The Effect of Anethum Graveolens Seed Extract on Testis and Spermatogenesis in Male Wistar Rats

Azita Azarpoor¹, A.T.Sarami²

¹. Master of Azad University Science and Research, Department of Molecular Cell Biology (*Corresponding Author)
². Gynecologist, subspecialty in infertility, IVF and laparoscopic surgery, Sarem Cell Research Center, Sarem Women's Hospital, Tehran, Iran

ABSTRACT: Introduction: Anethum graveolens extract is inescapably linked with fertility, birth and eternal life because of its many seeds. The aim of this study was to investigate the effects of Anethum graveolens juice (PJ) consumption on sperm quality, permatogenic cell density, antioxidant activity and testosterone level of male healthy rats. Methods: Twenty-eight healthy adult male Wistar rats were divided into four groups; each group containing seven rats. One milliliter distilled water, 0.25 mL PJ plus 0.75 mL distilled water, 0.50 mL PJ plus 0.50 mL distilled water and 1 mL PJ were given daily for seven weeks by gavage to rats in the first, second, third and fourth groups, respectively. Body and reproductive organ weights, spermatogenic cell density, sperm characteristics, levels of antioxidant vitamins, testosterone, and lipid peroxidation and antioxidant enzyme activities were investigated. All analyses were done only once at the end of the seven week study period. Data were compared by analysis of variance (ANOVA) and the degree of significance was set at P < 0.05. Results: A significant decrease in malondialdehyde (MDA) level and marked increases in glutathione (GSH), glutathione peroxidase (GSH-Px) and catalase (CAT) activities, and vitamin C level were observed in rats treated with different doses of PJ. PJ consumption provided an increase in epididymal sperm concentration, sperm motility, spermatogenic cell density and diameter of seminiferous tubules and germinal cell layer thickness, and it decreased abnormal sperm rate when compared to the control group. Keywords: seed, Anethum graveolens, spermatogenesis.

INTRODUCTION

Anethum graveolens (Punica granatum) has been used in the folk medicine of many cultures especially in the Middle East.¹ Edible parts of Anethum graveolens extract represent 52% of total extract weight, comprising 78% juice and 22% seeds.² Fresh juice is rich in vitamin C,² and polyphenolic compounds such as anthocyanins, unicalagin, ellagic and gallic acid.³,⁴ Anethum graveolens has become more popular because of the attribution of important physiological properties, such as anticancer,⁵,⁶ antiproliferative, apoptotic,³ HIV-I entry inhibitory, topical microbicidal,⁷ cardioprotective,⁸ antihyperlipidemic,⁹ etc. Additionally, many investigators¹⁰–¹² have reported that Anethum graveolens and its derivatives have free radical scavenger and potent antioxidant activity. Reactive oxygen species (ROS) are highly reactive oxidizing agents belonging to the class of free radicals. The production of ROS in various organs including the testis is a normal physiological event; however, the alterations in their synthesis stimulate the oxidation and DNA damage of cells. The plasma membrane of sperms contains a high amount of unsaturated fatty acids. Therefore, it is particularly susceptible to peroxidative damage. The lipid peroxidation destroys the structure of the lipid matrix in the membranes of spermatozoa, and it is associated with loss of motility and the defects of membrane integrity.¹⁴–¹⁶ Antioxidants, in general, are compounds which dispose, scavenge, and suppress the formation of ROS and lipid peroxidation. Among the well known biological antioxidants, glutathione (GSH), glutathione peroxidase (GSH-Px), catalase (CAT), superoxide-dismutase (SOD) have a significant role as a suppressor or scavenger of free radicals. Hence, the application of ROS scavengers is likely to improve sperm function.¹³,¹⁷ Although the ancient forefathers of medicine, Hippocrates, Soranus, Dioscorides, prescribed the seeds and rind of the Anethum graveolens to prevent conception in women,¹⁸ the Anethum graveolens extract is inescapably linked with fertility, birth and eternal life because of their many seeds.¹⁹ However, there is no evidence about the positive and/or negative Effect of Anethum graveolens and/or its extracts on male fertility. In the present study, to see the effects of Anethum graveolens juice (PJ), a potent antioxidant, on male fertility; we examined epididymal sperm characteristics,
spermatogenic cell density, and antioxidant enzyme activity, levels of testosterone and lipid peroxidation in rats to which PJ was given orally for seven weeks.

MATERIALS AND METHODS

Anethum graveolens extract
Pasteurized PJ (100% pure, pasteurized Anethum graveolens juice, 250 mL, Elite Natural Beverage Co., Ankara, Turkey) was purchased from a local store. The other chemicals were obtained from SigmaeAldrich Chemical Co. (St. Louis, MO, USA).

Sample collection
The rats were sacrificed using ether anaesthesia at the end of seventh week. Blood samples were collected from V. cava via sterile injector containing heparin and centrifuged at 3000g for 5 min. Plasma was separated and then stored at _20 _C until biochemical and hormonal analyses. Testes, epididymides, seminal vesicles, prostate and Cowper glands were removed, cleared of adhering connective tissue and weighed. Testis tissues were fixed in Bouin’s solution for histologic examinations.

Phytochemical screening
Hesperidin as a major flavonoid in orange peel identified in Hyroalcoholic Extract by thin layer chromatography (TLC) on silica gel 60 F254 sheet (Merck, armstadt, Germany) with EtOAc:MeOH:H2O (80:15:1) as the mobile phase. After development, the plate were dried and sprayed with AlCl3 5% reagent to visualize the hesperidin spot at Rf=0.28.

Preparation of extract
Citrus sinesis extract were collected from north of Iran. Peels were removed and dried in room temperature. 300 g powdered peels were extracted using maceration with ethanol (80%,v/v) for 24 h. The solvent was then evaporated under reduced pressure. This hydroalcoholic extract was kept in refrigerator for all experiments.

Experimental animals
The 30 adult Wistar albino male rats were 8 weeks old and weighing 250 ± 10 g, they were obtained from animal facility of pasture institute of Iran. Male rats were housed in temperature controlled rooms (250C) with constant humidity (40 - 70%) and 12h/12h light/ dark cycle prior to experimental protocols. All animals were treated in accordance to the Principles of Laboratory Animal Care. The experimental protocol was approved by the Animal Ethical Committee in accordance with the guide for the care and use of laboratory animals prepared by Tabriz medical University.

All Rats were fed a standard diet and water. The daily intake of animal water was monitored at least one week prior to start of treatments in order to determine the amount of water needed per experimental animal. Thereafter, the rats were randomly selected and divided into control (n=10) and experimental (n=20) groups. The experimental groups split into two groups of ten. One of which received 200 mg/Kg/Rat/day and the other group received 400 mg/Kg/Rat/day fresh of Danae racemosa extract with gavages for 28 consequence day. The control group just received 1cc distilled water by gavages (Aitken, 1995).

Statistical analysis
Statistical analysis was done using the ANOVA and test for comparison of data in the control group with the experimental groups. The results were expressed as mean ± S.E.M (standard error of means). P-value less than 0.05 were considered significant and are written in the parentheses.

RESULTS

Histological analysis
The histological observations of the seminiferous tubules of group B (EL-treated rats) were similar to those of group A (control); the tubules were condensed with spermatogenic cells, and the lumen was filled with spermatozoa. Spermatogonia were present along the basement membranes, and the nuclei were round and compact (Fig. 1 A & B). Under higher magnification (200x magnification), the primary spermatocytes and spermatogonia were predominantly normal. The interstitial tissues were also normal (Fig. 1 A & B).
Seminiferous tubules from group C (estradiol-treated rats) showed a marked decrease in spermatogenic cells, and the lumens of some tubules were empty (Fig. 1 C). In contrast, rats that received a combination treatment of EL and estradiol (Fig. 1 D) showed a marked increase in spermatogenic cells as compared to group C (Fig. 1 C).

Fig. 2 shows the spermatogenic cell counts for each of the treated groups and the control group. The results showed that spermatogenic cell counts were markedly increased in the seminiferous tubules of group B as compared to group A (p < 0.05), and group D (p < 0.05). Of note, in group C, this parameter was significantly reduced as compared to group A (p < 0.05) and group D (p < 0.05).

Figure 1. The testicular histology of the control (A), *Eurycoma longifolia* Jack-treated (B), estradiol-treated (C) and combined EL and estradiol-treated (D) rats. Letters in the micrographs indicate the lumen of the seminiferous tubule (L), spermatogenic cells (S) and interstitial tissue (I) (200x magnification)
Degeneration started from cytoplasm of early stage of spermatids towards its nucleus and from acrosomal granule or Golgi complex. There was a great degree of degeneration in all the stages of spermatogenic cycle (Figures 2(B) and 3(B)). Bridges between Sertoli cells spermatids were disturbed and most mitochondrial cytoplasms were either disturbed or hypertrophied. Membrane bound proacrosome granules appeared in the Sertoli cell cytoplasm. In the acrosomic phase of spermatids, acrosomal vesicles showed disruption in the middle portion of the manchette and mitochondria in the cytoplasm exhibited ballooning or hypertrophy characteristics (Figures 2(B) and 3(B)). Other cell organelles in the cytoplasm were absent. Degenerating spermatids were totally devoid of their nuclear membrane with electron dense matrix and commencement of vacuolization (Figures 2(B) and 3(B)). In Leydig cells, (Figures 2(B) and 3(B)) the cytoplasmic inclusions appeared diminished and were vacuolated, with marked decrease in organelle content. The nuclei were less chromatic; mitochondria in the cytoplasm were swollen.
contributing to vacuolization. Pinocytotic vesicles on periphery of the cells were clearly visible. Lipid droplets and lysosomes were seen scattered. Other interstitial cells showed different types of fibroblasts cells.

Table 2. Effect B. carterii and B. papyrifera smoke exposure on total count of seminiferous tubules, germ cells, Leydig cells and Sertoli cells in the testes of albino rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Seminiferous tubules in microscopic field (10^3)</th>
<th>Spermatogonia</th>
<th>Spermatocytes</th>
<th>Spermatids</th>
<th>Leydig cells</th>
<th>Sertoli cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Control</td>
<td>15.50 ± 0.21</td>
<td>130.70 ± 2.39</td>
<td>549.55 ± 29.34</td>
<td>986.35 ± 7.28</td>
<td>57.30 ± 0.41</td>
<td>29.63 ± 1.56</td>
</tr>
<tr>
<td>II B. papyrifera</td>
<td>21.13 ± 0.41 ***</td>
<td>90.50 ± 1.36 ***</td>
<td>427.31 ± 1.41 ***</td>
<td>672.30 ± 1.43 ***</td>
<td>21.79 ± 1.35 ***</td>
<td>20.81 ± 1.67 ***</td>
</tr>
<tr>
<td>III B. carterii</td>
<td>23.18 ± 0.40 ***</td>
<td>70.09 ± 2.36 ***</td>
<td>321.57 ± 4.21 ***</td>
<td>593.17 ± 3.3 ***</td>
<td>25.17 ± 1.77 ***</td>
<td>17.91 ± 1.39 ***</td>
</tr>
</tbody>
</table>

Table 3. The effect of the 400 and 600 mg/kg/rat citrus on Sperm parameters, TAC, SOD, MDA and testis weight of control and experimental groups in the rats.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=10)</th>
<th>Ct. 1, (400 mg/kg-per day citrus) (n=10)</th>
<th>Ct. 2 (600 mg/kg-per day citrus) (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testis (gram)</td>
<td>1.38±0.33</td>
<td>1.48±0.55</td>
<td>1.49±0.55</td>
</tr>
<tr>
<td>Sperm concentration (total count) (No of sperm/rat 10^8)</td>
<td>50.11±7.70</td>
<td>51.77±4.66</td>
<td>62.44±1.33*</td>
</tr>
<tr>
<td>Motility (%)</td>
<td>30.75±5.33</td>
<td>70±1.14*</td>
<td>82±0.33*</td>
</tr>
<tr>
<td>Viability (%)</td>
<td>69.15±4.56</td>
<td>94.80±1.66*</td>
<td>96.80±1.11*</td>
</tr>
<tr>
<td>Total Antioxidant capacity (TAC), (mmol/ml)</td>
<td>0.61±0.55</td>
<td>0.92±0.03*</td>
<td>0.90±0.11*</td>
</tr>
<tr>
<td>Malondialdehyde (MDA), (mmol/ml)</td>
<td>3.90±0.55</td>
<td>1.55±0.12*</td>
<td>0.81±0.12*</td>
</tr>
<tr>
<td>Super oxide dismutase (SOD), (u/g Hb)</td>
<td>1000±0.65</td>
<td>1500±0.55*</td>
<td>1550±0.55*</td>
</tr>
</tbody>
</table>

Discussion

In this study we evaluated the toxicity of B. papyrifera and B. carterii smoke exposure on the reproductive system in male Wistar rats. We found a significant increase in sperm anomalies with decreased sperm count, motility, sperm speed and the decreased fructose contents. The epididymis plays an important role in sperm development and sperm maturation, where it depends on the luminal environment of the epididymis; including its specific proteins. Extracts of plants like Ocimum sanctum leaves (O. ocimum) and Aegle marmelos have been reported to possess toxic effects on sperm parameters in rodent models. These findings are consistent with our study. It was suggested that these plant extracts cause androgen depletion at the target levels, particularly in the cauda epididymis thereby affecting physiological maturation of sperms. Present observations of increased abnormal sperms, reduced sperm count, motility and sperm speed with B. papyrifera and B. carterii suggested that sperm anomalies in rats might have resulted from the alteration in the epididymal milieu due to androgen deficiency and/or due to toxic effects on cellular levels. Fructose has been reported to be a source of energy for the motility of the gametes. Patel et al. demonstrated a positive correlation between seminal fructose and percentage of motile sperms. In this study abnormal sperm motility was directly correlated to decreased levels of fructose in seminal plasma and epididymal fluid. The oxidation of lipids was a crucial step in the pathogenesis of several diseases. Lipid peroxidation is a process generated naturally in small amounts in the body, mainly by the effect of several reactive oxygen species (hydroxyl radical, hydrogen peroxide, etc.) or by the action of several phagocytes.

Since lipid peroxidation is a self-propagating chain-reaction, the initial oxidation of only a few lipid molecules can lead to significant tissue damage. Despite extensive research in the field of lipid peroxidation it has not yet been precisely determined if it is the cause or an effect of several pathological conditions. Lipid peroxidation has been implicated in diseases such as atherosclerosis, IBD, ROP, BPD, asthma, Parkinson’s disease, kidney damage, preeclampsia and others. In this study, increased lipid peroxidation was correlated to damage in spermatozoa and testicular dysfunctions. Although cigarette smoke exposure to rats showed secretory dysfunction of the Leydig cells, deficiency in sperm maturation and spermatogenesis and significant reductions in epididymal sperm content, motility and infertility in vivo and in vitro, such effects have not been verified for incense smoke.
Oxidative stress (Aitken et al., 1995; Sies, 1991). Critical levels can overwhelm all antioxidants defense strategies of spermatozoa and seminal plasma causing improper balance between ROS generation and scavenging activities. Excessive ROS production that exceeds acting as free radical naturally present in the organism, they are mainly confined to cell compartments and counterbalanced by natural unpaired electron such as a superoxide ion (O₂), nitrogen oxide (NO) and hydroxyl radical (HO·). Even though Reactive oxygen species (ROS) are very reactive molecules ranked as free radicals owing to the presence of one unpaired electron such as a superoxide ion (O₂⁻), nitrogen oxide (NO) and hydroxyl radical (HO·). Even though naturally present in the organism, they are mainly confined to cell compartments and counterbalanced by natural antioxidant molecules, such as glutathione, glutathione peroxidase, superoxide dismutase, vitamin E and vitamin C, acting as free radical scavengers (Sharma et al., 1996; Miller et al., 1993). Cellular damage in the semen is the result of an improper balance between ROS generation and scavenging activities. Excessive ROS production that exceeds critical levels can overwhelm all antioxidants defense strategies of spermatozoa and seminal plasma causing oxidative stress (Aitken et al., 1995; Sies, 1991).

REFERENCES


