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Short communication

Astragalus membranaceus augment sperm parameters in male mice associated with cAMP-responsive element modulator and activator of CREM in testis

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ABSTRACT

Astragalus membranaceus BUNGE (AM; 黃芪 huáng qí) has been widely used as a medicinal herb for different kinds of diseases. AM treatment *in vitro* enhance sperm motility and ameliorates testicular toxicity, it has demonstrated the ability as a potential treatment for male infertility. In order to gain further insights on the molecular understanding of how AM enhances spermatogenesis, this study investigated whether AM has an affect on sperm parameters associated with cAMP response element modulator (CREM) and activator of CREM in testis (ACT) expression. Five-week-old male ICR mice were divided into four groups; control group and three different concentrations of AM treated groups. Each group was treated for 5 days a week for 5 weeks. Testis samples were collected for real time quantitative PCR and western blot analysis. Epididymis was taken out and used for sperm analysis using the computer assisted semen analysis (CASA) system. To facilitate expression of genes required for spermatogenesis, it is controlled by fine-tuning of CREM and its coactivator, ACT. AM treatment promotes CREM and ACT mRNA expression and also protein expression compared to control. AM enhances sperm values such as sperm count and motility compared to control. Overall, the study highlights, the ability of AM to increases CREM and ACT expression to facilitate sperm development and semen quality.

1. Introduction

Infertility is defined as failure to conceive after 12 or more months of regular unprotected intercourse.[1] Male-factor infertility is the sole cause of infertility in approximately 20% of infertile couples, and in 30–40% of couples, both male and female factors contribute.[2,3] Thus, half of all infertility can be attributed in part or completely to the male factor.[4] The pathogenesis of male infertility can be reflected by defective spermatogenesis due to failure in germ cell proliferation and differentiation.[5] Over the past decades growing evidences are also indicating a steady decline in human sperm counts and motility.[6] However, for many of these infertile men, no specific therapies are available to improve their fertility potential and only assisted reproductive technologies such as intrauterine insemination and *in vitro* fertilization will potentially help these men contribute to a pregnancy.[7] In the United States 29% of 428 infertile couples in northern California after 18 months of observation used complementary or alternative medicine as a fertility treatment, in which 18% used herbal therapy.[8]

Male reproduction is a complex process that involves the testes, epididymis, accessory sex glands, and associated hormones.[9] Testes perform two highly organized and intricate functions of spermatogenesis and steroidogenesis, which are crucial for the perpetuation of life.[9] Spermatogenesis, a highly dynamic and synchronized process, takes place within the seminiferous tubules of the testis with the support of somatic Sertoli cells, leading to the formation of mature spermatozoa from undifferentiated stem cells.[10] The interstitial compartment, which comprises Leydig cells, is the site of steroidogenesis in the testis.[11] Recent findings have shown that the testis has specialized transcription complexes that coordinate the differentiation program of spermatogenesis.[12] The cyclic adenosine monophosphate (cAMP) response element modulator (CREM) is an essential component of this program, and

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its activity is regulated through interactions with a germ cell-specific, CREM phosphorylation-independent transcriptional co-activator, activator of CREM in testis (ACT).[13] This precise and well-coordinated regulation of gene expression in post-meiotic germ cells is fundamental to male fertility.[13]

The root of *Astragalus membranaceus* BUNGE (AM; 黃芪 huáng qí), a perennial herb native to the northern province of China, has been cultivated in China, Korea, and Japan. AM has been widely used in East Asia as a medicine for different kinds of diseases, and has been reported to possess diverse biological activities, including anti-tumor, anti-inflammatory, anti-apoptotic, anti-oxidant, and immune-enhancing properties.[14–18] Hong *et al* used a trans-membrane migration method to screen 18 types of Chinese herbs and only AM aqueous extract showed a significant stimulatory effect on the motility of human spermatozoa *in vitro*.[19] Another study has demonstrated that AM aqueous extract incubation *in vitro* enhanced the sperm motility characteristics of 30 infertile male volunteers.[20] Our group and others have shown that AM ameliorates sperm factors from cyclophosphamide and cadmium induced toxicity.[21,22] However, its effects on the male reproductive functions have not been well investigated. The goal of this study is to investigate the effects of AM on sperm parameters in experimental systems associated with CREM and ACT gene expression.

2. Materials and methods

2.1. Preparation of A. membranaceus BUNGE (AM; 黃芪 huáng qí) extract

The root of AM was purchased from Wonkwang Herbal Drug Co. Ltd. (Korea). Extraction methods were performed using methods as described previously.[23] Two hundred and fifty grams of AM was boiled with 5 L 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 49.5 g (19.8%) of powder, which was kept at 4 °C. Before each experiments, dried extract was dissolved in distilled deionized water (Millipore, USA) and vortexed for 2 min at room temperature as described previously.

2.2. Animals and experimental protocol

Five-week-old male ICR mice were purchased from Nara Biotech Co. (Korea). The animals were housed in a specific pathogen-free environment with a 12/12-hour light/dark cycle at the Center for Laboratory Animal Care and Use at Kyung Hee University. 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). Animals had free access to standard rodent pellets (Purina, Korea) and water. After 7 days of adaptation to the environment, the mice were divided into four groups; control group (vehicle-treated, $n = 8$), and several concentrations of AM group (100, 500, 1000 mg/kg AM, $n = 8$) treated at the same period. Dosage of AM is derived from previous studies.[22,24] Oral gavage can result in passive reflux if the stomach is overfilled, aspiration pneumonia, esophageal and gastric rupture, and stress.[25] After repeated gavage procedure corticosterone levels return to baseline after the second day of gavage in mice and heart rate and blood pressure return to normal by the third day of gavage in rats.[26,27] Moreover, several studies administered herbal extracts 5 days per week in mice by gavage.[28–31] Thus AM was treated for 5 days a week for 5 weeks to minimize complications associated with delivery. At the end of the treatment period, the mice were anesthetized with urethane (100 mg/kg, i.p.). The testes were taken out, cleared of the adhering tissues, and weighed. Epididymis was taken out and used for sperm analysis. Testis samples were frozen for real-time quantitative PCR, and Western blot analysis.

2.3. Sperm analysis

To obtain sperm count, entire epididymis from the mouse was minced in sperm washing media incubated for 20 min at 37 °C. The sperm concentration outcomes produced by manual evaluation using the Computer Assisted Semen Analysis (CASA) system (Hamilton Thorne, USA). For assessment of sperm motility, sperms were recovered from excised caudal epididymis and allowed to capacitate for 20 min in sperm washing media 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.

2.4. RNA isolation and real-time quantitative PCR

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.*[32] RTQ-PCR was performed in a Step one plus System Thermal Cycler (Applied Biosystems, USA). RTQ-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 CREM, ACT and GAPDH were purchased from Applied Biosystems [assay ID: Mm00516346_m1 (CREM; gene symbol: Crem), Mm00480451_m1 (ACT; gene symbol: Fhl5) and Mm99999915_g1 (GAPDH; gene symbol: 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 of cDNA in each sample, and then all PCR products were normalized to that amount. Negative controls (without template) were produced for each run. 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, USA). For data analysis, we used the comparative Ct method. Data are expressed as relative quantity (RQ) and differences are shown in the figures as the expression ratio of the normalized target gene, according to the software results.

2.5. Western blot analysis

Proteins from homogenized testes were separated using nuclear extract kit according to manufacturer's protocol with minor modifications (Active & Motif, USA). Samples for protein extraction were half of the same testes used for RNA extractions. Equivalent amount (70 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 2 h at room temperature and incubated with the primary antibody (1:1000) for CREM-1 antibody (X-12, Santa Cruz Biotechnology, Inc., USA), ACT (FHL-5; sc-133581, Santa Cruz Biotechnology, Inc., USA), in PBS-T overnight at 4 °C. The membranes were then washed and developed with Western blotting chemiluminescent reagents (Thermo Scientific, Rockford, IL), and then exposed to X-ray films (Agfa, Mortsel, Belgium). The films were analyzed with AlphaEase FC software (Alpha Innotech).

2.6. Histological 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.

2.7. Statistical analysis

All quantitative data derived from this study were analyzed statistically. The results were expressed as the mean ± standard deviation (S.D.). Differences between groups were assessed by one way ANOVA using the SPSS software package for Windows. Statistical significance at p-values <0.05, <0.01, and <0.001 has been given respective symbols in the figures.

3. Results and Discussion

Hematoxylin and eosin stain was carried out to observe the testicular histological changes. Between the control group and *A. membranaceus* BUNGE (AM; 黃芪 huáng qí) (100, 500, 1000 mg/kg) treated group the morphology of spermatogonial cell layers,

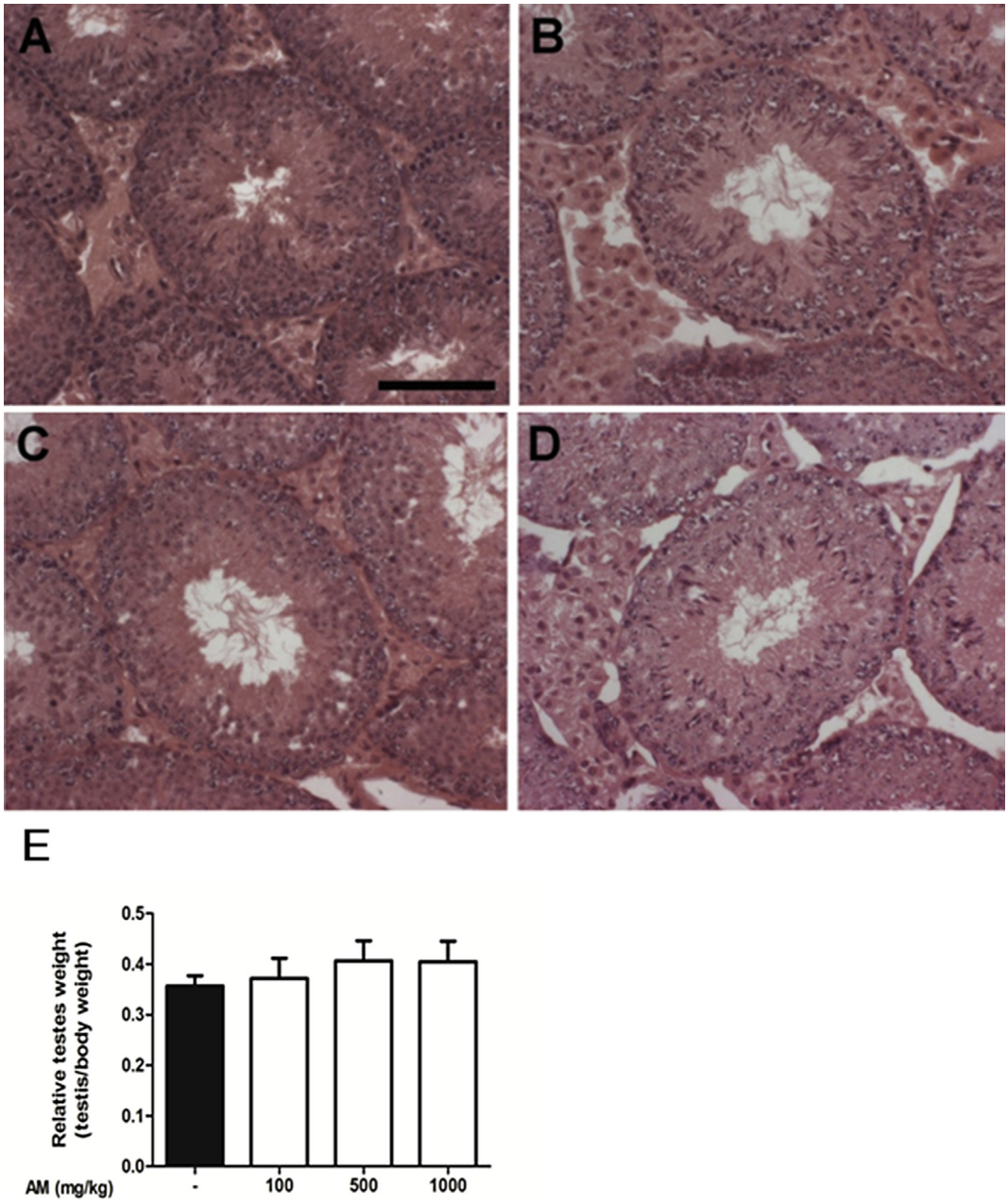

Fig. 1. Histological and weight examination on *Astragalus membranaceus* (AM) treated mice. (A) Control, (B) AM 100 mg/kg, (C) AM 500 mg/kg, (D) AM 1000 mg/kg, and (E) Relative testes weight (Testis/Body). Images were obtained at an objective magnification of 400×. Scale bars = 50 μm. Arrow indicates appearance of the marked interruption of spermatogenesis. Results are presented as mean ± S.D (n = 8).

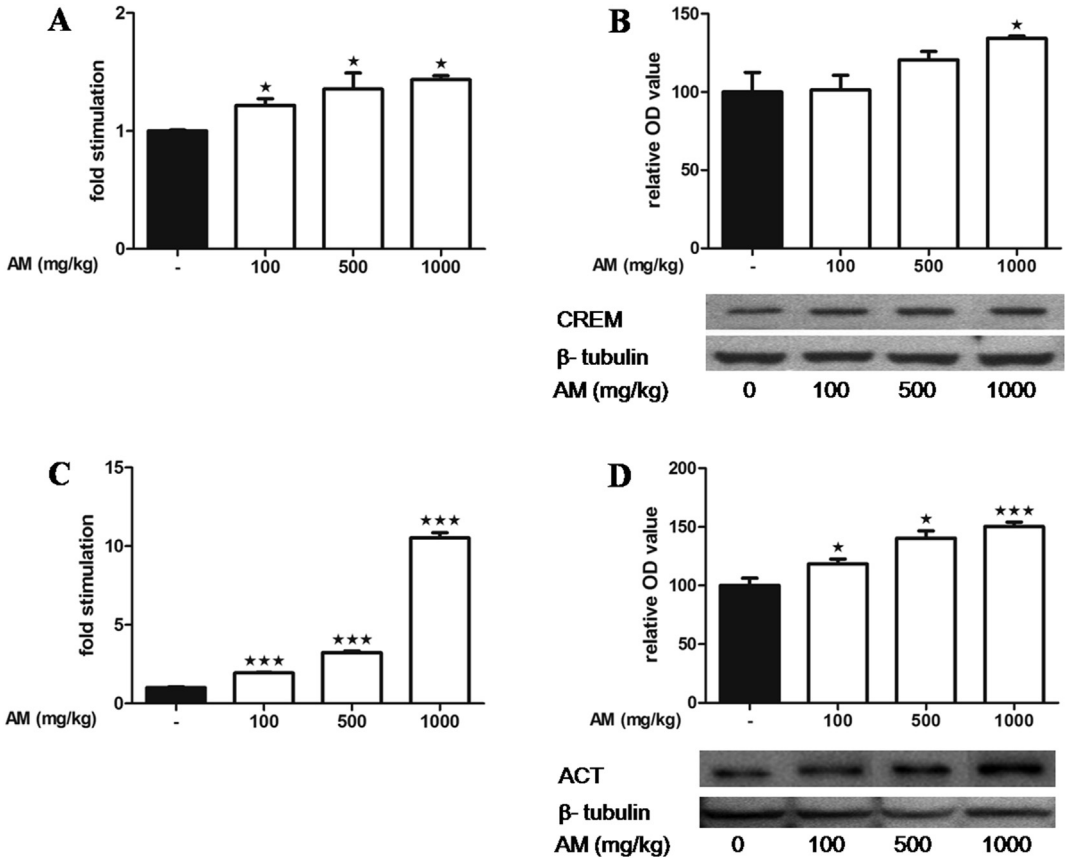

Fig. 2. Effect of *Astragalus membranaceus* (AM) on cAMP-responsive element modulator (CREM) and activator of CREM in testis (ACT). (A) Real-time quantitative PCR shows the relative expression of CREM mRNA levels. (B) Western blot analysis shows the relative expression of CREM protein levels. (C) Real-time quantitative PCR shows the relative expression of ACT mRNA levels. (D) Western blot analysis shows the relative expression of ACT protein levels. Results are presented as mean ± S.D. * Indicates the difference between the control group and the AM treated group (n = 3; *p < 0.05, ***p < 0.001).

spermatogenesis and multiple sperms in the lumen of the seminiferous tubules are analogous (Fig. 1A–D). The weights of body and testes were measured on the day after full treatments. The relative testes weight between the control group and AM (100, 500, 1000 mg/kg) treated group was not significant (Fig. 1E). The administration of AM did not show testicular toxic changes. In particular, our data are similar to prior findings that AM treatment did not show difference in food intake, behavior, body weight, and hematological parameters.[33] CREM and its coactivator, ACT, work together to facilitate expression of genes required for spermatogenesis.[34] In order to examine the effect of AM on the expression of the CREM mRNA levels in mouse testes, real-time quantitative PCR was carried out using total RNA of mouse testes. AM (100, 500, 1000 mg/kg) activates an increase in the CREM mRNA expression levels in mouse testes by 22%, 36%, and 44% compared to the control group (Fig. 2A). Western blot analysis was performed to determine the effect of AM on CREM protein expression in mouse testes. AM increased CREM protein levels dose-dependently, AM (1000 mg/kg) showed significant increase by 34% (Fig. 2B). The ACT mRNA expression levels in mouse testes showed 2, 3, and 10 fold increase by AM (100, 500, 1000 mg/kg) treatment (Fig. 2C). AM (100, 500, 1000 mg/kg) promotes ACT protein expression by 18%, 40%, and 50%, significantly (Fig. 2D). Previous study suggests that CREM plays an important role in ACT transcriptional regulation.[35] Interestingly, AM (1000 mg/kg) group that showed significant increase in protein levels of CREM showed a strong response to ACT

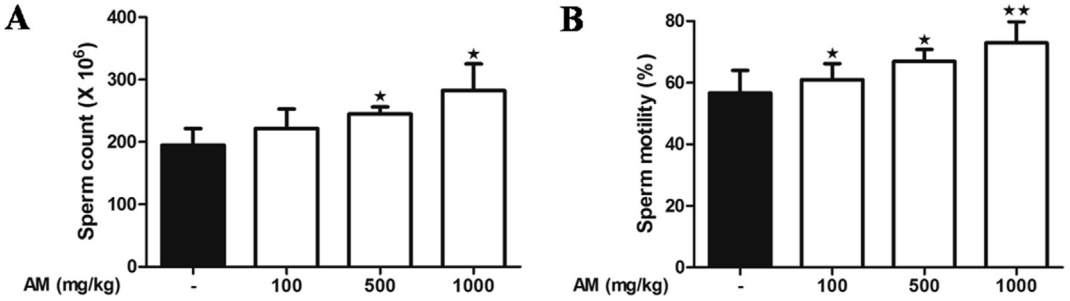

Fig. 3. Analysis of sperm parameters on *Astragalus membranaceus* (AM) treated mice. (A) Effect of AM on sperm count. (B) Effect of AM on sperm motility. Results are presented as mean ± S.D. * Indicates the difference between the control group and the AM treated group (n = 8; *p < 0.05, **p < 0.01).

expression. Although how CREM regulates ACT needs further study. The conventional parameters given most importance to determine semen quality have been the concentration, motility, and morphology of sperm. The mice treated with AM (100, 500, 1000 mg/kg) showed dose-dependent increase in epididymal sperm count as of 14%, 26%, and 45% compared to the control group (Fig. 3A). The sperm motility also showed dose-dependent increase by 8%, 18%, and 29% in AM (100, 500, 1000 mg/kg) treated groups compared to the control group (Fig. 3B). ACT is reported to be important for regulating transcription from a subset of CREM-dependent genes, likely targeting those genes associated with structural proteins that participate in the architecture of the flagellum.[13] But whether the increase in ACT expression by AM appears to regulate the formation of the flagellum, and increase sperm motility needs further study. As AM treatment *in vitro* is reported to significantly stimulate the motility of human spermatozoa.[19,20] It is interesting to speculate whether *in vitro* treatment of AM might have a wider role in regulating sperm motility. In the aggregate, these data suggest that AM treatment enhance sperm values. These results indicate that AM treatment improve the motility and total number of sperm, probably through CREM and ACT activation, with no severe side effects.

4. Conclusion

Our data suggest that *A. membranaceus* BUNGE (AM; 黃芪huáng qí) with its potential to improve sperm parameters could be recommended as a treatment for spermatogenesis-related male infertility. A molecular understanding of how AM enhances spermatogenesis would help broaden the therapeutic categories for male factors of infertility.

Conflicts of interest

All authors have no conflicts of interest to declare.

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References

1. Lindsay TJ, Vitrikas KR. Evaluation and treatment of infertility. *Am Fam Physician*. 2015;91:308–314.
2. Thonneau P, Marchand S, Tallec A, et al. Incidence and main causes of infertility in a resident population (1,850,000) of three French regions (1988–1989). *Hum Reprod*. 1991;6:811–816.
3. Mosher WD, Pratt WF. Fecundity and infertility in the United States: incidence and trends. *Fertil Steril*. 1991;56:192–193.
4. Patel ZP, Niederberger CS. Male factor assessment in infertility. *Med Clin N Am*. 2011;95:223–234.
5. Nayernia K, Li M, Jaroszynski L, et al. Stem cell based therapeutical approach of male infertility by teratocarcinoma derived germ cells. *Hum Mol Genet*. 2004;13:1451–1460.
6. Mitchell JH, Cawood E, Kinniburgh D, Provan A, Collins AR, Irvine DS. Effect of a phytoestrogen food supplement on reproductive health in normal males. *Clin Sci*. 2001;100:613–618.
7. Zini A, Fischer MA, Nam RK, Jarvi K. Use of alternative and hormonal therapies in male infertility. *Urology*. 2004;63:141–143.
8. Smith JF, Eisenberg ML, Millstein SG, et al. The use of complementary and alternative fertility treatment in couples seeking fertility care: data from a prospective cohort in the United States. *Fertil Steril*. 2010;93:2169–2174.
9. D'Cruz SC, Vaithinathan S, Jubendradass R, Mathur PP. Effects of plants and plant products on the testis. *Asian J Androl*. 2010;12:468–479.
10. Hess RA, Renato de Franca L. Spermatogenesis and cycle of the seminiferous epithelium. *Adv Exp Med Biol*. 2008;636:1–15.
11. Ge R, Chen G, Hardy MP. The role of the Leydig cell in spermatogenic function. *Adv Exp Med Biol*. 2008;636:255–269.
12. Sassone-Corsi P. Unique chromatin remodeling and transcriptional regulation in spermatogenesis. *Science*. 2002;296:2176–2178.
13. Hogeveen KN, Sassone-Corsi P. Regulation of gene expression in post-meiotic male germ cells: CREM-signalling pathways and male fertility. *Hum Fertil*. 2006;9:73–79.
14. Cho WC, Leung KN. In vitro and in vivo anti-tumor effects of *Astragalus membranaceus*. *Cancer Lett*. 2007;252:43–54.
15. Lai PK, Chan JY, Cheng L, et al. Isolation of anti-inflammatory fractions and compounds from the root of *Astragalus membranaceus*. *Phytother Res PTR*. 2013;27:581–587.
16. Kim MH, Kim SH, Yang WM. Beneficial effects of Astragaloside IV for hair loss via inhibition of Fas/Fas L-mediated apoptotic signaling. *PLoS One*. 2014;9:e92984.
17. He Y, Du M, Gao Y, et al. Astragaloside IV attenuates experimental autoimmune encephalomyelitis of mice by counteracting oxidative stress at multiple levels. *PLoS One*. 2013;8:e76495.
18. Hong F, Xiao W, Ragupathi G, et al. The known immunologically active components of *Astragalus* account for only a small proportion of the immunological adjuvant activity when combined with conjugate vaccines. *Planta Med*. 2011;77:817–824.
19. Hong CY, Ku J, Wu P. *Astragalus membranaceus* stimulates human sperm motility in vitro. *Am J Chin Med*. 1992;20:289–294.
20. Liu J, Liang P, Yin C, et al. Effects of several Chinese herbal aqueous extracts on human sperm motility in vitro. *Andrologia*. 2004;36:78–83.
21. Liang P, Li H, Peng X, Xiao J, Liu J, Ye Z. Effects of *Astragalus membranaceus* injection on sperm abnormality in Cd-induced rats. *Zhonghua nan ke xue = Natl J Androl*. 2004;10:42–45, 48.
22. Kim W, Kim SH, Park SK, Chang MS. *Astragalus membranaceus* ameliorates reproductive toxicity induced by cyclophosphamide in male mice. *Phytother Res PTR*. 2012;26:1418–1421.
23. Park WS, Shin DY, Kim do R, Yang WM, Chang MS, Park SK. Korean ginseng induces spermatogenesis in rats through the activation of cAMP-responsive element modulator (CREM). *Fertil Steril*. 2007;88:1000–1002.
24. Park EH, Kim do R, Kim HY, Park SK, Chang MS. Panax ginseng induces the expression of CatSper genes and sperm hyperactivation. *Asian J Androl*. 2014;16:845–851.
25. Turner PV, Brabb T, Pekow C, Vasbinder MA. Administration of substances to laboratory animals: routes of administration and factors to consider. *J Am Assoc Lab Animal Sci JAALAS*. 2011;50:600–613.
26. Okva K, Tamoseviciute E, Ciziute A, Pokk P, Ruksenas O, Nevalainen T. Refinements for intragastric gavage in rats. *Scand J Lab Anim Sci*. 2006;33:243–252.
27. Hoggatt AF, Hoggatt J, Honerlaw M, Pelus LM. A spoonful of sugar helps the medicine go down: a novel technique to improve oral gavage in mice. *J Am Assoc Lab Animal Sci JAALAS*. 2010;49:329–334.
28. Rider CV, Nyska A, Cora MC, et al. Toxicity and carcinogenicity studies of Ginkgo biloba extract in rat and mouse: liver, thyroid, and nose are targets. *Toxicol Pathol*. 2014;42:830–843.
29. National Toxicology P. *Toxicology and Carcinogenesis Studies of Kava Kava Extract (CAS No. 9000-38-8) in F344/N Rats and B6C3F1 Mice (Gavage Studies)*. National Toxicology Program technical report series. 2012, 1–186.
30. Omura M, Tanaka A, Hirata M, Inoue N. Testicular toxicity evaluation of two antimony compounds, antimony trioxide and antimony potassium tartrate, in rats and mice. *Environ Health Prev Med*. 2002;7:15–18.
31. Subbaramaiah K, Sue E, Bhardwaj P, et al. Dietary polyphenols suppress elevated levels of proinflammatory mediators and aromatase in the mammary gland of obese mice. *Cancer Prev Res*. 2013;6:886–897.
32. Saiki RK, Bugawan TL, Horn GT, Mullis KB, Erlich HA. Analysis of enzymatically amplified beta-globin and HLA-DQ alpha DNA with allele-specific oligonucleotide probes. *Nature*. 1986;324:163–166.
33. Fu J, Wang Z, Huang L, et al. Review of the botanical characteristics, phytochemistry, and pharmacology of *Astragalus membranaceus* (Huangqi). *Phytother Res PTR*. 2014;28:1275–1283.
34. Davidson I. Specific transcription regulatory mechanisms of male germ cells. In: Rousseaux S, Khochbin S, eds. *Epigenetics and Human Reproduction*. Heidelberg: Springer; 2011:291–309.
35. Steger K, Klonisch T, Gavenis K, et al. Round spermatids show normal testis-specific H1t but reduced cAMP-responsive element modulator and transition protein 1 expression in men with round-spermatid maturation arrest. *J Androl*. 1999;20:747–754.