CHANGES IN THE SPERMATOGENESIS AND cAMP- RESPONSIVE ELEMENT MODULATOR (CREM) GENE EXPRESSION IN RAT TREATED WITH *SALVIA HYPOLEUCA*

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Summary

Salvia species which are medicinal herbs have been used to improve male reproductive functions in traditional medicine. They also, have been used to treat several diseases. In this present study, effects of Salvia hypoleuca on male rat reproductive function were investigated by sperm analysis and assessment of CREM expression at mRNA and protein levels. Two hundred and fifty mg/kg/day of S. hypoleuca as treatment was given to 10-week old male wistar rats for 56 consecutive days. Sperm analysis, RT-PCR and western blot were carried out to investigate rat reproductive function. Results indicated significant increase in the weights of testis, epididymal sperm counts, and sperm motility compared to control group. RT-PCR and western blot showed an increase in the expression of both CREM mRNA and protein levels.

KEYWORDS: Spermatogenesis; CREM expression; Saliva hypoleuca.

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Introduction

Male infertility is a common problem today. About 13–18% of couples suffer from infertility and growing evidence from clinical and epidemiological studies suggests an increasing incidence of male reproductive problems (1). 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. The causes of male infertility include genetic problems, obstruction, variococele, infection, and exposure to toxins and radiation. Even with our increased knowledge about human reproductive functions, up to 23% of male infertility is idiopathic (2). Semen analysis, including sperm count, motility, and morphology, is the basis of investigations into male infertility. A number of genes participating in spermatogenesis have essential roles in human reproductive function, and when deleted or mutated they can cause male infertility (3). cAMP-responsive element modulator (CREM) is a key factor in the regulation of the expression of number of post-meiotic genes (4). CREM binds to transcription factor and regulates gene expression in spermatids (5). The genus Salvia, one of the most important genus of Lamiaceae family, is widely used in flavouring and folk medicine all around the world (6). Fifty-eight species of this genus are documented in the Flora of Iran; 17 of them are endemic (7).

The plants of the genus Salvia, which consist about 900 species (8) are generally known for their multiple pharmacological effects such as analgesic and anti-inflammatory (9), antioxidant (10), hepatoprotective (11), hypoglycemic activities (12), and antiischemia (13,14). The results of recent studies have shown that some species of Salvia impress fertility in rat. The data obtained from previous studies clearly demonstrated that Salvia hematodes enhances the orientation of males towards the female by increased anogenital behavior and enhance licking and grooming of the genitals (15). This plant also increases the ejaculation latency. Salvia fruticosa produces adverse effects on the fertility of male and female rats (16). In this study, we examined the effect of Salvia hypoleuca on male reproductive function in rats, using sperm analysis. We also investigated the effect of S. hypoleuca on spermatogenesis by assessingCREMexpression at themRNAand protein levels.

Materials and methods

Plant material

S. hypoleuca was collected from Guilan province (Iran), and authenticated at Medicinal Plants & Drugs Research Institute, Shahid-Beheshti University, Tehran, Iran. Its leaves and fruits were dried, under shade and powdered. The extract was prepared by maceration method (80% ethanol in 300 g/l for 48 h), and was filtered with a filter paper. Ethanol was removed by a rotary evaporator. The extract was dissolved in normal saline and administrated orally into rats.

Animals and treatment

Male wistar rats purchased from Razi Institute, (Karaj, Iran) and were divided randomly into two groups consisting eight rats per group: control (rats treated with normal saline) and experimental (rats treated with 250 mg/kg/body weight S. hypoleuca). Experimental group administered S. hypoleuca extract (250 mg/kg/day) for 56 days.

Tissue preparation

At end of treatment rats were anesthetized by pentobarbital sodium. The testis was removed, cleared and weighed. Weights were expressed in term of 100 mg of body weight. The epididymis was removed and used for sperm analysis.

Sperm analysis

Epididymis was exposed by scrotal incision and sperms were expressed out by cutting distal end of the caudal epididymidal tubule. Sperms with epididymal fluid were diluted with physiological saline and sperm motility and morphology were studied. Spermatozoa were counted according to Zaneveld and Polakoski (17). Sperm suspension was placed on both sides of Neubauer's hemocytometer and allowed to settle in a humid chamber for 1 h. The number of sperms in the appropriate squares of the hemocytometer was counted under the microscope of 100· magnification.

RNA isolation and RT-PCR

Total RNA isolation from rat testis

The testis was cut into small pieces. Fenazol was added to the testis tissue samples. The samples were homogenized and incubated for 5 min at 50 _C. Chloroform was added and the samples were centrifuged (12,000 rpm, 10 min, room temperature).

The aqueous phase was transferred to fresh tubes and isopropanol was added. The supernatants were incubated for 10 min at room temperature and centrifuged (12,000 rpm, 15 min, 4 _C); the RNA pellets were washed with 70% ethanol, air dried, and resuspended in diethylprocarbonation-treated water (DEPC-H2O). Total RNA samples were analyzed using gel electrophoresis. The final amount of RNA was estimated by determining the optical density at 260 nm.

cDNA synthesis and PCR-amplification

First strand cDNA synthesis with total RNA was performed using reverse transcriptase. Subsequently, PCR-amplification was performed by the method described Saiki et al. (18) the sequence of CREM primers were 50-GATTGAAGAAGAAAAATCAGA- 30 (forward primers, exon B) and 50-TTGACATATTCTTTCTTCTT- 30 (reverse primer, exon H), while for rat b-actin, 50-AGGCATCCTGACCCTGAAGAT-30 and 50-TCTTCATGAGGTAGTCTGT-CAG-30 were used. The PCR products were separated on 1.5% agarose gels, visualized under UV light, and analyzed using NTSYS software.

Western blot

Proteins from homogenized testis were separated using a nuclear extract kit. The samples for protein extraction were half of the same testis used for RNA extraction. The protein extracts were separated in 10% tris-glycine gels by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes electrophoretically. X-12 CREM-1 was used as the primary antibody against CREM-t, and conjugated goat anti-rabbit IgG was used as the secondary antibody. The antigens were detected using seePico CBB stain kit.

Statistical analysis

Data were expressed as mean± standard deviation. Student's t-test was used to compare means. A level of $p< 0.05$ was considered as statistically significant.

Results

Table 1 showed there were no significant changes in body weight between two groups however, there was a significant difference in testis weight compared with control group. Results showed that rats treated with S. hypoleuca increased sperm count or sperm concentration and motility and decreased sperm abnormalities compared with control group. RT-PCR and western blot were applied to determine effect of S. hypoleuca on CREM gene and protein expression in rat testis. CREM fragment was detected at 416 bp. Expression of CREM mRNA increased significantly in testis of rats treated with S. hypoleuca ($p < 0.05$, Fig. 1). b-Actin showed constant expression as internal control or housekeeping gene. Western blot of testicular homogenates revealed a major CREM-immunoreactive band with an approximate molecular weight of 35 kDa. There was significantly increased expression of CREM-immunoreactive bands in testis of rats treated with 250 mg/kg/day compared with control (Fig. 2).

Table 1: Body, testicular weight, and sperm parameters of control and experimental groups.

Parameters	Control	Experimental
Body weight (g)	378 ± 23.1	380 ± 17.5
Testis weight (g)	1.53 ± 0.06	1.62 ± 0.09*
Sperm count (10^6/ml)	10.27 ± 0.41	16.43 ± 0.27*
Sperm motility (%)	63.97 ± 3.27	71.66 ± 0.66*
Sperm abnormalities (%)	34.53 ± 0.97	31.82 ± 0.93

Values are mean ± SEM (n = 8).
* $p < 0.05$ (significantly different) vs. control.

Figure 1: RT-PCR analysis of CREM gene expression in testis from Salvia hypoleuca-treated rats. The left panel shows the expression of CREM and b-actin mRNA. The right panel, CREM mRNA levels are expressed as a ratio of those of the control group. Each column represents the mean ± S.D. *Shows that the mean differs significantly between the control and the Salvia hypoleuca-treated group (p<0.05).

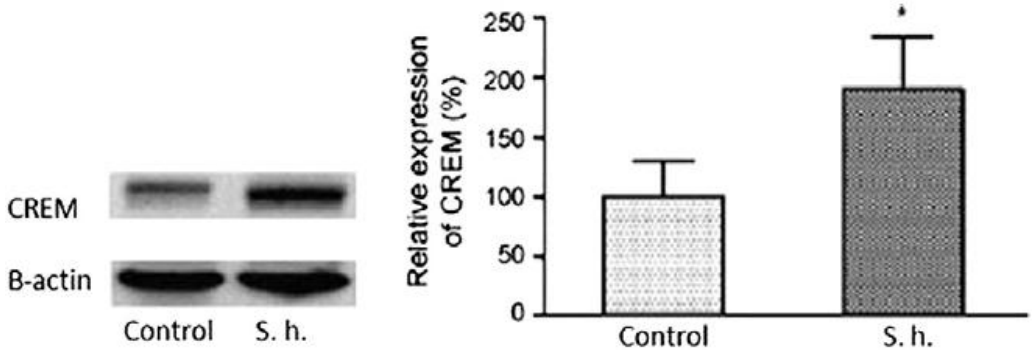

Figure 2: Western blot analysis of CREM protein from rat testis in the Salvia hypoleuca-treated group and control group.

Discussion

Results of our study indicated that S. hypoleuca affected sperm parameters and spermatogenesis in rat. In the present study treatment of rats with the extract of S. hypoleuca caused a significant increase in sperm count and motility, and decrease in sperm abnormalities compared with control group and increased testis weight of rats. CREM gene expression and protein level in rat testis treated with S. hypoleuca were higher compared with the control group. CREM is essential for spermatogenesis, and males lacking functional CREM gene are sterile due to their round spermatid maturation arrest (4,5,19). The CREM gene encodes the transcription activator CREM, which is highly expressed in male germ cells (20) and regulates the expression of many important post-meiotic genes (21). In addition, infertile men have substantial reduction or complete lack of both CREM protein and CREM mRNA. In present study, S. hypoleuca caused little change in body weight and no animal died during the study; no animal exhibited noticeable adverse effect from the administration of the extract. Results showed S. hypoleuca may increase male fertility by elevating sperm quality due to CREM expression at mRNA and protein levels.

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