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

Wonnam Kim¹, Do Rim Kim, Mun Seog Chang, Seong Kyu Park*

Department of Prescriptionology, College of Korean Medicine, Kyung Hee University, 1 Hoegi-dong, Dongdaemun-gu, Seoul 130-701, Republic of Korea

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

Keywords:
Activator of CREM in testis
Astragalus membranaceus (AM; 黃芪 huáng qí)
cAMP response element modulator
Sperm count
Sperm motility
its activity is regulated through interactions with a germ cell-specific, CREM phosphorylation-independent transcriptional co-activator, activator of CREM in testis (ACT).1 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 region 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.14–16 Hong et al used a transmembrane 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.24–26 Oral gavage can result in passive reflux if the stomach is overfilled, aspiration pneumonia, esophageal and gastric rupture, and stress.27 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 electrothermally 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), PS-C (sc-14621, 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 Astragalus membranaceus BUNGE (AM; 黃芪 huáng qí) (100, 500, 1000 mg/kg) treated group the morphology of spermatogonial cell layers,
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. CREM and its coactivator, ACT, work together to facilitate expression of genes required for spermatogenesis. 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. Interestingly, AM (1000 mg/kg) group that showed significant increase in protein levels of CREM showed a strong response to ACT.

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).

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 significant stimulator of human spermatozoa.10,11 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.

Acknowledgments

This work was supported by National Research Foundation of Korea (NRF) grant NRF-2014R1A2A1A11050419.

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 ZF, Niederberger CS. Male factor assessment in infertility. Med Clin N Am. 2011;55:223–234.
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 phytostrogen 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. Urolog. 2004;63:141–143.
8. Smith JP, Eisenberg ML, Millstein SC, 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.