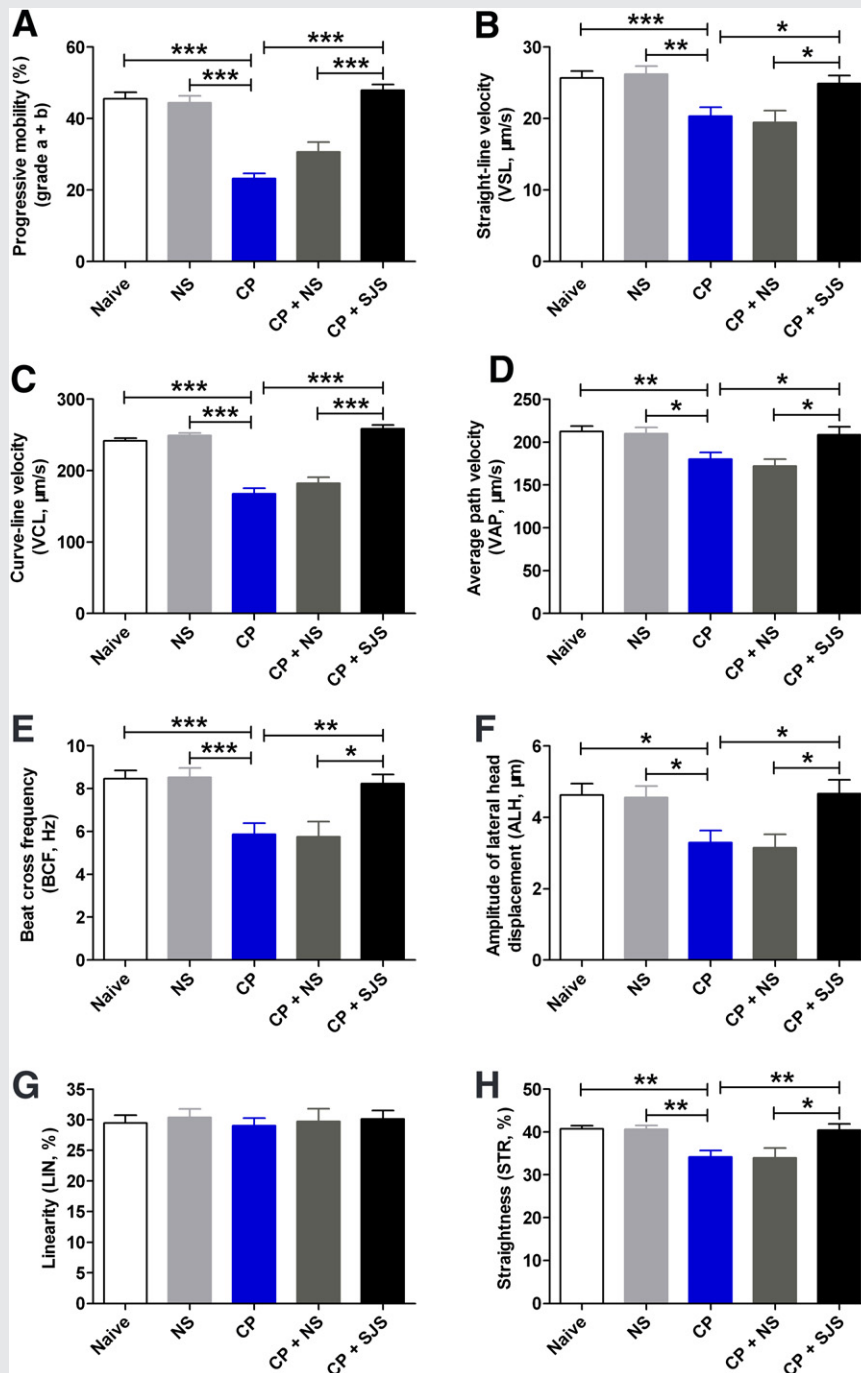
For the sperm swimming patterns, the straightness (STR) (see Fig. 1H) was approximately 16% diminished (*P* < .01), but no statistically significant alteration was observed in the linearity (LIN) (see Fig. 1G) in the CP-treated spermatozoa compared with the naïve or NS-treated spermatozoa. These results showed that CP induced a prominent decrease in the sperm motility, indicating the development of asthenozoospermia in rats.

We further examined the effect of soluble granules of SJS on CP-induced asthenozoospermia in rats. We found that all the CP-induced decreases in parameters of sperm motility were restored by SJS (as shown in the CP + SJS group) when compared with vehicle control (as shown in the CP + NS group) (*P* < .05-.001) (see Fig. 1). These results suggest that SJS exerts an ameliorative effect on CP-induced asthenozoospermia in rats. Moreover, we found that CP also induced a statistically significant reduction both in sperm concentration and in sperm viability, and these effects of CP were markedly reversed by SJS (*P* < .05-.001) (Fig. 2). These results imply that, apart from the sperm motility, CP also impaired the spermatogenesis and quality of spermatozoa and that SJS exerted a similar reversal effect on CP-induced decreases in sperm concentration and sperm viability.

Effects of CP and SJS on CatSper1 Protein and mRNA Expression

To further investigate whether the CP-induced asthenozoospermia in rats was due to a down-regulation of CatSper1 expression in spermatozoa, and whether SJS exerted its ameliorative effect on asthenozoospermia through restoring the CP-induced reduction of CatSper1 expression, we examined the protein and mRNA expression of CatSper1 in the rat spermatozoa treated with CP, SJS, and control using Western blot analysis and quantitative real-time RT-PCR assay, respectively. As shown in Figure 3A, Western blotting detection revealed that CP induced a prominent reduction of CatSper1 protein expression (0.77 ± 0.04) compared with naïve (1.07 ± 0.09) or NS (1.02 ± 0.08) controls (*P* < .05). The down-regulated effect of CP on CatSper1 protein expression was markedly restored by SJS (1.11 ± 0.10) but not by the vehicle control (0.91 ± 0.03) (*P* < .01, CP + SJS versus CP; *P* < .05, CP + SJS versus CP + NS). Similarly, the real-time RT-PCR assay revealed a statistically significant reduction of CatSper1

FIGURE 1



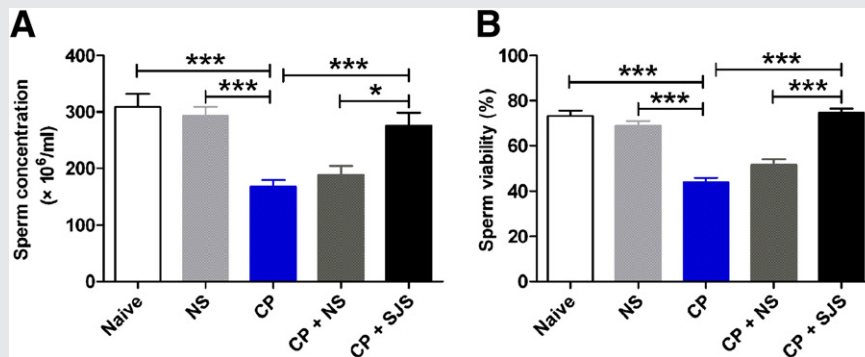
Computer-assisted analysis of sperm motility in control and experimental rats. (A) Progressive motility (grades A + B). (B) Straight-line velocity (VSL). (C) Curve-line velocity (VCL). (D) Average path velocity (VAP). (E) Beat cross frequency (BCF). (F) Amplitude of lateral head displacement (ALH). (G) Linearity (LIN). (H) Straightness (STR). Note that except for linearity, cyclophosphamide (CP) induced a statistically significant reduction in all parameters of sperm motility and the effects of CP were markedly restored by soluble granules of Sheng-Jing-San (SJS). * $P < .05$; ** $P < .01$; *** $P < .001$, one-way ANOVA followed by Bonferroni multiple comparison test, $n = 12$ rats in each group. Grade A, rapid progressive motility; grade B, slow progressive motility.

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mRNA expression in the epididymal spermatozoa of CP-treated rats (0.41 ± 0.05) compared with naïve (0.62 ± 0.05) or NS (0.61 ± 0.03) control rats ($P < .05$) (see Fig. 3B).

The CP-induced down-regulation of *CatSper1* mRNA expression was also dramatically restored by SJS (0.60 ± 0.02) but not by the vehicle control (0.39 ± 0.07) ($P < .05$) (see Fig. 3B).

FIGURE 2



Analyses of sperm concentration and viability in control and experimental rats. (A) Sperm concentration. (B) Sperm viability. Note that cyclophosphamide (CP) induced a statistically significant reduction both in sperm concentration and sperm viability, and the effects of CP were markedly restored by soluble granules of Sheng-Jing-San (SJS). * $P < .05$; *** $P < .001$, one-way ANOVA followed by Bonferroni multiple comparison test, $n = 12$ rats in each group.

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These results demonstrate that CP induced a statistically significant reduction of CatSper1 protein and mRNA expression, and SJS effectively restored the down-regulated effects of CP on CatSper1 expression. Together with the aforementioned data, our results suggest that repression of CatSper1 channels in spermatozoa likely contributes to the CP-induced asthenozoospermia in rats, while the up-regulation of CatSper1 expression by SJS was probably involved in the ameliorative effects of SJS on asthenozoospermia.

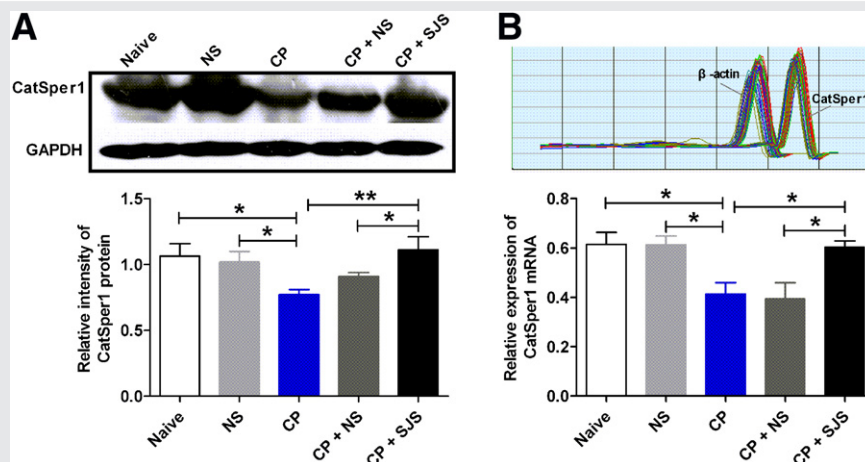
DISCUSSION

In our present study, we have demonstrated for the first time to our knowledge that CatSper1, a family of sperm-specific

CatSper channels, is statistically significantly down-regulated in CP-induced asthenozoospermia in rats. Moreover, we also have clarified that soluble granules of Sheng-Jing-San (SJS), a traditional Chinese medicine recipe, can restore the CP-induced reduction of CatSper1 expression in epididymal spermatozoa, and hence exerts an ameliorative effect on asthenozoospermia. These data indicate that the down-regulation of CatSper1 channels in spermatozoa is associated with the pathogenesis of asthenozoospermia in rats; thus, the up-regulation of CatSper1 channels by SJS appears to ameliorate sperm motility and work against the CP-induced asthenozoospermia.

We have validated the CP-induced rat model of asthenozoospermia by providing direct evidence that, except for

FIGURE 3



Expression of CatSper1 protein and mRNA in epididymal spermatozoa in control and experimental rats. (A) Western blotting detection of CatSper1 protein expression. Upper: representative of Western blotting bands. Lower: analysis of the relative intensity of CatSper1 protein. We used GAPDH as an internal control. (B) Real-time RT-PCR assay of CatSper1 mRNA expression. Upper: representative of melting curve. Lower: summary of relative CatSper1 mRNA expression that was normalized to the internal control, β -actin. Note that cyclophosphamide (CP) induced a statistically significant down-regulation of CatSper1 protein and mRNA expression in rat epididymal spermatozoa, and the effects of CP were markedly restored by soluble granules of Sheng-Jing-San (SJS). * $P < .05$; ** $P < .01$, one-way ANOVA followed by Bonferroni multiple comparison test, $n = 5$ rats in each group.

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linearity (LIN), all parameters of sperm motility are statistically significantly decreased in CP-exposed rats. As an anti-cancer alkylating agent, CP is known to be a male reproductive toxicant (34). Male cancer patients treated with CP exhibit an increased incidence of oligospermia and azoospermia (35, 36). Studies in rats or mice also have shown that animals exposed to CP become oligospermic and azoospermic, manifested as dramatic decreases in sperm count and motility (30, 34, 37–39). Hence, the diminished parameters of sperm motility in our current findings confirm the development of the CP-induced rat model of asthenozoospermia.

Using the established model of CP-induced asthenozoospermia in rats, we documented a prominent reduction in the expression of the CatSper1 channel in epididymal spermatozoa. We provide direct evidence showing that both of the CatSper1 protein and mRNA expression statistically significantly decrease in epididymal spermatozoa in CP-exposed rats. Certainly, as there were fewer sperm in the CP-treated rats, and the analysis of CatSper1 protein and mRNA was performed on homogenated epididymis, we could not make sure that the decrease in CatSper1 protein and mRNA was not simply due to fewer sperm being homogenized relative to other epididymal cell types. Hence, further studies on purified sperm will be needed.

Several studies have now demonstrated that the CatSper1 channel is required for depolarization-evoked Ca^{2+} entry and for hyperactivated movement, a key flagellar function of spermatozoa (6, 15). It is well accepted that Ca^{2+} signaling serves as a central regulator in many key activities of sperm cells, including capacitation, hyperactivation, chemotaxis, and acrosome reaction (1). Therefore, repression of the CatSper1 channel in sperm cells impairs Ca^{2+} signaling, which in turn causes impairment of the sperm motility and hyperactivation associated with asthenozoospermia and male subfertility (2, 3). In support of this notion, male mice deficient in the *Catsper1* gene have been shown to be impaired both in sperm motility and hyperactivation, and they are completely infertile (6, 9). Mutations in the *Catsper1* channel protein have also been identified as a cause of human male infertility, probably resulting from diminished sperm motility (14).

In our study, we only detected a reduction of CatSper1 protein and mRNA expression in the epididymal spermatozoa of CP-induced asthenozoospermic rats. However, whether the functional characteristics or intrinsic electrophysiologic properties of the CatSper1 channel also decrease in CP-exposed rats needs to be further studied. In addition, because there is no selective antagonist targeting for the CatSper1 channels yet, up-regulation of the CatSper1 channels in CP-exposed rats through overexpression of the CatSper1 gene and down-regulation of CatSper1 channels in naïve rats via mutation or knockdown of the CatSper1 gene need to be studied to provide direct evidence that functional down-regulation of CatSper1 channels contributes to CP-induced asthenozoospermia in rats.

Although we have identified a remarkable down-regulation of CatSper1 expression in the epididymal spermatozoa of CP-induced asthenozoospermic rats, other members of

the CatSper family such as the CatSper2, CatSper3, and CatSper4 channels may also play important roles in the pathogenesis of asthenozoospermia. It has been documented that in addition to the *Catsper1* channel, expression of CatSper2, CatSper3, and CatSper4 channels are detected exclusively in the testis (7, 8, 40). Coincidentally, *Catsper3* and *Catsper4* knockout male mice demonstrate infertility from a quick loss of motility and a lack of hyperactivated motility under capacitating conditions (41). The involvement of *Catsper2* in asthenoteratozoospermia has also been identified in a human autosomal gene defect associated with nonsyndromic male infertility, which indicates that disruption of *Catsper2* might underlie highly the reduced sperm motility found in humans (42).

In fact, considerable evidence has accumulated that all four CatSper ion channel proteins are required for male fertility and sperm cell hyperactivated motility (43, 44). For instance, male mice with the CatSper-null mutant have been shown to be infertile because their spermatozoa fail to hyperactivate (44). A significant reduction in the level of CatSper gene expression has also been found among patients who lack sperm motility (45). Accordingly, pharmacologic blockade of the CatSper channels not only prevents the development of sperm hyperactivated motility by capacitating incubations but also causes a reversible loss of flagellar waveform asymmetry to prehyperactivated sperm (46).

Taken together, our present study provides a novel molecular mechanism underlying the pathogenesis of asthenozoospermia, where the down-regulation of CatSper channels in the spermatozoa may contribute to the impairment of sperm motility and eventually to the development of asthenozoospermia. As one of the important factors that cause infertility, asthenozoospermia, which is characterized by reduced sperm motility, has no effective treatment so far (47). In this study, we have provided experimental evidence that soluble granules of Sheng-Jing-San (SJS), a traditional Chinese medicine recipe, exert an ameliorative effect on CP-induced asthenozoospermia in rats.

In a rat model of CP-induced asthenozoospermia, we have documented that all the CP-induced decreases in sperm motility parameters are restored by SJS. In addition, we found a reversal effect of SJS on the CP-induced decreases in sperm concentration and sperm viability. These data are line with previous clinical observations that SJS treatment can significantly improve oligozoospermia and asthenozoospermia (16–19). The effect of SJS on spermatogenesis has been documented in murine models of oligozoospermia (20–22). We provide convincing evidence that SJS can effectively restore the CP-induced down-regulation of CatSper1 expression in epididymal spermatozoa in rats with asthenozoospermia. These findings therefore validate our hypothesis that SJS exerts its therapeutic effect on asthenozoospermia by up-regulating CatSper1 channels in the spermatozoa.

Previous studies have documented that SJS contains a large number of trace elements, including Se, Zn, Mn, Fe, Cu, Ti, and Ca (23), all of which are required for spermatogenesis, and maturation and quality of spermatogenic cells (24–28). A separate study has demonstrated that Se treatment in male mice can up-regulate the expression of

CatSper genes, thus resulting in elevation of sperm motility (29). Supplemental Se and vitamin E have been reported to potentially improve semen quality and to have beneficial and protective effects, especially on sperm motility (48). Selenium is now suggested as useful for the treatment of idiopathic male infertility, diagnosed as asthenoteratospermia or asthenospermia by semen analysis (25, 48, 49). With these findings together with our data, we would propose that SJS treatment's improvement of asthenozoospermia may occur via up-regulation of CatSper1 channels in the spermatozoa. Whether the treatment of asthenozoospermia via SJS administration involves other members of the CatSper channel family such as CatSper2, CatSper3, or CatSper4 needs to be further studied. In addition, because the CP presumably also acts at the testicular level, we cannot exclude the possibility that SJS exerts some effect at the testicular level with other CatSper channels.

In summary, we have found a statistically significant reduction of the CatSper1 channel, which is associated with an impairment of sperm motility in epididymal spermatozoa in CP-induced asthenozoospermic rats. Moreover, we have shown that SJS may improve the sperm motility of asthenozoospermic rats by restoring the CP-induced decrease in CatSper1 expression in epididymal spermatozoa. This study suggests that down-regulation of the CatSper1 channel in epididymal spermatozoa likely contributes to the pathogenesis of asthenozoospermia, while up-regulation of the channel by SJS improves sperm motility and therefore can be used as an effective therapeutic strategy for the treatment of male infertility diagnosed as asthenozoospermia.

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