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ORIGINAL ARTICLE

Sperm Biology

Panax ginseng induces the expression of *CatSper* genes and sperm hyperactivation

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The cation channel of sperm (*CatSper*) protein family plays important roles in male reproduction and infertility. The four members of this family are expressed exclusively in the testis and are localized differently in sperm. To investigate the effects of *Panax ginseng* treatment on the expression of *CatSper* genes and sperm hyperactivation in male mice, sperm motility and *CatSper* gene expression were assessed using a computer-assisted semen analysis system, a Fluoroskan Ascent microplate fluorometer to assess Ca^{2+} influx, real-time polymerase chain reaction, Western blotting and immunofluorescence. The results suggested that the Ca^{2+} levels of sperm cells treated with *P. ginseng* were increased significantly compared with the normal group. The *P. ginseng*-treated groups showed increased sperm motility parameters, such as the curvilinear velocity and amplitude of lateral head displacement. Taken together, the data suggest that *CatSper* messenger ribonucleic acid levels were increased significantly in mouse testes in the *P. ginseng*-treated group, as was the protein level, with the exception of *CatSper2*. In conclusion, *P. ginseng* plays an important role in improving sperm hyperactivation via *CatSper* gene expression.

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INTRODUCTION

Fertilization is the process in which sperm and egg combine. The sperm penetrates the zona pellucida of the egg, initiating the development of a new organism.¹ Hyperactivated motility assists in the process of fertilization *in vivo* by allowing sperm to reach the oocyte through mucus-filled passages, in addition to helping the sperm penetrate the zona pellucida.² A computer-assisted semen analysis (CASA) system has been developed to detect hyperactivation and to confirm the percentage of hyperactivated sperm in a sample. It measures the following motion parameters: curvilinear velocity (VCL, $\mu\text{m s}^{-1}$), average-path velocity, $\mu\text{m s}^{-1}$, straight-line velocity, $\mu\text{m s}^{-1}$, beat cross frequency, Hz, straightness, amplitude of lateral head displacement (ALH, μm) and linearity. An increased VCL and ALH are indicative of hyperactivation.^{3,4}

Cations – such as Na^+ , K^+ and Ca^{2+} – are involved in regulating sperm motility and fertility. A sperm-specific Na^+/H^+ exchanger located at the principal component of the flagellum is required for motility and fertility.⁵ A rapid change in sperm motility is accomplished by the rapid diffusion of K^+ and Ca^{2+} and Ca^{2+} across the sperm plasma membrane through selective ion channels.⁶

Calcium ion signaling affects all aspects of cellular life and death. Ca^{2+} regulates mitochondrial function, innate immunity, motility, transcription, viability and apoptosis.⁷ Ca^{2+} is commonly required for motility in epididymal sperm samples and Ca^{2+} regulates the activated and hyperactivated motility of ejaculated sperm.^{8–13} Intracellular Ca^{2+} stores are the main concern, particularly in hyperactivated motility regulation. Flagellar wave symmetry in permeabilized sperm is increased by Ca^{2+} , which, at sufficiently high levels, inhibits motility.¹⁴ Further, Ca^{2+} is required for hyperactivation.^{15,16}

Members of the *cation channel of sperm (CatSper)* family are expressed solely in spermatozoa. *CatSper1* is localized to the principal piece of sperm and is required for evoked Ca^{2+} entry and hyperactivation control in sperm.¹⁶ *CatSper2* is located in the sperm tail and is essential for regulating hyperactivation.¹⁷ *CatSper3* and 4 are localized in the testes and sperm and are required for the motility of hyperactivated sperm.¹⁸ Studies have localized *CatSper* messenger ribonucleic acids (mRNAs) exclusively to the testes, while *CatSper* proteins were expressed in the testes and sperm.^{15,19,20}

Korean *Panax ginseng* C. A. Meyer is a traditional medicinal plant. In Asia, it is considered the most precious of all medicinal plants. Originally, the efficacy of *P. ginseng* was based on oriental medical science theory.²¹ We reported previously that Korean ginseng induces spermatogenesis in rats via the activation of the cAMP-responsive element modulator. Rats treated with ginseng had a significantly increased epididymal sperm count and sperm motility.²²

However, there are few studies of the effects of *P. ginseng* on sperm hyperactivation in male mice. Therefore, this study investigated the effects of *P. ginseng* treatment on sperm motility and hyperactivation with reference to *CatSper* expression in male mice.

MATERIALS AND METHODS

Chemicals and medium

For the analysis of sperm parameter, the medium consisted of M199 medium (GIBCO, Big Cabin, OK, USA), 0.5% bovine serum albumin (Sigma-Aldrich Co., St. Louis, MO, USA) and 1 mmol l^{-1} pyruvic acid (Sigma-Aldrich Co., St. Louis, MO, USA). CASA (Hamilton Thorne, Beverly, MA, USA), 2X-CEL disposable

sperm analysis chambers (in depths of 80 μm) (Hamilton Thorne, Beverly, MA, USA) were used for analysis of sperm motility, parameters of sperm quality. TaqMan[®] Gene expression master mix (Applied Biosystems, Inc., Woburn, MA, USA) was used for the quantitative polymerase chain reaction (PCR) (Applied Biosystems, Inc., Woburn, MA, USA). And *CatSper* antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were used for Western blot analysis and the immunofluorescence with hematoxylin staining.

Preparation of *P. ginseng* extract

P. ginseng, the root of *P. ginseng* C. A. Meyer was purchased from Won Kwang Herbal Drug Co. Ltd. (Seoul, Korea). Three hundred grams of dried *P. ginseng* were boiled with six liter of water for 2 h at 100°C and then the suspension was filtered and concentrated under reduced pressure. The filtrate was lyophilized and yielded 76.5 g (25.5%) of powder, which was kept at 4°C. Before each experiment, dried extract was dissolved in distilled deionized water (Millipore, Billerica, MA, USA) and vortexed for 2 min at room temperature.

Animal experiment

Animals

Five-week-old male C57BL/6J mice were purchased from SLC Inc. (Hamamatsu, Japan). The animals were housed in a specific pathogen-free environment with a 12/12-h light/dark cycle. Animals had free access to standard rodent pellets (Purina, Bundang-gu, Gyeonggi-do, Korea) and water. Animal care and experimental procedures followed the requirements in the "Guide for the Care and Use of Laboratory Animals" (Department of Health, Education and Welfare, National Institutes of Health, 1996), which was approved by Institutional Review Board of College of Korean Medicine in Kyung Hee University.

Treatment of *P. ginseng*

After 7 days of adaptation to the environment, the mice were divided into two groups: normal group (vehicle-treated, $n = 8$) and *P. ginseng* group (PG) (100, 500, 1000 mg kg^{-1} , $n = 8$). *P. ginseng* was treated for 5 days a week for 5 weeks. The animals were weighed weekly in order to adjust the gavages volume and to monitor their general health.

Sperm preparation

Mice were killed by CO_2 asphyxiation and cervical dislocation. Sperm were collected as previously described.¹⁰ Briefly, epididymal caudal and ductus deferens sperm were punctured with a 30-gauge needle and incubated at 37°C to allow sperm to disperse into surrounding medium.

Sperm analysis

Epididymal motility was evaluated using the method described by Connolly *et al.*,²³ with some modifications. For assessment of sperm motility, sperms were recovered from excised ductus deferens, caudal epididymides and allowed to capacitate for 90 min in media at 37°C. For the confirmation of *P. ginseng* effect on sperm motility, sperms were incubated in medium containing 10 mmol l^{-1} 1, 2-bis-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetra-acetic acid (BAPTA) for 1 min. Sperms were scored as motile if any movement was detected and used to analyze the motility, VCL and ALH by CASA system.

Ca^{2+} flux assay

Epididymal caudal and ductus deferens sperm were used for intracellular Ca^{2+} levels measurement, as previously described.²⁴

Epididymal caudal sperm from the mouse were minced in sperm washing media incubated for 90 min at 37°C. The Ca^{2+} levels outcomes produced by manual evaluation using the Fluoroskan Ascent Microplate Fluorometer (Thermo, Marietta, OH, USA). Epididymal caudal sperm suspensions were loaded with Fluo-4 NW Calcium assay kits. For Fluo-4 NW, emission intensity was monitored at 485 nm and 538 nm as the wavelength pair.

RNA isolation and real-time polymerase chain reaction

One milliliter of trizol was added to the testis tissue samples. RNA samples were analyzed by denaturing formaldehyde/agarose/ethidium bromide gel electrophoresis. The final amount of RNA was estimated by spectrophotometer (Molecular Devices, Downingtown, PA, USA) at 260 nm. First strand cDNA synthesis with 5 μg of total RNA was performed using Moloney Murine leukemia virus 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.*²⁵

Real-time PCR was performed in a Step one plus System Thermal Cycler (Applied Biosystems, Inc., Woburn, MA, USA). Real-time PCR was performed on a volume of 20 μl containing 2 μl (200 ng) of cDNA and 10 μl of PCR master mix, 1 μl of each taqman probe and 7 μl of diethyl pyrocarbonate-treated water. Gene expression assay mixes for *CatSper1-4* and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Applied Biosystems [assay ID: Mm00460530_m1 (*CatSper1*), Mm00467632_m1 (*CatSper2*), Mm00712792_m1 (*CatSper3*), Mm01190761_m1 (*CatSper4*) and Mm99999915_g1 (GAPDH)]. The program was set at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 60 s. Samples were amplified with GAPDH primers for determination of the initial relative quantity (RQ) of cDNA in each sample and then all PCR products were normalized to that amount. Samples were amplified in triplicate, averages were calculated and differences in cycle threshold (Ct) data were evaluated by Sequence Detection Software V1.3.1 (Applied Biosystems, Inc., Woburn, MA, USA). For data analysis, we used the comparative Ct method with the following formula: $\Delta\text{Ct} = \text{Ct} (\text{Target, TLR}) - \text{Ct} (\text{Endo, GAPDH})$. Data are expressed as RQ and differences are shown in the figures as the expression ratio of the normalized target gene, according to the software results.

Western blot analysis

Proteins from homogenized testes were separated using nuclear extract kit according to manufacturer's protocol with minor modifications (Active and Motif, Carlsbad, CA, USA). The protein concentrations were determined by Bradford method.²⁶ The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed as described previously.²⁷ 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 mmol l^{-1} Tris and 250 mmol l^{-1} glycine containing 20% methanol, pH 8.3. Transfer was performed at a constant voltage of 20 mA for 1 h. After transfer, membranes were blocked in phosphate buffered saline (PBS) containing 0.05% Tween PBS-T with 5% skim milk for 2 h at room temperature and incubated with the primary antibody for *CatSper 1* (sc-21180, 1:1000), *CatSper 2* (sc-98539, 1:1000), *CatSper 3* (sc-98818, 1:500) and *CatSper 4* (sc-83126, 1:500) in PBS-T overnight at 4°C. After incubation, the membranes were rinsed 3 times with 1 \times PBS and incubated with conjugated donkey anti-goat

IgG (*CatSper1*, 4) and conjugated anti-rabbit IgG (*CatSper2*, 3) for 1 h at room temperature followed by three rinses with $1 \times$ PBS. *CatSper* antibodies were validated by immunofluorescence staining using mouse spermatozoa.

Immunofluorescence detection with hematoxylin staining

Immunofluorescence detection with hematoxylin staining was performed according to the procedure described previously.²⁸ For immunofluorescence detection with hematoxylin staining studies, the testes were fixed overnight in Bouin's solution, dehydrated in 70%, 80%, 95%, 100% ethanol, xylene and embedded in paraffin, and 7 μ m thick tissue sections. The sections were deparaffinized and rehydrated in xylene, 100%, 95%, 80%, 70% ethanol. The sections were then treated in a microwave oven in 10 mmol l⁻¹ citrate buffer, pH 6.0, for 12 min. After three washes in PBS, endogenous peroxidase activity was quenched by 3% hydrogen peroxide in PBS for 20 min and again washed 3 times in PBS. Sections were then incubated in a blocking (saponin 0.5 mg in gelatin 2 mg ml⁻¹) for 1 h in order to block nonspecific binding. Subsequently, sections were incubated for overnight at room temperature with *CatSper 1* (sc-21180, 1:100), *CatSper 2* (sc-98539, 1:100), *CatSper 3* (sc-98818, 1:100) and *CatSper 4* (sc-83126, 1:100) in a humidified chamber. Sections were washed 3 times in PBS before being incubated with the appropriate secondary antibody [Cy3-conjugated anti-rabbit 1:500 (*CatSper2*, 3), Cy3-conjugated anti-biotin 1:500 (*CatSper1*, 4)] for 1 h at room temperature. Samples were washed 3 times in PB and covered with microscopy coverslips on mounting. All samples were counterstained with hematoxylin stain (Sigma-Aldrich Co., St. Louis, MO, USA).

Statistical analysis

All quantitative data derived from this study were analyzed statistically. The results were expressed as the mean \pm standard deviation.

Differences between groups were assessed by one-way ANOVA using the SPSS software package for Windows (SPSS Inc., Chicago, IL, USA). Statistical significance at $P < 0.001$, <0.01 and <0.05 has been given respective symbols in the tables or figures.

RESULTS

Effects of *Panax ginseng* on sperm motility parameters

The sperm motility values of normal and *P. ginseng*-treated (100, 500 and 1000 mg kg⁻¹) groups were 52.96% \pm 4.09% versus 63.84% \pm 5.33%, 64.51% \pm 6.09%, and 66.23% \pm 4.63%, respectively; all $P < 0.05$. The *P. ginseng* treatment increased sperm motility significantly compared with the normal group. The sperm motility in mice treated with 10 mmol l⁻¹ BAPTA was decreased significantly compared to the normal group (10.12% \pm 2.72% vs. 52.96% \pm 4.09%, $P < 0.001$). The groups treated with *P. ginseng* (100, 500 and 1000 mg kg⁻¹) showed increased sperm motility compared to the BAPTA (10 mmol l⁻¹) control group (10.12% \pm 2.72% vs. 30.38% \pm 5.41%, 23.81% \pm 4.12% and 27.48% \pm 4.26%, $P < 0.05$, 0.05, 0.01, respectively; **Figure 1a**). The VCL of the normal and *P. ginseng*-treated (100, 500 and 1000 mg kg⁻¹) groups were 106.36 \pm 5.08 versus 118.48 \pm 11.15, 116.98 \pm 11.10 and 114.89 \pm 9.99 μ m s⁻¹, respectively; all $P < 0.05$. Sperm treated with BAPTA had a significantly decreased VCL compared with the normal group (106.36 \pm 5.08 vs. 50.91 \pm 6.01 μ m s⁻¹, $P < 0.001$). In addition, the BAPTA (10 mmol l⁻¹) and *P. ginseng* (100, 500 and 1000 mg kg⁻¹) groups had higher curvilinear velocities than the control group (50.91 \pm 6.01 vs. 80.67 \pm 7.72, 69.24 \pm 3.24 and 78.16 \pm 3.32 μ m s⁻¹, $P < 0.01$, 0.05, 0.01, respectively; **Figure 1b**). The ALH of the normal and *P. ginseng*-treated (100, 500 and 1000 mg kg⁻¹) groups were 8.12 \pm 0.25 versus 8.35 \pm 0.20, 8.56 \pm 0.34 and 8.33 \pm 0.28 μ m, respectively; all $P < 0.05$. The *P. ginseng* treatment significantly increased the ALH compared to the normal group. Sperm treated with BAPTA had a significantly decreased

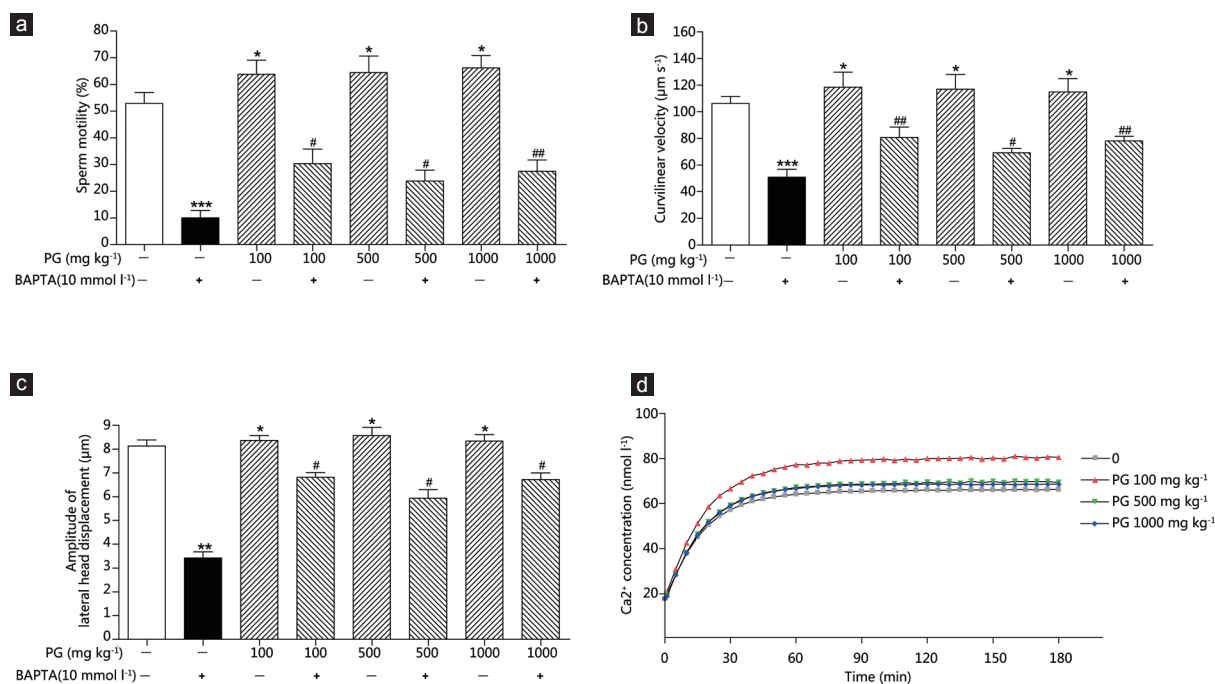


Figure 1: Effects of *Panax ginseng* on sperm motility parameters. Sperm motility are the results for the normal (N) and *P. ginseng*-treated (100, 500 and 1000 mg kg⁻¹) groups in the presence or absence of 10 mmol l⁻¹ 1,2-bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetra-acetic acid. (a) Sperm motility; (b) curvilinear velocity (μ m s⁻¹); (c) amplitude of lateral head displacement (μ m) and (d) Ca²⁺ levels. *Significantly different from the normal value ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$). #The mean is significantly different from the control value ($#P < 0.05$, $##P < 0.01$).

ALH compared to the normal group (3.42 ± 0.25 vs. 8.12 ± 0.25 μm , $P < 0.01$). Furthermore, the BAPTA (10 mmol l^{-1}) and *P. ginseng* (100, 500 and 1000 mg kg^{-1}) groups had a higher ALH than did the control group (3.42 ± 0.25 vs. 6.81 ± 0.20 , 5.93 ± 0.34 and 6.71 ± 0.28 μm , respectively; all $P < 0.05$, **Figure 1c**). In addition, *P. ginseng* treatment increased the intracellular Ca^{2+} levels compared to the normal. The sperm cell Ca^{2+} levels in the *P. ginseng*-treated (100 mg kg^{-1}) group were increased significantly (about 20%) at 60 min and were maintained up to 180 min (**Figure 1d**).

Effect of Panax ginseng on CatSper messenger ribonucleic acid levels by real-time polymerase chain reaction

To examine the effects of *P. ginseng* on *CatSper* mRNA levels in mouse testes, real-time PCR was performed. The total RNA of mouse testes in the 100, 500 and 1000 mg kg^{-1} *P. ginseng*-treated groups was examined. Real-time PCR showed that the *CatSper*1-4 mRNA levels were increased significantly in the *P. ginseng*-treated (100, 500 and 1000 mg kg^{-1}) groups. The 100, 500 and 1000 mg kg^{-1} *P. ginseng*-treated groups had significantly increased *CatSper*1 mRNA levels compared with the normal group (1.00 ± 0.06 vs. 1.22 ± 0.13 , 1.31 ± 0.17 and 1.35 ± 0.14 , $P < 0.05$, $P < 0.01$, respectively; **Figure 2a**). The *CatSper*2 mRNA levels in the *P. ginseng*-treated (100, 500 and 1000 mg kg^{-1}) groups were also increased significantly compared to that in the normal group (1.00 ± 0.03 vs. 1.39 ± 0.08 , 1.23 ± 0.06 and 1.40 ± 0.02 , $P < 0.01$, $P < 0.001$, respectively; **Figure 2b**). Likewise, the *CatSper*3 mRNA levels were higher in the *P. ginseng*-treated (100, 500 and 1000 mg kg^{-1}) groups compared to the normal group (1.0 ± 0.05 vs. 1.19 ± 0.09 , 1.38 ± 0.09 and

1.17 ± 0.04 , $P < 0.05$, 0.01 , 0.01 , respectively; **Figure 2c**). In addition, the 100, 500 and 1000 mg kg^{-1} *P. ginseng*-treated groups showed enhanced *CatSper*4 mRNA levels (1.0 ± 0.10 vs. 1.73 ± 0.15 , 1.52 ± 0.21 and 2.18 ± 0.08 , $P < 0.01$, 0.05 , $P < 0.001$, respectively; **Figure 2d**).

Effects of Panax ginseng on CatSper protein levels in mouse testes by Western blotting

Western blotting was used to determine the effects of *P. ginseng* on *CatSper*1, 2, 3 and 4 protein levels in mouse testes. β -Tubulin was used as the internal control. There were dose-dependent increases in the *CatSper*1 protein levels in the *P. ginseng*-treated (100, 500 and 1000 mg kg^{-1}) groups compared to the normal group (100% vs. 133.31%, 161.20% and 162.34%, respectively, all $P < 0.01$; **Figure 3a**). However, the *CatSper*2 protein levels in the *P. ginseng*-treated (100, 500 and 1000 mg kg^{-1}) groups increased slightly (100% vs. 101.37%, 102.27% and 106.92%, respectively; **Figure 3b**), but the increase did not reach statistical significance. The *CatSper*3 protein levels in the *P. ginseng*-treated (500 and 1000 mg kg^{-1}) groups increased in a dose-dependent manner (100% vs. 134.87% and 139.57%, respectively; both $P < 0.05$), as shown in **Figure 3c**. In addition, the *CatSper*4 protein level was increased significantly by *P. ginseng* treatment (100, 500 and 1000 mg kg^{-1}) compared to the control (100% vs. 119.35%, 126.56% and 125.63%, respectively, all $P < 0.05$; **Figure 3d**).

Effect of Panax ginseng on CatSper protein levels based on immunofluorescence with hematoxylin staining

Immunofluorescence detection of *CatSper*1, 2, 3 and 4 with hematoxylin staining was performed in mouse testes. The *P. ginseng*-treated

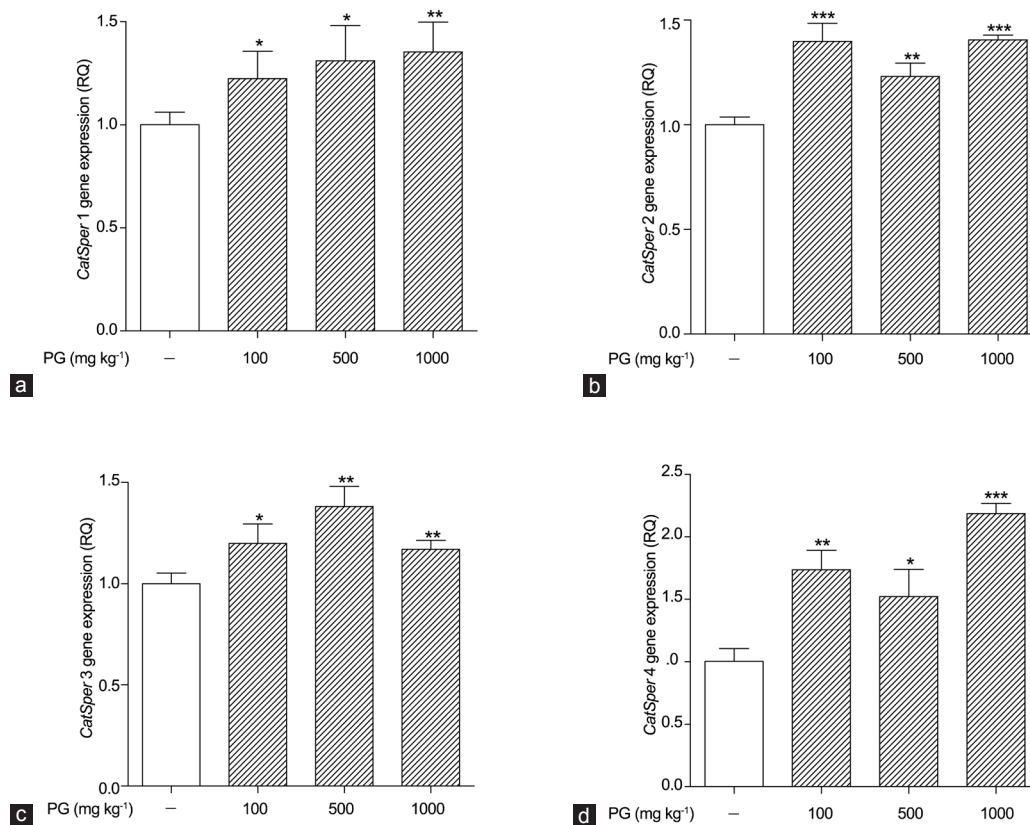


Figure 2: Effect of *Panax ginseng* treatment on *CatSper* messenger ribonucleic acids levels in mouse testes as determined by real-time polymerase chain reaction. Normal and *P. ginseng*-treated (100, 500 and 1000 mg kg^{-1}) groups. (a) *CatSper*1; (b) *CatSper*2; (c) *CatSper*3 and (d) *CatSper*4. Each column represents the mean \pm standard deviation ($n = 3$). The normal group was used as the control (relative quantity, RQ = 1). *Significantly different from the vehicle-treated group (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).

groups (100, 500 and 1000 mg kg⁻¹) had significantly increased dose-dependent levels of *CatSper*1, 3 and 4 proteins. In particular, *P. ginseng* treatment induced *CatSper* expression in mouse testes predominantly in spermatids and spermatozoa, as observed by fluorescence staining. By contrast, *P. ginseng* treatment did not increase *CatSper*2 levels, as determined by Western blotting (Figure 4). In the mouse testes, *CatSper* proteins were in a rounded, positive form (Figure 4a, 4d, 4g and 4j). All samples were counterstained with hematoxylin (Figure 4b, 4e, 4h and 4k). Overlaid *CatSper* immunofluorescence and hematoxylin-counterstained images suggested induction of *CatSper* proteins (Figure 4c, 4f, 4i and 4l).

DISCUSSION

During fertilization, sperm require high-amplitude flagellar bends associated with hyperactivation to penetrate the oocyte zona pellucida.^{29,30} Hyperactivated motility is characterized by asymmetrical flagellar bends of high amplitude and lower frequency, revealed as the swimming pattern shown by most spermatozoa.^{2,10,31,32} The initiation and maintenance of hyperactivated motility is related to an increase in Ca²⁺ concentration in the flagellum.^{8,33–35} Ca²⁺ signaling in sperm is important during fertilization. Ca²⁺ uptake is a process whereby mammalian sperm gain the capacity to undergo the acrosome reaction and fertilize oocytes.^{13,36} Motility activation occurs when sperm are released from the cauda epididymis. Flagellar Ca²⁺ levels during capacitation induce hyperactivation^{34,37} by increasing the amplitude of the principal flagellar bend, which produces asymmetrical beating.⁸ Intracellular Ca²⁺ levels were shown to regulate sperm motility and hyperactivation, capacitation and the acrosome reaction and were regulated by Ca²⁺ chelators, such as BAPTA.^{34,35,38–41} BAPTA reduced the elevation of Ca²⁺ and hyperactivation.⁴² Moreover, BAPTA-treated

sperm had a lower VCL and smaller ALH compared with the normal group.⁴³ In this study, in the presence of BAPTA, the sperm motility parameters were improved with *P. ginseng* treatment, as estimated using the CASA system. *P. ginseng* treatment induced sperm motility in male mice. The sperm motility and related parameters were increased significantly with *P. ginseng* treatment compared with the normal group. The epididymal sperm motility and subsequent parameters in mice treated with BAPTA decreased significantly compared to the normal group. Furthermore, mice treated with *P. ginseng* showed increased sperm motility parameters compared to the BAPTA (10 mmol l⁻¹) control group. These results suggest that *P. ginseng* not only increased sperm motility but also promoted VCL and ALH hyperactivation (Figure 1a–c). In addition, intracellular Ca²⁺ levels were measured by a Fluoroskan Ascent Microplate Fluorometer after sperm were isolated from the ductus deferens and cauda epididymis. Sperm cell Ca²⁺ levels of the *P. ginseng*-treated groups were increased compared to the normal group (Figure 1d). Therefore, Ca²⁺ was important for sperm motility and hyperactivation. Moreover, calcium chelator treatment decreased the apparent sperm motility and hyperactivation. Therefore, increasing the concentration of calcium is critical for maintaining high sperm motility and hyperactivation. Furthermore, compared to the normal group, all *P. ginseng* concentrations significantly increased the calcium concentration, even in the presence of BAPTA.

CatSper is a cation-channel plasma membrane protein necessary for normal sperm motility, in addition to sperm penetration of the zona pellucida.¹⁵ *CatSper*s 1–4 proteins are found only in the testes and are localized to the principal component of the flagellum.^{15,19,30,44} *CatSper*1, 3 and 4 are restricted to late-stage germ-line cells (spermatids) in the testes, while *CatSper*2 transcription begins during the early stages of spermatogenesis (pachytene spermatocytes).^{15,17,19,44} *CatSper*3

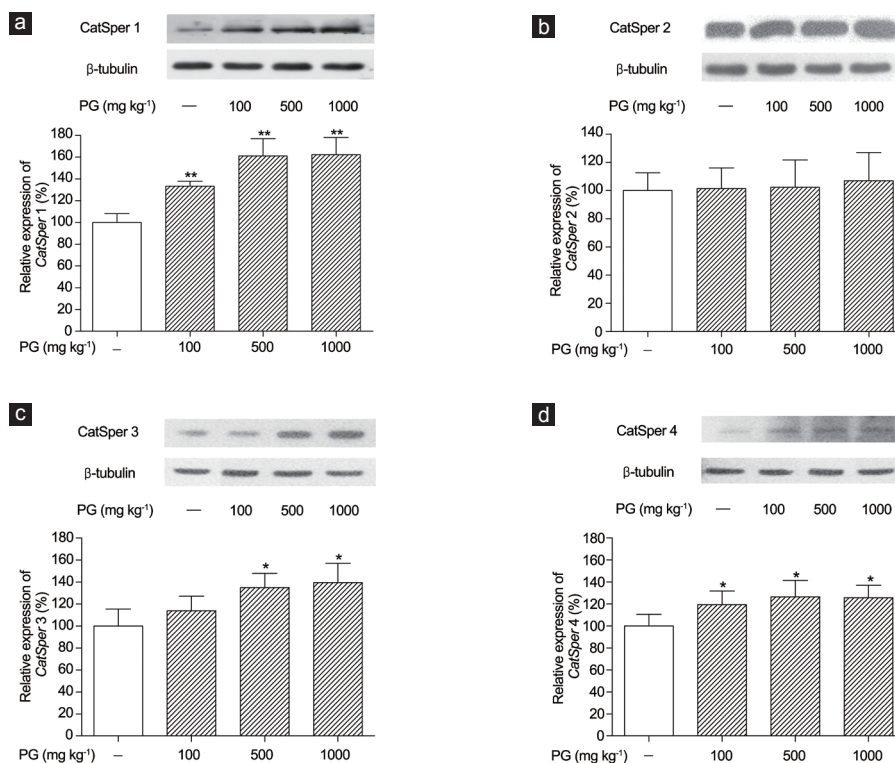


Figure 3: Effects of *Panax ginseng* treatment on *CatSper* protein levels in mouse testes by Western blotting using anti-*CatSper* and β -tubulin antibodies. Data are the results from the normal (N) and *P. ginseng*-treated (PG) (100, 500 and 1000 mg kg⁻¹) groups. (a) *CatSper*1; (b) *CatSper*2; (c) *CatSper*3 and (d) *CatSper*4. Each column represents the mean \pm standard deviation ($n = 3$). *Significantly different from the vehicle-treated group (* $P < 0.05$, ** $P < 0.01$).

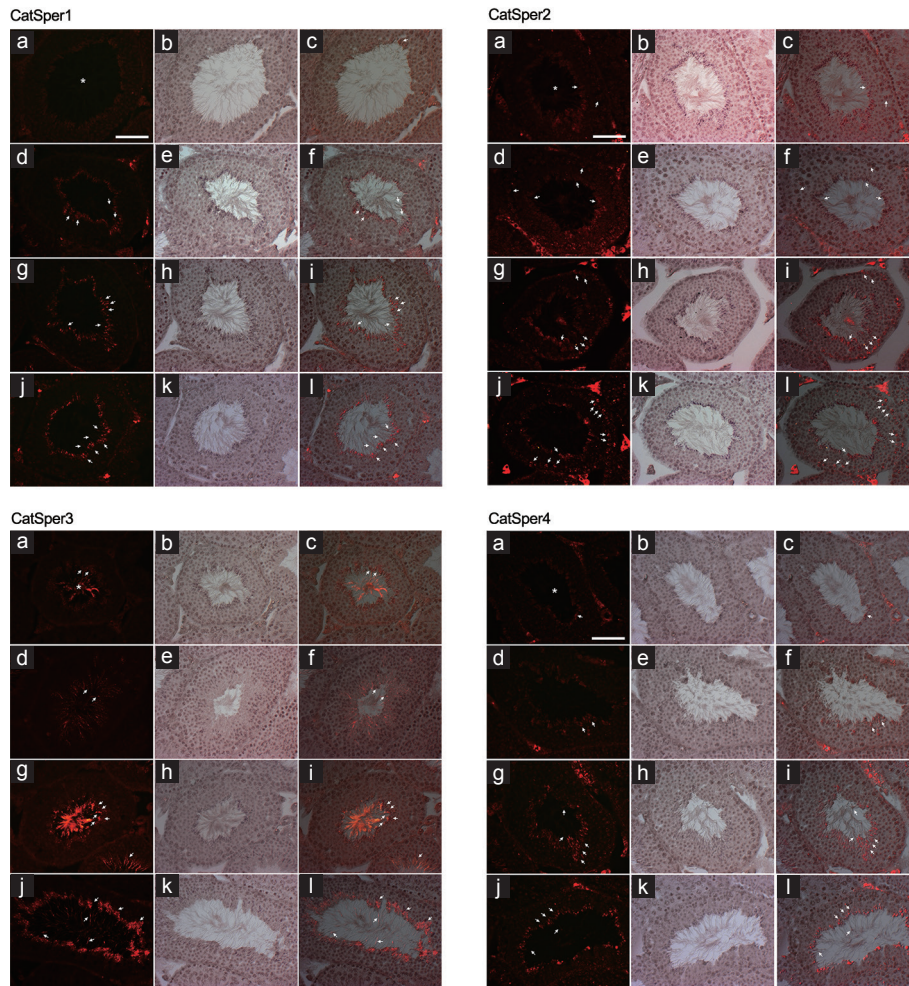


Figure 4: Effect of *Panax ginseng* on *CatSper* protein levels based on immunofluorescence with hematoxylin staining. *CatSper*1, 2, 3, and 4 levels are the results from normal (N) and *P. ginseng*-treated (100, 500 and 1000 mg kg⁻¹) groups. *CatSper* proteins had a positive rounded form (a, d, g, j) in the mouse testes. All samples were counterstained with hematoxylin (b, e, h, k). Overlaid *CatSper* immunofluorescence and hematoxylin-counterstained images showing induction of *CatSper* proteins (c, f, i, l). In each column, a-c is the normal group and d-f, g-i and j-l are the *P. ginseng*-treated groups (100, 500 and 1000 mg kg⁻¹), respectively. Images were obtained at a magnification of $\times 40$. Scale bars = 50 μ m.

and 4 proteins are necessary for hyperactivated sperm motility during capacitation. Moreover, *CatSper*s1–4 form a tetramer cation channel, which is required for the development of hyperactivated motility during sperm capacitation in the female reproductive tract.⁴⁵ *CatSper*s1–4-null mice have normal testicular histology, epididymal sperm counts and sperm morphology, indicating normal progression of spermatogenesis. By contrast, the phenotype of all *CatSper*s1–4-null mice is complete male infertility.^{46,47}

The pharmacological effects of Korean *P. ginseng*, as demonstrated by modern science, include enhancement of immune system function, liver function and sexual function.^{48,49} To investigate the effects of *P. ginseng* on *CatSper* expression, we performed real-time PCR and Western blotting. *CatSper*s1–4 mRNA levels in the *P. ginseng* groups were increased significantly compared with the normal group (Figure 2). Western blot analysis was performed to determine the effect of *P. ginseng* on *CatSper*s1–4 protein levels in mouse testes. The *CatSper* 1, 3 and 4 protein levels in the *P. ginseng* groups were increased significantly in a dose-dependent manner (Figure 3). Furthermore, immunofluorescence detection with hematoxylin staining showed that the *CatSper*1, 3 and 4 protein levels were higher in the testes of

P. ginseng-treated mice (Figure 4), whereas the *CatSper*2 mRNA level was increased, but the protein level remained unchanged. We believe that this is because *CatSper*1, 3 and 4 are expressed during the late stage of spermatogenesis (spermatids) in the testes, while *CatSper*2 transcription begins during the early stages of spermatogenesis. *P. ginseng* might affect the expression of the genes related to sperm motility at the late stage of spermatogenesis. These results suggest that *P. ginseng* treatment induces production of functional *CatSper*1, 3, and 4 mRNA and protein, which might be required to maintain and enhance sperm motility and hyperactivation via the VCL and ALH.

CONCLUSIONS

P. ginseng plays an important role in the improvement of sperm motility and hyperactivation via induction of *CatSper* expression. This suggests that *P. ginseng* could be used to treat reproductive dysfunction and male infertility.

AUTHOR CONTRIBUTIONS

EHP, SKP and MSC conceived of the study and participated in its design and wrote the manuscript. DRK, EHP and SKP carried out the animal studies, participated in the sperm analysis. HYK carried out

the immunoassays and performed the statistical analysis. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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