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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).