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Effects of *Psoralea corylifolia* on the cAMP-responsive element modulator (CREM) expression and spermatogenesis in rats

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ABSTRACT

Aim of the study: Psoralea corylifolia (PC) is a medicinal herb used to improve male reproductive function in Korean traditional medicine. It has been used for treatment of male infertility including sexual dysfunction by improving kidney function.

Materials and methods: To investigate the effect of PC on spermatogenesis, the cAMP-responsive element modulator (CREM) in rat testes was evaluated using sperm analysis, the reverse-transcription polymerase chain reaction, and Western blot analysis. PC was administered to 10-week-old male Wistar rats for 56 consecutive days, the sperm formation period of the rat.

Results and conclusions: The PC-treated rats had increased sperm counts with enhanced levels of CREM messenger RNA and protein, suggesting that PC induces spermatogenesis via CREM activation in rat testes.

1. Introduction

About 13–18% of couples suffer from infertility, and growing evidence from clinical and epidemiological studies suggests a rising incidence of male reproductive problems (Iammarrone et al., 2003). Male factors are responsible for 51.2% of conjugal infertility, and for idiopathic reasons, the males in 39% of these couples have abnormal semen analyses (Hassun Filho et al., 2005). Male infertility can be caused by various genetic lesions such as gross chromosomal aneuploidies, rearrangements, microdeletions, and single-gene defects. It affects not only on genes controlling the male germ line, but also on the network involved in male gonadal development and male somatic development (Vogt, 2004).

Recent studies suggest an increased incidence of genetic disorders related to male infertility, which may act at different levels, interfering with germ cell generation and maturation, or leading to the production of nonfunctional spermatozoa (Iammarrone et al., 2003). Several spermatid-specific genes contain a cAMP-responsive element (CRE), which serves as binding site for the transcription factor cAMP-responsive element modulator (CREM) (Sassone-Corsi, 1995). This pathway primarily uses cAMP-response element binding protein (CREB) and CREM to mediate gene expression. Moreover, CREB/CREM proteins and other potential members of the CREB family are key molecular regulators during all stages of spermatogenesis (Don and Stelzer, 2002). Combinations of genetic changes in the human CREM gene can explain some forms of male infertility (Vouk et al., 2005). CREM plays an essential role in primate spermatid maturation (Behr and Weinbauer, 2001), and a compelling amount of data has established that CREM, a transcription factor that responds to the cAMP signal transduction pathway, drives the expression of key testis-specific genes (Kimmins et al., 2004).

The fruit of *Psoralea corylifolia* (PC; Leguminosae), known as "Boh-Gol-Zhee" in Korea, has been used in the treatment of kidney Yang-deficiency patterns, such as impotence, premature ejaculation, lower back pain, spermatorrhea, frequent urination, and urinary incontinence (Bensky, 1992). In addition, an extract of PC is also thought to be useful as a remedy for bone fractures, osteomalacia, and osteoporosis (Zhao et al., 2005), and a variety of biological activities of its constituents or extracts has been reported (Haraguchi et al., 2002). PC contains coumarins, flavonoids, and meroterpenes such as psoralen, isopsoralen, neobavaisoflavone, bavachin, bavaisoflavone, bavachromene, psoralidin, corylifolinin, bavachinin, and bavachalcone. Many experiments have examined the effective components of PC. For example, psoralen and isopsoralen have been applied clinically to treat skin diseases such as psoriasis and vitiligo (Chakraborty et al., 1996); they can also inactivate viruses in blood and are commonly used to treat kidney dysfunction, while bavachinin and bavachin have anti-oxidative activities and strongly inhibit mitochondrial and microsomal lipid peroxidation (Chen et al., 2005).

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However, the relationship between PC and its effects on male reproductive tract malfunction associated with CREM gene expression and spermatogenesis *in vivo* have not been elucidated. To investigate the effects of PC on male reproductive functions, we measured spermatogenic parameters, including testicular weight, the sperm count, and sperm motility. In addition, we assessed CREM expression at the mRNA and protein levels.

2. Materials and methods

2.1. Preparing the PC extract

Psoralea corylifolia Linnaeus fruits were purchased from Wonkwang Herbal Drug (Seoul, Korea). PC (300 g) was boiled in water (6 L) for 2 h at 100 °C, and then the suspension was filtered and concentrated under reduced pressure. The filtrate was lyophilized and yielded 44.70 g (14.9%) of powder, which was kept at 4 °C. A voucher specimen (PC001) has been deposited in our laboratory. Before each experiment, dried extract was dissolved in distilled deionized water and vortexed for 2 min at room temperature.

2.2. Animals and experimental protocol

2.2.1. Animals

Ten-week-old male Wistar rats were purchased from SLC Japan (Hamamatsu, Japan). The animals were housed in a specific pathogen-free environment on a 12/12-h light/dark cycle at the Center for Laboratory Animal Care and Use at Kyung Hee University, Seoul, Korea. The rats had access to standard rodent pellets (Purina Korea, Seoul, Korea) and water *ad libitum*. The 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).

2.2.2. Treatment with cyclophosphamide and PC

After allowing 7 days for adaptation to the environment, the rats were divided into three groups of eight animals each: the normal (vehicle-treated), control (treated with cyclophosphamide only), and PC (treated with cyclophosphamide and PC) groups. Cyclophosphamide was administered for the first 14 days (20 mg kg^{-1} day^{-1}, p.o.), while PC was given for 56 consecutive days (1.0 g kg^{-1} day^{-1}, p.o.). In the PC group, cyclophosphamide and PC were both given for the first 2 weeks. The animals were weighed weekly to adjust the gavage volume and to monitor their general condition.

2.2.3. Tissue preparation

At the end of the treatment period, the rats were anesthetized with Zoleti 50 (25 mg/kg, i.p.; Virbac, Carros, France). The testes were removed, cleared of the adhering tissues, and weighed. The relative testes weight was calculated as the absolute testes weight/body weight. The epididymis was removed, and used for the sperm analysis. Testis samples were frozen before use in the reverse-transcription polymerase chain reaction (RT-PCR) and Western blotting assays.

2.3. Sperm analyses

The epididymal sperm count and motility were evaluated using the method described by Connolly et al. (2005), with some modifications. For the sperm count, the epididymis was minced in M199 media containing 0.5% bovine serum albumin (BSA) and incubated for 5 min at 37 °C. The sperm concentration was determined manually using a hemocytometer (Neubauer Zählkammer, Gehrden, Germany). To assess sperm motility, sperm were recovered from the excised caudal epididymis and allowed to capacitate for 5 min in M199 media containing 0.5% BSA at 37 °C. Sperm were counted as motile when any movement was detected. The sperm motility was converted into a percentage.

2.4. RNA isolation and RT-PCR

2.4.1. Total RNA isolation from rat testes

Fenozol (800 μl) was added to the testis tissue samples (0.1 g), and the samples were homogenized and incubated for 5 min at 50 °C. Then 200 μl of chloroform was added and the samples were centrifuged at 12,000 × *g* for 10 min at room temperature. The aqueous phase was transferred to fresh tubes and 400 μl of isopropanol was added. The supernatants were incubated for 10 min at room temperature and centrifuged at 12,000 × *g* for 15 min at 4 °C. Then, the RNA pellets were washed with 70% ethanol, air-dried, and resuspended in diethylpyrocarbonate-treated water (DEPC-H_2O). Total RNA samples were analyzed using denaturing formaldehyde/agarose/ethidium bromide gel electrophoresis. The final amount of RNA was estimated spectrophotometrically (Molecular Devices, Sunnyvale, CA, USA) at 260 nm.

2.4.2. cDNA synthesis and PCR-amplification

First strand cDNA synthesis with total RNA (2 μg) was performed using MMLV reverse transcriptase and oligo dT primer for 1 h at 42 °C. The PCR-amplification was carried out using a modification of the method (Saiki et al., 1986). The cDNA was added to 10× PCR buffer containing 25 mM $MgCl_2$, 2.5 mM dNTP, polymerase (1 U), each primer (4 pmol), and DEPC-H_2O to give a final volume of 25 μl. The mixture was heated at 95 °C for 5 min, followed by 35 cycles at 95 °C for 1.5 min, 52–56 °C for 3 min, and 72 °C for 1.5 min. The PCR products were separated on 1.5% agarose gels, visualized with ethidium bromide using the i-MAX gel image analysis system (CoreBioSystem, Seoul, Korea), and analyzed using Alpha Easy™ FC software (Alpha Innotech, San Leandro, CA, USA).

2.5. Western blotting assay

Proteins from homogenized testes were separated using a nuclear extract kit (Active Motif, Carlsbad, CA, USA). The samples for protein extraction were half of the same testes used for RNA extraction. The protein extracts (50 μg) were separated in 10% Tris–glycine gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes electrophoretically. X-12 CREM-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as the primary antibody against CREM-t, and conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO, USA) was used as the secondary antibody. The antigens were detected using a SeePico CBB stain kit (Benebiosis, Seoul, Korea).

2.6. Statistical analysis

The results are expressed as the mean ± S.D. Statistical differences between groups were assessed using Student's *t*-test. Statistical significance at $p < 0.001$, <0.01, and <0.05 is indicated in the tables and figures.

3. Results

The body and testicles were weighed the day after the last of the 56 treatments with PC. The relative weights of the testes in the PC group were significantly higher than those of the controls ($p < 0.01$).

The sperm count of the PC-treated rats was 23% higher than that of the control group ($p < 0.01$), while the sperm motility in the PC