Physalis Alkekengi Reduces Spermatogenesis and Camp – Responsive Element Modulator Gene Expression In Rat.

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Summary

Physalis alkekengi has a large history of herbal use because of its pharmacological characteristics.The objectives of this study are to investigate effects of Physalis alkekengi extract on the structure of testis, its sperm parametic data, hormone levels(progesterone) and expression of cAMP-responsive element modulator(CREM) of adult rats. Physalis alkekengi extract was administered to adult male wistar rats (n=8) for 56 consecutive days (150 mg/kg/body weight/day). At the end of the treatment period, Sperm analysis, histological and hormonal studies, and CREM gene expression were studied. Results demonstrate disorganized germinal epithelium, degenerated seminiferous tubules, decrease in the number of sperms and their motilities, increase in the number of abnormal sperms, significant decrease in the level of testosterone and nonsignificant increase in the level of concentration of plasma LH, in experimental group. The level of FSH was normal between two groups. Reverse transcription polymerase chain reaction (RT-PCR) showed a decrease in the expression of CREM in experimental group. These results suggest that Physalis alkekengi has an antispermatogenic and antifertility effects in adult male rats.

Keywords: Physalis alkekengi, Spermatogenesis, CREM gene expression.

Introduction

It is undeniable that the increasing of the population in the developing countries is an anxiety, therefore finding safe and effective contraceptive materials can be very useful.Over 3/4 of the world population relies mainly on plants and their extracts for health care (1). Undoubtedly, the lack of medicinal plants utilization creates good feelings. Fertility control through natural products is being given great attention by WHO (2). *Physalis alkekengi*, of the family *Solanaceae*, has several medical uses. Biochemical studies demonstrated the presence of physalin, citric acid and vit C as the major components in P. alkekengi extract. Physalin is a compound with various pharmacological characteristics and is known as anti bacterial, anti leishmanial and anti tumor chemical (3-5). The whole plant is anti phlogistic, anti pyretic, anti tussive and expectorant (6-8). Its extract has been used for treatments of wide ranges of skin, urinary,kidney , bladder stone, fibrile disease, inflammation, general edema, arthritis and rheumatism diseases (8, 9). The antifertility activity of *P. alkekengi* has been vastly described in Persian traditional medicine (8). The results of recent studies have shown that P. alkekengi has antiimplantation activity and reduces the number of neonates. The data obtained from previous studies clearly demonstrated the presence of an estrogen antagonist in the aqueous extract of *P. alkekengi* which produces diestrus and

diminishes uterine glucose 6- phosphate dehydrogenase activity (10-12). The aqueous extract of P. alkekengi, containing steroidal compounds with known estrogen antagonistic properties, probably interferes with the function of estradiol in inducing ovarian 3β-hydroxysteroid dehydrogenase (3 β-HSD) synthesis. It may also contain components which inhibit this enzyme, thus reducing progesterone synthesis required for maintaining pregnancy (13). Spermatogenesis is well-coordinated developmental program in which the different steps are defined by a cell type – and stage – specific induction or repression of expression of specific genes (14). Many of these genes are expressed predominantly or exclusively in spermatogenic cells, and their regulation can involve control at the transcriptional or post- transcriptional levels, various hormones, signaling pathways, transcription factors;Interactions with the local environment play critical roles in regulating different stages of processes of differentiation (15, 16). cAMP-responsive element modulator (CERM) is a key factor in regulating the expression of number of postmeiotic genes().CREM also binds to cAMP response elements, modulates transcription of cAMP-responsive genes and regulates gene expression in spermatids (17, 18). Since, no research has so far been implemented about the possible effects of *P. alkekengi* on male reproductive system, this study was undertaken to elucidate the influences of *P. alkekengi* on the rats' male reproductive activities, by investigating any changes in testes structures, sperm parameters, hormone levels and CREM expression at mRNA level.

Materials and Methods

Plant material

Physalis alkekengi 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 gr/lit for 48 hours), filtered with filter paper. Ethanol was removed by rotary evaporator. The extract was dissolved in normal salin and administrated oraly into rats.

Animals and treatments

Healthy adult male wistar rats (200g-300g), purchased from Razi Institute (Karaj, Iran), were housed in animal house, at ambient room temperature with a controlled light and dark period of 12 hours. The animals were fed with a standard laboratory food (pellets) and provided adlibitum. They were weighted before and after the study. After 7 days for adapting to the new environment, the rats were randomly divided into two groups of control (treated with normal salin for the period of 56 days, n=8) and experimental (treated with 150 mg/kg/day of *P. alkekengi* extract for 56 days, n=8) groups.

Tissue preparation and Hormone assay

At the end of the treatment period, the pentobarbital sodium (40 mg/kg i. p.) was administered for anesthesia. Blood samples was collected from abdominal aorta, separated after centrifugation (3000 rpm) and stored at -80 C°, to carry out the hormonal assays. Hormone levels were measured by radioimmunoassay coat-A-count

kit (diagnostic products corporation,LA,Calif) using Packard Cobra gamma-counter.Testes were removed, leared of adhering tissue,and weighed. The epididymis was removed, used for sperm analysis. Some of the testes samples were frozen before RT-PCR and others were fixed in formalin 10%. Then, tissue processing for H&E staining and histopathological studies, were carried out.

Sperm analysis

For analysis, epididymis was exposed through scrotal incision and sperms were expressed out by cutting the distal end of the caudal epididymal tubule. Spermatozoa (with epididymial fluid) were diluted with physiological salin and their motilities and morphologlical structures were studied. Countings were carried out as per the method of Zaneveld and Polakoski (19). Sperm suspension was placed on both sides of Neubauer's hemocytometer and allowed to settle in a humid chamber,for 1 hour.The number of spermatozoa, in the appropriate squares of the hemocytometer, was counted.

RNA isolation and RT-PCR

Total RNA isolation from rat testes

The testes were cutted into small pieces. Fenozol was added to the samples, homogenized and incubated for 5min at 50 C°. Chloroform was added, samples were centrifuged (12000 rpm, 10min, room temperature). The aqueous phase was transferred to fresh tubes and isopropanol was added, supernatants were incubated for 10min, at room temperature and centrifuged (12000 rpm, 15min, 4C°). RNA pellets were washed with 70% ethanol, air-dried, and resuspended in diethylpyrocarbonated-treated water (DEPC-H2O). Total RNA samples were analyzed by 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 by reverse transcriptase,and PCR-amplification was performed (Saiki et al.). The sequences of the CREM primers were 5'-GATTGAAGAAGAAAAATCAGA-3' (forward primer, exon B) and 5'-TTGACATATTCTTTCTTCTT-3'(reverse primer, exon H),and for rat β-actin,5'-AGGCATCCTGACCCTGAAGAT-3' and 5'-TCTTCATGAGGTAGTCGTGCAG-3', were used (20). The PCR products were separated on 1.5% agarose gel, visualized under UV light and analyzed using NTYSIS software**.**

Statistical analysis

Mean and standard error of mean [SEM] were calculated, significant differences were analyzed by student T-test. The values were considered significant at $P<0.05$.

Results

Body and organ weights: Treatments with *Physalis alkekengi* had no effect on the survival of the male rats. Results showed no significant differences between body weights of the rats of control and experimental groups (Table 1). However, there was a significant reduction in the weights of testes of experimental group, compared with control group (Table 1).

Sperm analysis: Administration of *P. alkekengi* extract (150 mg/kg/day) for 56 consecutive days, has significantly reduced sperm counts and motility and increased sperm abnormalities in experimental group in comparison with control group ($P<0.05$) (Table 1).

Histopathological studies: Histological studies showed normal seminiferous tubules in control group(Fig 2a),but indicated, as the result of *P. alkekengi* treatments, spermatogenic cells had become vacuolated in experimental group and also a less compact arrangements of the number of spermatids was observed (Fig 2b). It also revealed disorganized and degenerated seminiferous tubules, and large number of metaphasic cells in germinal epithelium of rats of experimental group (Fig 2C, 2D).

Plasma hormonal levels: Plasma level of testosterone was significantly decreased in treated group compared to the control ($P<0.05$). There was an increase in the plasma level of LH in treatment group,but not significant. Results also showed a normal level of FSH in two groups (Table 2).

CREM mRNA levels in rat testes: Reverse transcription polymerase chain reaction (RT-PCR) was performed to evaluate the effect of P. alkekengi extract (150 mg/kg/day) on CREM gene expression in rat testes. The CREM fragment was detected at 520 bp. CREM mRNA levels decreased significantly in testes from the experimental group in compared to control (Fig 2).

Table1: The effects of Physalis alkekengi extract on body weight, testicular weight and sperm parameters of adult male rat.

Parameters	Control	Experimental
Initial Body Weight (g)	218.38 ± 1.22	220.00 ± 1.41
Final Body Weight (g)	219.50 ± 1.21	217.25 ± 1.77
Testicular Weight (g)	1.58 ± 0.0087	1.51 ± 0.00 79*
Sperm Count (× 10^6/ml)	17.25 ± 0.52	10.75 ± 0.45*
Sperm Motility (%)	71.37 ± 0.59	61.87 ± 0.54*
Sperm Abnormalities (%)	29.75 ± 0.59	35.75 ± 0.49*

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

Table 2: Effect of 150 mg/kg/day Physalis alkekengi extract (for 56 days) on hormonal levels in adult male rat.

Parameters	Control	Experimental
Testosterone Level (ng/ml)	6.015 ± 0.037	4.608 ± 0.11*
FSH (MIU/M)	11.894 ± 0.055	8.848 ± 0.172*
LH (MIU/M)	9.777 ± 0.031	9.834 ± 0.016

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

Figure 1. Micrographs of rat's testicular tissue. Sections are stained with H&E method, showing the testis structure in different groups of rats. (a) Normal structure of testis in control group. (b) Vacuolated spermatogenic cells (stars) arranged in a less compact order and smaller number of spermatids (arrows) in the testis of experimental group.disorganized and degenerated seminiferous tubules, and large number of metaphasic cells (arrows) in germinal epithelium of testis in experimental group.

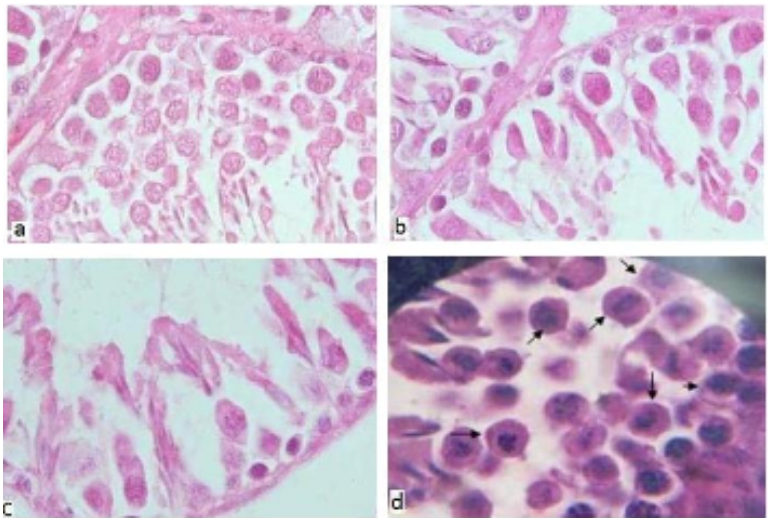
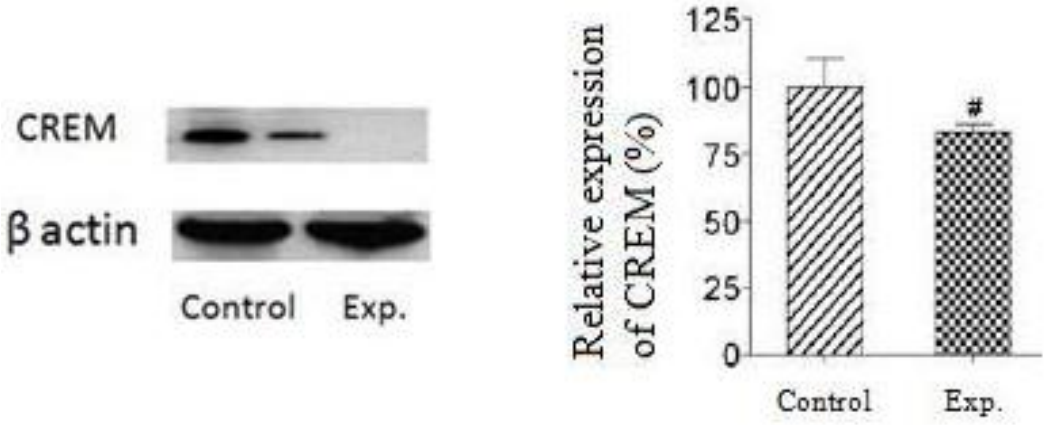


Figure 2. RT-PCR analysis of CREM mRNA expression in rat testes. Results showed that CREM mRNA expression was significantly decreased in the testes of experimental group in compare with control group.



Discussion

Present study was undertaken to evaluate effects of *P. alkekengi* extract on male fertility including, spermatogenesis and CREM gene expression in the testes of rats. Interestingly, although some studies demonstrated the effects of *P. alkekengi* on female mouse and rat fertility, no attempt appears to have been made, so far, to determine the effects of this plant on male fertility. *P. alkekengi* has a large history of herbal use. The anti fertility potential of *P. alkekengi* has been described in Persian traditional medicine, by having contraceptive and abortive properties (7, 8, 10). Antifertility effects of *P. alkekengi* on female rats have been proved by Montaserti et al., 2007, which affected by inhibiting implantation (11). Vessal et al., 1991, demonstrated the presence of an estrogen antagonist in the extract of *P. alkekengi* fruits which probably create interferences with the functions of estradiol in inducing ovarian 3β-HSD synthesis. It may also contain components inhibiting this enzyme, thus reducing progesterone synthesis required for maintaining pregnancy (10).

Our results indicated that administration of *P. alkekengi* extract creates marked reduction in the testes weights, sperm counts and motilities compared with controls. Increase in the number of abnormal and disorganized sperms were remarkable. Reduction in testicular weight, which is known to be mostly related to the number of spermatozoa. Considering the results of sperm analysis and histopathological studies, a decrease on the weights of testes of experimental groups can be expected. A significant reduction in the number and motility of sperm ($P<0.05$) was observed in experimental group, which could be due to the influence of the extract on the cell cycle, cell division, and expression of genes necessary for the spermatogenesis. It is also possible that these changes might be result of changes in the microenvironment of epididymis and creation of a toxic microenvironment presence in *P. alkekengi* extract, thus influencing sperm count and motility. A large number of metaphasic cells were observed in the germinal epithelium of treated group, possibly caused by cell cycle blockage.

Masao et al. (1988) showed that physalin M isolated from *P. alkekengi* exhibits cytotoxicity against tumor cells (5). It is also possible that effects of *P. alkekengi* extract on the reproductive system and function of male rats, has been results of the existence of physalin. Data from hormonal essay showed a significant reduction of the level of testosterone synthesized by lydig cells, under the control of LH. The mechanism by which LH stimulates lydig cells, involves the increase formation by cAMP via serpentine LH receptor (21). Testosterone exerts an inhibitory feedback effect on pituitary LH secretion (21). It appears that reduction of testosterone level makes a reduction as inhibitory action of testosterone on LH secretion, thus the secretion of LH will increased. The level of FSH was normal in both groups. Along with testosterone, FSH is responsible for the maintenance of gametogenesis and acts on the Sertoli cells to facilitate the spermatogenesis (21). CREM is essential for spermatogenesis, and males lacking functional CREM gene are sterile due to their round spermatid maturation arrest (17, 18, 22). The CREM gene encodes the transcription activator CREM, which is highly expressed in male germ cells (23) and regulates the expression of many important post-meiotic genes (24). In the present study, testes of rats treated with *P. alkekengi* exhibited significant reduction in CREM mRNA level, in comparison with control group. Given the results, it is concluded that *P. alkekengi* has specific effects on sperm parameters and reproductive functions of male rats which is probably performed by a reduction of the level of CREM expression. The decreasing effect of *P. alkekengi* on spermatogenesis may lead to the manufacturing of new drugs for regulation of spermatogenesis and fertility in males.

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