The effect of aqueous extract of Salep Tubers on the structure of testis and sexual hormones in male mice

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Abstract:
Introduction:
Salep tubers have been used in traditional medicine as a drug for improving sexual function and vigor. We did not find sufficient scientific evidence to support it. The present study was designed to evaluate the effect of Salep tuber extract on the structure of testis and sexual hormones in adult male mice.

Materials and Methods:
In this experimental study, we used 18 adult male mice (mean age of 4 weeks) and randomly divided them into three groups: the control group did not receive anything, the placebo group received only 200 µl deionized water, and the experimental group received 40 mg salep extract/200µl deionized water intraperitoneally for 7 days. Two weeks after the last injection, we took blood sample for biochemical analysis of LH, FSH and Testosterone and removed the testis of animals for histopathological study.

Results:
The results showed a significant increase in LH (ng/ml) in the experimental group (0.6 ± 0.09) compared to the placebo (0.39 ± 0.006) and control (0.38 ± 0.007) groups (p<0.05). Also, the level of testosterone hormone (ng/ml) showed a significant difference in the experimental group (0.38 ± 0.52) compared to placebo (0.37 ± 0.03) and control (0.36 ± 0.02) groups (P<0.05). The number of germinal and leydig cells revealed a significant difference in the experimental group compared to the placebo and control groups (P<0.01). Amongst the other parameters, no significant differences were observed in all groups.

Conclusion:
Injection of salep tuber extract could significantly be effective on spermatogenesis and concentration of LH and testosterone hormones.

Keywords: Plant Extracts, Testis, BALB C Mice

Introduction
Infertility is one of the problems of mankind. According to the World Health Organization (WHO) report, 10 to 15% of young couples are faced with infertility problem. Male infertility factors contribute to approximately 40% of all infertility cases (1-2). There are many different causes in the incidence of male infertility including: genetic disorders, obstruction of the genital tract, varicocele, reduced sperm production, reduced semen quality parameters, erectile dysfunction, and men’s sexual impotence (3). Studies have
shown that semen parameters in 25 to 40% of young people are below the WHO standard indicators (4). Treatment is performed through surgery, chemical and herbal medication, and laboratory assisted reproductive techniques. In Iran and other countries, prescribing herbal drugs by herbal medicine practitioners is commonplace for treatment of many diseases. One of these plants that is highly used in India, Nepal, China, Europe and other parts of the world is the “Salep” (wild orchid) (5-6).

Salep plant or marshland finger tuber (Dactylorhiza lancibracteata (C.koch) Renz) with the old name of “Orchis maculata L.”, which belongs to the Orchid family, has many species and grows almost all over the world. Its tuber is usually harvested in early summer and maintains its medicinal properties for two years (7-9). This plant contains such chemicals as: glucomannan, nitrogenous substances, starch, protein, sugar, hydroxy benzaldehyde, ferulic acid, Quercetin, Daucosterol, Cirsilineol, and steroid (5-10, 12). In traditional medicine, this plant is used for dressing and treating glottal inflammation and in treatment of bowel disorders, tuberculosis, diarrhea, Parkinson’s, cancer, fever, and especially as a regenerative in sexual activity and erectile dysfunction, and it is also prescribed for increased stamina and as an energizer (13-14, 5-6). This plant is also used in ice-cream, soft drinks, and confectionary industries (15-16).

Studies of Thakur et al. on rats showed that consumption of salep plant root extract increases attraction to the opposite sex, frequency of erection and ejaculation, the animal’s body weight, reproductive organs weight, fructose and testosterone (17-19). In a review study by Shamloul, it was reported that despite increased tendency to use medicinal plants to improve sexual activity and erectile dysfunction, but, there are no scientific evidence to support such effects (20).

Given all the above, this study was conducted to examine effects of Salep tubers aqueous extract on the testis tissue and sexual hormones in adult male mice.

Materials and Methods

Collection and extraction method:
Samples of Salep plant or marshland finger tuber were collected from suburbs near Khomain city heights and after preparation of herbarium specimens, they were identified by the Kashan’s Barij Essence Company’s botanist and registered with code number 195-1. The roots of this tuber plant were collected in early summer and after washing and dust removal, dried in the shade in laboratory. Fully dried samples were turned into a powder using an electric herb grinder. The resulting powder was mixed with ethanol 96% at a rate of 5 times volume of plant and thoroughly stirred in a Roto-Mix mixer for 24 hours at ambient temperature until a homogenous solution was obtained. Next, the solution was filtered through and dried in ambient conditions for 48 hours to transform into a solid alcohol-free extract. To prepare the aqueous extract, one gram of the obtained extract was dissolved in 5cc double distilled water to achieve a solution containing 20% extract (200 mgr in 1 cc) and it was kept refrigerated.

Grouping of animals:
In this experimental study, 18 adult male mice (one month old) of the Balb/c strain with mean weight of 21-23 grams were used. The mice were procured from the Pasteur Institute, and accommodated in Animal Care Center of Kashan University.
of Medical Sciences for a week to adapt to the environment. During the study, animals were kept under 12 hours light and 12 hours dark conditions at ambient temperature (20-25 °C) and had free access to food and water. Animals were randomly divided into 3 equal groups of: experimental, placebo, and control. Experimental group received intraperitoneal injections of 40 mg Salep root extract in 200 µl double distilled water daily for one week. The placebo group received 200 µl double distilled water intraperitoneally, and control group did not receive any substance.

**Blood collecting and hormonal tests:**
Two weeks after the last injection and after weighing the animals, blood samples were obtained from their heart. After separating the blood serum; FSH, LH, and testosterone hormones were measured with Immunoradiometric Assay using Gamma counter LB951 Berthold model purchased from Beckman Coulter Company-Germany.

**Microscopic examination of testis tissue:**
Following the completion of test period, mice were killed by cervical dislocation. In sterile conditions, an incision was made in the lower abdomen, right and left testes were removed and placed in a physiological serum. After removing the surrounding fat, they were weighed separately using Sartorius scale (made in Germany, with accuracy of 0.0001 grams), and their volume was measured by submerging them into physiological serum in a 5 ml graduated cylinder.

For the histological studies, testes were fixed in Bouins solution for 24 hours and preparation stages were performed with standard procedure, and finally they were embedded in paraffin. 5-micron serial sections perpendicular to the longitudinal axis of testes were prepared and stained using Hematoxilin-Eosin method. Ten slices were randomly selected from each animal tissue sections, and in each slice, 5 round or nearly round seminiferous tubules (50 in total) that were in stages VII and VIII of seminiferous epithelium cycle, were assessed. To measure seminiferous tubule diameters, Zeiss microscope fitted with an eye piece micrometer with 100×magnification and calibrated by a stage micrometer was used. Measurements were expressed in microns. Number of seminiferous tubules’ cells including: spermatogonia, spermatocyte I of pachytene stage, spermatids, and mature sperm were counted using an light microscopy (with 400×magnifications) in 50 round seminiferous tubules in each animal, and mean number of these cells in each group was compared with other groups. Leydig cells were counted using light microscopy (400×magnification) in 10 visual fields (50 visual fields in total for each animal), and average number of cells in each group was compared with other groups.

**Statistical analysis:**
Mean variables between the three groups of experimental, placebo, and control were performed using ANOVA and Tukey’s test with confidence level of 95%. Differences were considered significant at P<0.05.

**Results**
All the results obtained in this study are presented in table 1. Comparison of animal’s weight, testes weight, testes volume, and seminiferous tubule diameters between study groups revealed insignificant differences. No significant difference was observed in mean FSH hormone between the 3 groups.
Mean level of luteinizing hormone showed a significant increase (P<0.05) in the experimental group (0.6±0.09) compared to placebo group (0.39±0.006) and control group (0.38±0.007). Also, mean level of testosterone hormone showed a significant increase (P<0.05) in the experimental group (1.25±0.52) compared to the placebo group (0.37±0.03) and control group (0.36±0.02).

Number of spermatogonia cells with (P<0.01) and spermatocytes I in the stage of pachytene, spermatids, and mature sperm cells (P<0.001) in every seminiferous tubule in the experimental group showed significant differences compared to the placebo and control groups. However, number of Sertoli cells per tubule was not significantly different between the three groups. Leydig cells in the experimental group (22±2.09) showed a significant difference (P<0.01) compared to placebo (15±1.46) and control (16±1.01) groups. Comparison of study variables between placebo and control groups revealed insignificant differences.

**Discussion**

The results indicate a significant increase induced by Salep plant root aqueous extract injections in number of spermatogonia, spermatocyte I, spermatid, and mature sperm cells.

This is the first experimental study on mice that shows effect of Salep plant on the structure of testis tissue. Studies conducted by Thakur *et al.* rather focused on the sexual behavior effects of Salep in mice, which caused an increase in attraction to the opposite sex, frequency of erection and ejaculation, testosterone hormone, sperm count in epididymis, and semen fructose (17, 19). On the other hand, use of this plant in traditional medicine is commonplace in various societies and it is believed that this plant is nourishing and energizing and effective in improving male sexual libido (11, 15).

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control</th>
<th>Placebo</th>
<th>Experimental</th>
<th>Test results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal weight (gr)</td>
<td>22±1.2</td>
<td>22±1.6</td>
<td>23±1.1</td>
<td>NS**</td>
</tr>
<tr>
<td>Testes weight (gr)</td>
<td>0.73±0.003</td>
<td>0.7±0.009</td>
<td>0.7±0.004</td>
<td>NS</td>
</tr>
<tr>
<td>Testes volume (Cm³)</td>
<td>0.10±0.003</td>
<td>0.10±0.008</td>
<td>0.10±0.003</td>
<td>NS</td>
</tr>
<tr>
<td>Seminiferous duct diameter (µm)</td>
<td>180.160±5.10</td>
<td>182.72±4.36</td>
<td>188.79±4.52</td>
<td>NS</td>
</tr>
<tr>
<td>Spermatogonia cells (number/duct)</td>
<td>44±3.49</td>
<td>43±3.78</td>
<td>61±3.09</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Spermatocyte I cells (number/duct)</td>
<td>46±2.10</td>
<td>45±2.57</td>
<td>62±2.84</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Spermatid cells (number/duct)</td>
<td>126±4.90</td>
<td>125.7±2.96</td>
<td>180.5±7.45</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Mature sperm cells (number/duct)</td>
<td>53±5.3</td>
<td>60±8.33</td>
<td>134±7.04</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Sertoli cells (number/duct)</td>
<td>15±0.47</td>
<td>14±1.46</td>
<td>16±0.79</td>
<td>NS</td>
</tr>
<tr>
<td>Leydig cells (number/microscope field)</td>
<td>16±1.01</td>
<td>15±1.46</td>
<td>22±2.09</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>FSH (ng/ml)</td>
<td>0.39±0.23</td>
<td>0.41±0.03</td>
<td>37±0.03</td>
<td>NS</td>
</tr>
<tr>
<td>LH (ng/ml)</td>
<td>0.38±0.007</td>
<td>0.39±0.006</td>
<td>0.6±0.09</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>Testosterone (ng/ml)</td>
<td>0.36±0.02</td>
<td>0.37±0.03</td>
<td>1.25±0.52</td>
<td>P&lt;0.05</td>
</tr>
</tbody>
</table>
* Findings are presented as mean±SD.
** NS stands for Not Significant
Other results of this study include increased sexual hormones of luteinizing (LH) and testosterone in the experimental group compared to the control group (P<0.05). This increase has also been reported in other studies (17).

An increase in the number of Leydig cells as a testosterone secreting cell in response to luteinizing hormone could explain changes created in the testis tissue and increasing trend of spermatogenesis through effects of chemicals in Salep plant on the hypothalamic-pituitary-testis axis. Increased testosterone could release dopaminergic mediator chemicals in the brain. It has been demonstrated that there is a significant relationship between dopamine release in the nucleus accumbens and improving sexual activities (21-23).

One of the factors proven to have a role in increasing sperm count and sexual activity is elimination of the oxidants in semen. Quercetin and ferulic acid are chemicals found in the Salep plant and are considered to have an anti-inflammatory, anti-cancer, and antioxidant role (10, 24-26). Young et al. stated that consumption of natural antioxidants protects sperm cells against oxidative stress induced by lysed cells and ultimately improve fertility (27).

Other compositions in the Salep plant include glucomannan whose level in various species varies from 7 to 61%. Glucomannan is a polysaccharide that provides the required energy for sperm production in the seminiferous tubules. This substance has a role in weight loss, blood glucose control, and cholesterol reduction (12, 28). Lack of animals’ weight gain could be due to relatively high proportion of this substance in the Salep root extract or short experiment duration. In contrast to the results obtained in this study, other studies have reported body and sexual organs’ weight gain induced by use of this plant (16). They believe that anabolic activity of the body increases with increasing testosterone, which leads to increased body and sexual organs’ weight.

**Conclusion**

This study showed that Salep root aqueous extract increased spermatogenesis and improved libido by increasing testosterone and LH. It is recommended that further studies be conducted in order to find the mechanism and the effect of this substance, so that it can be used as an effective medicine for improved sexual activity and fertility.

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**References:**

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