두자 투약기간이 수컷 생쥐의 생식능력에 미치는 영향

경희대학교 대학원 한의학과 부인과학교실
박대순, 이진우, 이창훈, 조정훈, 장준복, 이경섭

ABSTRACT

Administration Term-dependent Effects of Allii Tuberosi Semen Extract Solution on the Reproductive Capacities in the Mice

Dae-Soon Park, Jin-Moo Lee, Chang-Hoon Lee, Jung-Hoon Cho,
Jun-Bock Jang, Kyung-Sub Lee
Dept. of Oriental Gynecology, College of Oriental Medicine,
KyungHee Univ., Seoul, Korea

목 적: 본 연구는 기간별 두자 투약이 생쥐의 생식능력에 미치는 영향을 알아보고자 하였다.

방 법: 이를 위해 30일, 60일, 90일 및 120일간의 두자 투약이 생쥐의 총 정 자수, 활동 정자수, 정상형태 정자수, 고환조직 및 hyaluronidase activity를 측정 하여, 다음과 같은 결론을 얻었다.

결 과:
1. 총 정자수는 60일과 90일 투여 후 대조군에 비하여 증가하였다.
2. 활동 정자수는 60일, 90일 및 120일 투여 후 대조군에 비하여 증가하였다.
3. 정상 형태 정자수는 60일, 90일 및 120일 투여 후 대조군에 비하여 증가하였다.
4. 고환 세정관의 조직학적 변화는 60일 투여 후에 가장 현저히 나타났다.
5. Hyaluronidase activity는 모든 투여군에서 대조군에 비하여 증가하였다.

결 론: 이상의 결과를 통해 60일 이상의 두자 투약이 남성 생식능력을 활성화 할 수 있다고 사료된다.

교신저자(박대순) : 경기도 부천시 오정구 원종2동 229-7 원종한의원
전화 : 032-678-3114 이메일 : emdonejong@daum.net
I. Introduction

Although there is controversy on the variation in the quality of semen between different studies\(^{1,2}\), a number of publications have reported a global decline in the semen quality of infertile or subfertile men\(^{3,4}\).

In 2002, Jensen et al. reported that the worldwide decreasing semen quality among young men could be a factor in the declining fertility rates among teenagers and might be the cause of the decreasing birthrate\(^{5}\). Moreover, various male factors are involved in approximately 30-50% of infertile couples\(^{6}\).

The suggested risk factors that affect semen quality are age\(^{7}\), obesity\(^{8}\), various kinds of environmental and occupational chemical\(^{9,10,11}\) and smoking\(^{12,13,14}\). Although many aspects of male factor infertility have remained as yet unclear, several causes can interfere with spermatogenesis and reduce sperm quality and production. Many clinicians now accept the importance of good quality sperm to ensure IVF success, with or without ICSI\(^{15,16}\).

To improve the semen quality, a number of drugs or nutritional therapies have been studied\(^{17}\) and recently some kinds of Oriental medicinal herb or prescription have showed satisfactory results\(^{18,19,20}\).

Previous studies confirmed the favorable antioxidant effect of Allii tuberosi Semen on the sperm and dose-dependent effect on the sperm index\(^{21,22}\).

Therefore, we investigated the administration term-dependent effects of Allii tuberosi Semen extract solution on the sperm index, the histological changes of testis and the activity of hyaluronidase in male mice.

II. Materials and methods

1. Herb

We used the dried mature seeds of Allium tuberosum Rottler (Allii tuberosi Semen) purchased from the department of oriental pharmacy, at the hospital of Oriental medicine, Kyung-Hee university.

2. Experimental animals

Eight-week-old male ICR mice (31.42±1.57g) were purchased from Samtaco. Co. (Seoul, Korea) and housed in an animal room at 23°C temperature with a light:dark cycle of 12h:12h. All mice were given access to standard laboratory chow and tap water ad libitum.

3. Experimental preparation

The water-soluble extract of Allii tuberosi Semen was prepared by boiling 1000g of dried Allii tuberosi Semen with 1L distilled water (Ultrapure
Water Systems. Milli-Q, USA) at 60°C for 48 hours. The dissolution was physically stimulated with an ultrasonic cleaner and the collected supernatant was filtrated with filter paper (Whatman No. 5, USA). The remaining solid materials received further physical stimulation for 30 min with 1L distilled water and filtrated. The two kinds of filtrated solution were mixed and the concentration was compressed (below 60°C, reduced pressure) by rotary vacuum evaporator (Eyela, Japan). The compressed solution was preserved -60°C for 48 hours (Temphold Hanil, Korea) and freeze-dried by freeze dryer (CleanVac 8S, Hanil, Korea) for 72 hours. Finally, 48.81g of water-soluble extract of Allii tuberosi semen was obtained.

4. HPLC of experimental solution

To confirm the constituents of the experimental solution, 50ml of ethanol (50%) was added to 500mg of Allii tuberosi semen extract and dissolved for 1 hour before being centrifuged. A further 50ml of ethanol was added again to the remaining solid materials and ultrasonic extraction was repeated twice for 15 min. All solutions were mixed to obtain the compressed concentrated ethanol extract of Allii tuberosi semen. Before liquid chromatography, 50ml of ethanol (50%) was added and the mobile phase was 0.1M H₃PO₄:CH₃CN (72:28, v/v). An aliquot was injected into the Waters Spherisorb ODS1 column (40mm×250mm) and analysed on by high performance liquid chromatography (HPLC: Waters 996 Photodiode Array Detector. USA) with a UV detector (254nm). The HPLC chromatogram of the extract of Allii tuberosi semen is shown in Fig. 1.

Fig. 1. HPLC chromatogram of Allii tuberosi semen

5. Experimental design and administration

Forty mice were divided into four experimental groups of ten mice each. In groups A, B, C and D, Allii tuberosi semen extract solution was administered for 30, 60, 90 and 120 days, respectively. The water-soluble extract of Allii tuberosi semen was dissolved in water and the daily treatment dose was controlled at 0.3mg/g.

Twenty mice were divided into four corresponding control groups of 5 in each group and received the same
amount of saline for the same days, respectively.

6. Sampling and procedure

One day after the last administration of extract of Allii tuberosi Semen or saline, the mice were sacrificed by decapitation. For sperm sampling, the cauda epididymis was cut with a surgical blade and the sperm were obtained from the vas deference with a microtube under the microscope.

Ten milliliters of collected sperm were suspended in M2 media and incubated in a CO₂ chamber (Forma, USA) for 1 hour. Aliquots of 5ul were suspended in a Makler sperm counting chamber (Sefi Medical Instruments, Haifa, Israel) for the total numbers of sperm and motile sperm to be calculated under the microscope (×200).

On a slide glass (Fisher, USA) washed with 70% ethanol, a 10ul sperm suspension was dripped and smeared with a cover slip (Fisher, USA) and then treated in order with the Diff-Quick kit (National Chemical, Japan) fixative for 15 sec. solution I for 10 sec and solution II for 5 sec. After drying at room temperature, we observed the morphology of the sperm and among 400 sperm counted the numbers of sperm showed normal head, body and tail.

7. Histological analysis

The testes from each mouse were fixed in 10% formalin (Junsei, Japan) and dehydrated by ethanol (Merck, USA) from low density to high density. Each stage was restricted to 1 hour and the testes were additionally dehydrated twice in 100% ethanol for 1 hour. After overnight cleaning by xylene (Junsei, Japan), the testes were embedded in paraffin wax (Oxford, USA) for 2 hours in every stages. The mounted testes were cut into 0.1mm sections with a rounding microtome (Reichert-Jung Co., Germany) and stained with hematoxylin-eosin (Sigma, USA) after the deparaffinization. After the mounting with Canada balsam (Junsei, Japan), the stained glass samples were observed under a light microscope (Nikon, Japan).

8. Hyaluronidase activity

The sperm suspension was diluted 5 times with 0.14M sodium chloride, after which 0.1ml acetate buffer (0.3mol/L containing 0.45mol/L sodium chloride) and 0.1ml hyaluronic acid substrate (4mg hyaluronic acid dissolved in 1L water) were added to 1ml diluted sperm suspension and the mixture was preincubated for 24 hours at 37°C. To the mixture, 60ul potassium tetraborate (0.8mol/L in water, pH 10) was added and reacted in a 100°C heating block (Fisher, USA) for 5 min. The reaction was stopped by ice and the mixture was then incubated in the warm bath for 20 min at 37°C after the addition
of 2ml p-dimethylaminobenzaldehyde. After incubation, the mixture was centrifuged (1500×g) for 10 min and the supernatant was collected. The optical density was read at 582nm with a spectrophotometer (Beckman, Germany).

9. Statistical analysis
The results were compared between the control and experimental groups using Student's t-test. The statistical significance was assessed among the experimental groups using one-way analysis of variance (ANOVA) followed by Tukey B multiple comparison test. A p-value less than 0.05 was considered statistically significant.

III. Results

1. Effects on the total number of sperm
After the administration of Allii tuberosi Semen extract solution, the total number of sperm was significantly increased in groups B and C compared with their respective control groups (p<0.01).

Among the experimental groups, the sperm count in group B was significantly more than in groups A and D (Table 1).

2. Effects on the number of motile sperm
After the administration of Allii tuberosi Semen extract solution, the number of motile sperm was significantly increased in groups B, C (p<0.01) and D (p<0.05) compared with their respective control groups.

Among the experimental groups, the number of motile sperm in groups B and C was significantly more than in groups A and D. Moreover group B showed a significant increase compared with group C (Table 2).

3. Effects on the number of morphologically normal sperm
After the administration of Allii tuberosi Semen extract solution, the number of morphologically normal sperm was significantly increased in groups B, C and D compared with their respective control groups (p<0.01).

Among the experimental groups, the number of morphologically normal sperm in groups B and C was significantly more than in groups A and D (Table 3).

4. Changes on histological analysis
The histological sections of both control and experimental mice, after the administration of saline or Allii tuberosi Semen extract solution, respectively, retained normal morphological characteristics. However, in group B mice, seminiferous tubules contained markedly more spermatozoa than did control group (Fig. 2).
5. Effects on the activity of hyaluronidase

After the administration of Allii tuberosi Semen extract solution, the
hyaluronidase activity was significantly increased in groups A (p<0.05), B, C
and D (p<0.01) compared with their respective control group.

Among the experimental groups, there were no significant differences (Table 4).

<table>
<thead>
<tr>
<th>Group</th>
<th>Control (n=5)</th>
<th>Experimental (n=10)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>20.8±5.9&lt;sup&gt;2&lt;/sup&gt;</td>
<td>20.4±3.9&lt;sup&gt;3&lt;/sup&gt;</td>
<td>ns</td>
</tr>
<tr>
<td>Group B</td>
<td>15.2±3.3</td>
<td>29.9±6.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>Group C</td>
<td>17.6±3.6</td>
<td>27.0±5.0&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>Group D</td>
<td>19.2±4.7</td>
<td>21.9±4.3&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>ns</td>
</tr>
</tbody>
</table>

1) Statistical significance was tested by Student’s t-test between the control and experimental groups.
2) Results represent mean±standard deviation.
3) The same letters indicate non-significant difference by Tukey B multiple comparison test.

Group A: administered for 30 days
Group B: administered for 60 days
Group C: administered for 90 days
Group D: administered for 120 days
Control: administered saline
Experimental: administered Allii tuberosi Semen extract solution at 0.3mg/g/day

Table 2. Effect of Allii tuberosi Semen Extract Solution on the Number of Motile Sperm in the Mice (x106/ml)

<table>
<thead>
<tr>
<th>Group</th>
<th>Control (n=5)</th>
<th>Experimental (n=10)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>11.2±3.3&lt;sup&gt;2&lt;/sup&gt;</td>
<td>12.8±2.0&lt;sup&gt;3&lt;/sup&gt;</td>
<td>ns</td>
</tr>
<tr>
<td>Group B</td>
<td>8.0±0.0</td>
<td>21.8±4.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>Group C</td>
<td>8.8±3.3</td>
<td>17.9±3.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>Group D</td>
<td>8.4±2.7</td>
<td>12.3±2.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>p&lt;0.05</td>
</tr>
</tbody>
</table>

1) Statistical significance was tested by Student’s t-test between the control and experimental groups.
2) Results represent mean±standard deviation.
3) The same letters indicate non-significant difference by Tukey B multiple comparison test.

Group A: administered for 30 days
Group B: administered for 60 days
Group C: administered for 90 days
Group D: administered for 120 days
Control: administered saline
Experimental: administered Allii tuberosi Semen extract solution at 0.3mg/g/day
Table 3. Effect of Allii tuberosi Semen Extract Solution on the Number of Morphologically Normal Sperm in the Mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Control (n=5)</th>
<th>Experimental (n=10)</th>
<th>p-value (^{1)})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>10.8±4.2 (^{2)})</td>
<td>14.7±3.2 (^{2)})</td>
<td>ns</td>
</tr>
<tr>
<td>Group B</td>
<td>9.2±2.2</td>
<td>20.8±6.4 (^{b})</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>Group C</td>
<td>10.4±1.1</td>
<td>21.3±5.5 (^{b})</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>Group D</td>
<td>11.0±2.9</td>
<td>15.6±2.6 (^{a})</td>
<td>p&lt;0.01</td>
</tr>
</tbody>
</table>

1) Statistical significance was tested by Student’s t-test between the control and experimental groups
2) Results represent mean±standard deviation
3) The same letters indicate non-significant difference by Tukey B multiple comparison test

Group A: administered for 30 days
Group B: administered for 60 days
Group C: administered for 90 days
Group D: administered for 120 days
Control: administered saline
Experimental: administered Allii tuberosi Semen extract solution at 0.3mg/g/day

Fig. 2. Histological change in the testis of mice administrated Allii tuberosi Semen extract solution (×200)
Table 4. Effect of *Allii tuberosi* Semen Extract Solution on the Activity of Hyaluronidase in the Mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Control (n=5)</th>
<th>Experimental (n=10)</th>
<th>p-value$^{1)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>0.106±0.004$^{a,b}$</td>
<td>0.175±0.053$^{a,b}$</td>
<td>$p&lt;0.05$</td>
</tr>
<tr>
<td>Group B</td>
<td>0.086±0.041</td>
<td>0.186±0.042$^{a,b}$</td>
<td>$p&lt;0.01$</td>
</tr>
<tr>
<td>Group C</td>
<td>0.106±0.005</td>
<td>0.190±0.026$^{a,b}$</td>
<td>$p&lt;0.01$</td>
</tr>
<tr>
<td>Group D</td>
<td>0.104±0.003</td>
<td>0.208±0.013$^{a,b}$</td>
<td>$p&lt;0.01$</td>
</tr>
</tbody>
</table>

1) Statistical significance was tested by Student's t-test between the control and experimental groups.
2) Results represent mean±standard deviation.
3) The same letters indicate non-significant difference by Tukey B multiple comparison test.

Group A: administered for 30days
Group B: administered for 60days
Group C: administered for 90days
Group D: administered for 120days
Control: administered saline
Experimental: administered *Allii tuberosi* Semen extract solution at 0.3mg/g/day

IV. Discussion

Infertility is defined as one year of unprotected intercourse without conception and the male factor accounts for 20%-50% of such infertility. To evaluate infertility in males, various systemic approaches such as history taking, physical examination, semen analysis and hormone assessment are typically conducted. Nevertheless available evidence has demonstrated that male infertility is an easily neglected issue of reproductive health$^{23)}$.

Moreover, although many treatments focus on the female partner, the various assisted reproductive technologies (ART) all carry the danger of direct transportation of genetic disorders and raise the risk for obstetrical and perinatal complications caused by multiple pregnancies$^{24)}$.

Recovery of testicular spermatozoa is therefore a useful therapeutic strategy for infertile males, irrespective of whether ART is scheduled or not.

Recently various kinds of surgical operation and internal medication have been developed and worldwide efforts made to gain the benefits of numerous nutritional therapies$^{61)}$$^{15)17)20)}$. Acupuncture has been suggested to improve some kinds of male infertility$^{25)26)}$, as have some kinds of Oriental medicinal herb and prescription$^{18)19)23)}$.

According to Oriental medicinal theory, the kidneys and liver, which are very closely related, are the two organs most commonly involved in male infertility. The kidney stores the essence Qi, which roughly corresponds to the western concept of male and
female gametes. The liver stores blood, which is closely related to the reproductive essence. Both the essence Qi and blood are Yin in nature and the liver and kidney are therefore considered to be of the same source. A weakness in one organ is often associated with an imbalance in the other. In addition, it is the liver’s function to make Qi move smoothly through all the organs, thereby ensuring the proper functioning of all bodily processes. If the liver is unable to promote this movement, pathology will arise in the affected organs.

The dried mature seeds of *Allium tuberosum* Rottler. *Allii tuberosi semen* were recorded to have a tonifying effect on the Yang of the kidney. In previous studies, *Allii tuberosi semen* was confirmed to have a favorable antioxidant effect on the sperm and a dose-dependent effect on the sperm index. Therefore, we investigated the administrative, term-dependent effects of *Allii tuberosi semen* extract solution on the sperm index and the activity of hyaluronidase in the mice.

After the administration of *Allii tuberosi semen* extract solution, the sperm count was significantly increased in 60- and 90-days administered groups compared with their respective control groups. Among the experimental groups, 60-days administered group showed significantly elevated sperm count compared with 30- and 120-days groups.

After the administration of *Allii tuberosi semen* extract solution, the number of motile sperm was significantly increased in 60-, 90- and 120-days administered groups compared with their respective control groups. Among the experimental groups, 60- and 90-days administered groups showed significantly elevated results compared with 30- and 120-days administered groups. Moreover 60-days administered group showed a significant increase compared with 90-days administered group.

After the administration of *Allii tuberosi semen* extract solution, the number of morphologically normal sperm was significantly increased in 60-, 90- and 120-days administered groups compared with their respective control groups. Among the experimental groups, 60- and 90-days administered groups showed significantly elevated morphologically normal sperm count compared with 30- and 120-days administered groups.

These sperm index results confirmed the satisfactory effect of *Allii tuberosi semen* extract solution on the quality of mice semen and suggest that the best results on semen index are achieved after 60-days administration of *Allii tuberosi semen* extract solution.

After 60-day administration of *Allii tuberosi semen* extract solution.
seminiferous tubules of the experimental mice were found to contain more spermatozoa than the control groups did. This suggests that the improved semen index was caused by the activation of testis tissue functions.

The activity of hyaluronidase showed a statistically significant increase in all experimental groups compared with their respective control groups, but the differences among the experimental groups were not significant. Therefore, the benefit of Allii tuberosi Semen extract solution on the hyaluronidase activity might start within 30 days but could not be increased after that.

These study results support the use of Allii tuberosi Semen to assist or treat infertile couples for whom the infertility is caused by male factors. However, purification of the active component(s) from Allii tuberosi Semen as well as its clinical application awaits further investigation.

V. Conclusions

1. Allii tuberosi Semen extract solution increases the total number of sperm at 60- and 90-day administration.
2. Allii tuberosi Semen extract solution increases the number of motile sperm at 60-, 90- and 120-day administration.
3. Allii tuberosi Semen extract solution increases the number of morphologically normal sperm at 60-, 90- and 120-day administration.
4. Allii tuberosi Semen extract solution improves the functions or mechanisms on seminiferous tubules at more than 60 days of administration.
5. Allii tuberosi Semen extract solution activates the hyaluronidase activity within 30 days.

Therefore, we suggest that Allii tuberosi Semen extract solution controls the sperm index and the reproductive capacities in the mice via a yet-to-be determined mechanism.

References


