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Full Length Research Paper

The effect of aqueous extract of Salep prepared from root-tubers of *Dactylorhiza maculate* (Orchidaceae) on the testes and sexual hormones of immature male mice

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The use of the salep plant as a fortifying agent and sexual energy is common in traditional medicine. However, the unavailability of sufficient scientific evidence on the effects of sex hormones on the testis and the survey was designed and implemented. In this experimental study, 18 male rats breed immature BALB/C (mean age 4 weeks) were selected and divided randomly into three groups: 1) Control (no feeding), 2) Placebo (receiving 200 µl of distilled water), and 3) experimental (receiving 200 µl distilled water containing 40 mg of salep extract). Two weeks after the last injection, blood sampling to investigate the testosterone, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were done and mice' testes were also removed for histological studies. The average animal weight, testicular weight, volume and diameter of seminiferous tubules and also the number of Spermatogonia, Primary Spermatocyte, Spermatid, mature sperm (in Pachytene stage), Sertoli and Leydig cells between different groups were determined. Statistical analyses were done by using ANOVA and Tukey' test. Injection of aqueous salep extract significantly increased the levels of LH and testosterone hormones and also Spermatogonia, Spermatocyte1, Spermatid, mature sperm and Leydig cells in the experimental group rather than in the placebo group was evaluated. Some parameters such as the animal and testicular weight, the volume and diameter of seminiferous tubules, the number of Sertoli cells and the amount of the FSH hormone, did not show significant changes. One week injection of aqueous salep extract increases the LH and testosterone hormones, which can be effective on spermatogenesis cycle as well as sperm production and would be proposed as a booster drug of sexual activities.

Key words: Aqueous salep extract, sexual hormones, testis tissue, immature male mice Balb/c.

INTRODUCTION

Infertility is one of the problems of human society. According to the World Health Organization (WHO), 10 to 15% of young couples are faced with infertility difficulty that its 50% is related to the male factor causes (WHO, 1999; Henkel et al., 2005). The occurrence of male reproductive disorders, including genetic disorders, reproductive tubules obstruction, varicocele, reduced sperm production, reduced parameters of semen quality and erectile dysfunction leads to the male infertility (Zargari, 1989). Studies have shown that semen parameters in 25 to 40% of young people are below the WHO standard index (Andersen et al., 2000). Treatments are performed by surgery, chemical and herbal drugs and assisted reproductive laboratory procedures. Traditional

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herbal medicines prescribed commonly by physician in Iran and other countries for the treatment of many patients. One of these plants that used highly in India, Nepal, China, Europe and other regions of the world is *Dactylorhiza maculata* (Bhattarai, 1996; Freudenstein and Rasmussen, 1997). The powder form of its dried root-tubers is known as salep.

The tuber usually gets up in early summer and maintains its medicinal properties until the next year (Grieve, 1989) *D. maculata* contains different compounds such as glucomannan, nitrogenous substances, starch, protein, sugar, hydroxybenzaldehyde, Ferulic acid, Quercetin, Daucosterol, Cirsilineol and Steroids (Rojhan, 2002; Azamkhan, 2002). In traditional medicine, salep has been prescribed for dressing and treating of glottal inflammations and intestine disorders, tuberculosis, diarrhea, Parkinson, cancer, fever, and especially used to strengthen the sexual activity, erectile disfunction therapy, physical strength enhancement and increase vigorousness (Thakur and Dixit, 2007; Tekinsen and Guner, 2010).

Salep is used in Ice Cream, beverage industry and confectionery (Kaya and Tekin, 2001). Thakur studies on mice showed that consumption of salep root extract increases the tendency of opposite sex, number of erection and ejaculation, animal's body weight, weight of reproductive organs, fructose and testosterone hormone (Thakur et al., 2009, 2011). In a review study that was conducted by Shamloul (2010), he reported that although the tendency of using herbs to improve sexual activity and erectile dysfunction is growing, there is not enough documented scientific evidence to confirm it.

Concerning above objects, the effect of aqueous extracts of salep root (*D. maculata*) on sexual hormones and testes was conducted in immature male mice.

MATERIALS AND METHODS

Extraction methods

Extraction according to standard protocols was done by maceration method in Plant Research Institute, Karaj, Iran. Briefly, after collecting the root-tubers *D. maculata* from rural areas of Markazi province, Iran, plant parts were cleaned, dried and powdered, then mixed with 96% ethanol and thoroughly stirred for 24 h at room temperature to obtain a homogenous solution. The solution was filtered and dried for 48 h in dry conditions to produce non alcoholic dry extract. For preparing of aqueous extract, 1 g of dry extract was solved in 5 cc of warm double distilled water to achieve 20% extract solution (200 mg in 1 cc) and was kept in the refrigerator.

Grouping of animals

In this experimental study 18 immature male mice balb/c (age: two weeks) with an average weight of 14-16 g were used. Mice were purchased from Pasteur Institute of Iran and accommodated in Animal Care Center of Kashan University of Medical Sciences, for one week. During research animals were kept consequently in 12 h light and dark conditions and moderate temperature (20-25°C) and they had free access to food and water. All of the clinical trials that conducted on animals were approved by Animal Care Committee of Kashan University of Medical Sciences.

Before starting the main work, based on clinical experiments, different salep extract doses were injected to immature mice (to select the most appropriate drug dose). The dose of 40 mg gave the best results and therefore this amount was used as an appropriate dose in the main study.

Animals randomly divided into three equal groups: Experimental, Placebo and Control groups. In the experimental group, 40 mg salep root extract solved in 200 µl distilled water, were injected intraperitoneally daily for a week, Placebo group intraperitoneally received 200 µl distilled water daily for a week and the control group did not receive any material.

Blood collection and hormonal tests

Two weeks after the last injection and after weighing the animals, blood samples were obtained from mice heart. After separating the blood serum, FSH, LH and testosterone hormones were measured by Radio Immune Assay method in the Reference laboratory of Kashan University of Medical Sciences.

Microscopic study of the testis

Following the completion of tests period, mice were killed through the excision of cervical spinal cord. In sterile conditions by creating a gap in the lower abdomen, right and left testes were removed and placed in normal saline medium.

After removing of testis's fats, cleaned testes were weighed by Sartorius machine (made in Germany, with accuracy of 0.0001). Testicular volume was measured by dipping of testes into cylindrical measure contains normal saline.

For histological studies, testes initially were fixed in Bouins solution for 24 h and preparing stages were done by standard procedure and were finally embedded in paraffin. 5-micron sections of testes were prepared serially then were stained by Hematoxilin and Eosin method. 10 Tissue cross sections were dissected randomly and in each section, round or nearly round five seminiferous tubules at VII and VIII stage were studied (totally 50 seminiferous tubules).

Measurement of seminiferous tubules diameter

To measure the diameter of seminiferous tubuls, Zeiss microscope with eye piece micrometer and 100 × magnifications was used and measured as microns. 50 seminiferous tubules cells per mouse, including spermatogonia, primary spermatocyte, spermatid and mature sperm at the Pachytene stage with light microscopy (400× magnification) were counted and averaged, then after each group compared with others.

Leydig cells were counted by using of light microscopy (400 × magnifications) and the average numbers of cells in each group were compared with other groups.

Statistical analysis

Mean variables between the three groups; experimental, placebo and control were performed by using of ANOVA and Tukey' test. Difference of 5% >P is considered as significant.

RESULTS

All results are shown in Table 1. The animal weight, testicular weight, and testicular volume between the studied groups showed no significant difference.

Table 1. Ontogenetic and reproductive parameters in the three groups of mice groups.

Parameters	Experimental	Placebo	Control	Test results
Animal weight (g)	22±1.2	22±1.6	23±1.1	Ns
Testis weight (g)	0.065±0.004	0.066±0.006	0.78±0.005	Ns
Testicular volume (m^3)	0.098±0.005	0.10±0.003	0.10±0.003	Ns
Seminiferous tubules diameter (µm)	173.20±4.057	177.64±2.73	194.001±3.784	P< 0.01
Spermatogonia cells (no/tubules)	34±2.01	34±2.96	56±3.61	P< 0.001
Spermatocyte 1 (no/tubules)	43±1.92	44±3.20	67±2.50	P< 0.001
Spermatid cells (no/tubules)	96±4.25	104.7±5.57	175±4.05	P< 0.001
Mature sperm (no/tubules)	67±2.39	70±1.83	145±7.45	P< 0.001
Sertoli cells (no/tubules)	15±0.79	15±0.33	18±0.33	P< 0.001
Leydig cells (no/shown)	12±0.75	14±0.615	26±2.87	P< 0.001
FSH (ηg / ml)	0.107±0.01	0.093±0.011	0.09±0.015	Ns
LH (ηg / ml)	0.397±0.13	0.39±0.014	0.57±0.1	P< 0.05
Testosterone (ηg / ml)	0.47±0.011	0.52±0.039	1.02±0.055	P< 0.01

However, the seminiferous tubules diameter increased in the experimental group in comparison with other groups significantly (p <0/01).

In biochemical investigations there was no significant difference in the FSH level between three groups, but the level of LH and testosterone hormones was significant in the experimental group rather than in the placebo and control groups.

Number of spermatogonia, primary spermatocyte, spermatid, mature sperm and sertoli cells in seminiferous tubuls showed significant difference (p <0.001).

The average number of Leydig cells in the experimental group compared to the placebo and control groups showed significant difference (p <0.001). The parameters for comparison between the placebo and control groups were not confirmed to be statistically different.

DISCUSSION

This is the first experimental study on immature mice that shows the effect of salep root on the testis tissue and spermatogenesis cycle during the early ontogenesis. Studies that have done by Thakur and colleagues mostly focused on the impact of salep plant on sexual behavior of mouse that cause increasing of mice tendency to the opposite sex, number of erection, ejaculation, sperm count and semen fructose (Thakur and Dixit, 2007; Thakur et al., 2011). On the other side consumption of salep has been prevalent in traditional medicine of different societies and it believes this plant is energetic and tonic and is effective in improving of sexual male strength (Farnoosh and Riazi, 2007; Esteves et al., 2011).

The results of this research shows that the injection of aqueous extract of salep root in the experimental group increased the number of spermatogonia, primary spermatocyte, spermatid, mature sperm and sertoli cells rather than in the placebo and control groups that results increasing of seminiferous tubules diameter (Table 1).

One of the interesting points of this study is a significant increase of Sertoli cells number at immature mice. Based on the studies Sertoli cells proliferation occurs only on days of 10 to 18 (pre-maturation and Mid-maturation), and in days of 15-20 Sertoli cells proliferation fall down, and Gap Junction is created among Sertoli cells (Emerson, 2003). Sertoli cells improve the spermatogenesis under the effect of testosterone hormones (Mclahan et al., 1995). This study shows that salep extract affects mostly on proliferative cells (spermatogonia, spermatocyte and spermatid) and does not have effect on sertoli cells of mature mouse, which have lost its proliferative property.

Other result of this study was the increase in the rate of LH and testosterone hormones in experimental group rather than the control and placebo groups. The effect of testosterone on testicular cells is done via growth factors such as Integrins or Stem cell factor. These factors by mediated receptors on the proliferative cells cause the stimulation of Spermatogonial mitosis (Mclahan et al., 1995). Increasing the number of Leydig cells as a cell that secrete testosterone in response to the LH secretion, could be identified as the reason of testicular changes and extension of spermatogenesis period trough the salep component effects on the hormonal axis of Gonado-Hypophysis (Singh et al., 1995; Spaliviero et al., 2004). In this process LH acts via the Leydig surface cells so that following its attachment to the receptors, increases the rate of interacellular c.AMP (Contreras et al., 1996; Qian et al., 2009), this increase leads to the proteins phosphorylation of cells through the activation of a particular protein kinase A (Siu et al., 2005; Estrada et

al., 2003). Finally, Pregenelone is synthesized from cholesterol and driven by activation of steroid molecules (Griffin et al., 2010; Monder et al., 1994). This step is the most important reaction in the production of testosterone, then after testosterone attached to the androgenic receptors inside the Sertoli cells and spermatogenesis is activated (Lei et al., 2001; Zhang et al., 2001). Thus, the increase of LH through the Salep extract injection leads to the Leydig cells proliferation and finally causes the rise of testosterone hormone.

Salep also enhances the ATP (Farnoosh and Riazi, 2007) and it is probably salep receptor directly activated protein kinase activity inside the Leydig cell. So either increase of LH in the pituitary and enzymatic agent may cause increase of testosterone secretion.

The conversion of Spermatid to Spermatozoid (Spermiogenesis) is also affected by testosterone that secreted by Leydig cells (Mclahan et al., 1995). Probably the increase of spermatids and testosterone and LH hormones would increase Spermiogenesis development pathway, and sperm isolated from spermatids. In this study, the number of sperm in the seminiferous tubules increased significantly.

However, researchers have reported the presence of steroids in salep from *Dactylorhiza*-species) and it is believed that the augmentation of the level of sexual hormones are dependent on steroids (Lien and Lien, 1996; Wheeler and Garleb, 1991). The increase in the level of testosterone could release chemical Dopaminergic mediators. It is confirmed that there are significant relationship between the release of dopamine in accumberis nucleus and improvement of sexual activity.

The oxidant deletion from the semen fluid is one of the factors that have been proven its role in improving of sexual activity and increasing the number of sperm cells. Querectine and Ferulic acid are chemical substances that are found in the salep plant, and have shown anti-inflammatory, anti cancer and antioxidant roles (Cozzolino and Widmer, 2005). According to the previous studies presence of Quercetin, Daucosterol, Cirsilineol and Ferulic acid has been proven in salep plant (He et al., 2005), which is supporting our results as well. Ferulic acid has anti oxidant activity that its combination with C and E vitamins has shown anti cancer effect.

Glucomannan is another compound that has found in salep, which varies in different species between 7 to 61%. Glucomannan is a polysaccharide that provides adequate energy to sperm production in the seminiferous tubules. This material controls the weight, blood sugar and the amount of cholesterol (Thakur et al., 2009). The researchers believe that the rise of the level of testosterone promotes the anabolic processes and positively influences growth and body weight.

Conclusions

This study showed that the aqueous extract of salep root by the increase of the level of testosterone and LH hormones causes the improvement of spermatogenesis and the health of sexual organs. Further research on the influence of salep may introduce this substance as an effective supplementary drug for sexual activity and fertility improvement.

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