Effects of Dietary Phytoestrogens on Mouse Testis: Evaluation by Electron Microscopy and Caspase-3 Immunostaining

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Abstract

Objective: The purpose of this study was to evaluate the effects of dietary phytoestrogens on mouse testis using light and electron microscopy and caspase-3 immunostaining.

Materials and Methods: Eighteen male Swiss Albino mice of 3-weeks-old were separated into three groups, each including six mice, after weaning at postnatal 21st day. They were fed by three different diets; a phytoestrogen-free diet (phyto-0 group), a diet containing 500 µg/g phytoestrogen (phyto-500 group) or a diet containing 1000 µg/g phytoestrogen (phyto-1000 or phyto-rich group) for 6 weeks. After completing their sexual maturity on day 63, all were sacrificed under anesthesia. Extracted testes were prepared for investigation by light and electron microscopy and caspase-3 immunostaining was used to demonstrate the apoptosis.

Results: During the study period, all mice in three dietary groups increased their weights regularly, but there was no statistically significant difference among groups for each week. Histological examination was normal in phyto-0 diet group by light and electron microscopy, and caspase-3 immunostaining showed no increased apoptosis. The phyto-500 and phyto-1000 diet caused a series of changes in the testis including increased apoptosis in the germ cells, increased edema in interstitial area, deposition of hyaline-like substance and increased lipid deposition in Leydig cells. These changes were more obvious in phyto-1000 group. The results of immunostaining by caspase-3 showed that apoptosis significantly increased in primary spermatocytes in both phyto-500 and phyto-1000 groups when compared to phyto-0 group (p=0.000).

Discussion: Our results suggested that phytoestrogens might have detrimental effects on male reproductive function in a dose dependent manner.

Keywords: phytoestrogen, diet, mouse, testis, male reproduction

Özet

Diyette Bulunan Fitööstrojenlerin Fare Testisi Üzerindeki Etkileri: Elektron Mikroskopisi ve Kaspad-3 Immün Boyama ile Yapılan Değerlendirmesi

Amaç: Bu çalışmanın amacı, diyette bulunan fitööstrojenlerin fare testisi üzerindeki etkilerini ışık ve elektron mikroskopu ve kaspad-3 immün boyama ile araştırmaktır.

Materiale ve Metot: On sekiz Swiss Albino erkek fare 21 günlük iken süren kesilerek üç gruba ayrıldılar. Bu üç gruptaki färler fitööstrojen içermeyen (fito-0 grubu) diyet, 500 µg/g fitööstrojen içeren diyet (fito-500 grubu) ve 1000 µg/g fitööstrojen içeren diyet (fito-1000 grubu) olmak üzere 6 hafta süreyle beslendiler. Altıncı üçüncü günden sonra tüm färlerin anestezi altında iken çıkarılan testisleri, ışık ve elektron mikroskopu ile incelemek ve apoptozis göstermek için kaspad-3 immün boyama yapmak üzere hazırlarlandı.

Sonuçlar: Her üç diyet grubundaki färlerin çalışma süresince ağırlıkları düzenli olarak artış gösterdi, ancak her bir hafta için gruplar arasında istatistiksel olarak anlamlı bir fark gözlemmedi. Fito-0 grubunda ışık ve elektron mikroskopu bulguları normaldaki ve kaspad-3 ile artmış apoptozis gözelemedi. Fito-500 ve fito-1000 diyet ise testislerde germ hücrelerinde artış apop-
Introduction

Estrogen has traditionally been known as the female hormone, but this idea has been challenged in early 1990’s and an essential physiological role for estrogen in male fertility was identified (1). The demonstration that male fertility is impaired in mice lacking estrogen receptor-alpha (ER-α) along with the discovery of a second estrogen receptor-beta (ER-β), which is widely expressed in the male reproductive tract, has clearly showed the role of estrogens in male (1,2). The importance of estrogen in the adult testis was also highlighted by phenotype of aromatase knockout (ArKO) mouse, where the inhibition of estrogen biosynthesis resulted in spermatogenic abnormalities (3-5). Because the estrogen receptors are expressed in the developing reproductive tract from fetal life through adulthood and estrogen receptor-β is predominant in the seminiferous epithelium, estrogen may act directly on the seminiferous tubules to mediate spermatogenesis (5).

On the other hand, exposure to estrogens in the environment may have detrimental effect on male reproductive development and health. The administration of estrogens and xeno-estrogens during fetal, neonatal or adult period has been reported to be associated with a series of male reproductive disturbances, such as cryptorchidism, epididymal defects, impaired sperm production and maturation and an increased incidence of testicular cancer (1,6). Therefore, compounds that are potentially able to disrupt this hormonal homeostasis are of increasing concern (5,7-8).

Phytoestrogens are naturally occurring non-steroidal plant chemicals that can act like the female hormone estrogen. Over 300 plants and plant products including soy-containing foods may have some potential adverse effects. They can cause impaired reproductive function in some animal species (12-14). However, in the absence of endogenous phytoestrogens, they can act as partial estrogen agonists (5,15). Robertson et al. (5) showed that soy consumption clearly had a beneficial agonistic effect on the testis of ArKO mice, which lacks endogenous aromatase products like estradiol and estrone, particularly in terms of the maintenance of testis weight, germ cell development and seminiferous tubule epithelial and luminal volume. Therefore, it is mandatory to speculate that phytoestrogens may have direct effects on male reproductive function. All these facts together clearly warrants to make a research into the effects of dietary phytoestrogens on the male reproductive system. The purpose of this study was to evaluate the effects of dietary phytoestrogens on mouse testis. Testicular histology was evaluated by light and electron microscopy, and caspase-3 immunostaining was used to demonstrate the apoptosis.

Materials and Methods

This study was carried out at Gazi University Department of Histology and Embryology, after having permission from local ethics committee. According to the study design, the diets used in this study were obtained from Research Diet, Inc (New Brunswick, NJ, 08901, USA). The following three diets including various concentrations of phytoestrogens were prepared for the three study groups: 1) Phyto-free diet (phyto-0 diet), was a casein based AIN-76A diet containing no added genistein or daidzein, 2) Phyto-500 diet included 350 µg/g genistein and 150 µg/g daidzein. The composition of this diet is somewhat higher than Purina 5001, which is a standard rodent diet in USA. To prepare this diet 0.35 g genistein and 0.15 g daidzein were added per kg of AIN-76 diet 3) Phyto-rich diet (phyto-1000 diet), included 700 µg/g genistein and 300 µg/g daidzein. For this diet, 0.7 g of genistein and 0.3 g daidzein, were added per kg of AIN-76A. So, in our study a phytoestrogen-free diet, a diet similar to normal rodent diet and a phytoestrogen-rich diet –which differed from each other by phytoestrogen concentrations– were used. Table 1 shows the dietary concentrations and the calculated metabolizable energy values for each diet group.

In this experiment, eighteen 3-weeks-old male Swiss Albino mice were used. Mice were obtained from Laboratories of Hifzusshha Institute of Ankara after weaning at postnatal 21st day. These 18 mice were separated into three groups, each
including six mice. All mice in three groups were weighed and feeding was started by three distinct diets. The first group (phyto-free group) was fed by a casein based AIN-76A diet. Group 2 and group 3 were fed by phyto-500 and phyto-1000 diets, respectively. All mice were housed in polycarbonate cages (6 mice/cage) and were maintained on daily light/dark cycle of 12:12 h, with ambient temperature and humidity set at 21°C (22±2°C) and 50% (40-70%), respectively, in the Animal Laboratory of Ankara University Medical School. All mice were allowed unrestricted access to food and water. Cages were cleaned every other day and animals were weighed every week during study period.

When they completed their sexual maturity on day 63, all mice were weighed and sacrificed under intraperitoneal 0.1 mg/g Ketamine HCL (Ketamidor, Richter Pharma, Austria) and Xylazine HCL 0.004 mg/g (Rompun, Bayer, Turkey) anesthesia. After a total perfusion and fixation process, both testes were dissected out, weighed and labeled on small bottles including 2.5% phosphate buffered gluteraldehyde. Tissue preparation for light and electron microscopy and immunohistological studies were carried out at Gazi University, Department of Histology and Embryology. Testicular tissue samples were cut into small pieces. They were fixed in 2.5% phosphate buffered gluteraldehyde for 2 hours and postfixed in 1% osmium tetroxide, dehydrated in serial alcohol, and then embedded in araldite. The semi thin sections were stained with toluidine blue and examined with a photomicroscope (BH2 Olympus). After the selection of appropriate specimens, thin sections were obtained and stained with uranyl acetate and lead citrate. They were examined with an electron microscope (Carl Zeiss EM 900). The number of apoptotic cells was evaluated using caspase-3 immunostaining. Germ cells were classified into four major groups including spermatagonia, spermatocytes, round spermatids and elongated spermatids.

The differences between the mean weights of organs and mice and the number of apoptotic cells by caspase-3 immunostaining. Germ cells were classified into four major groups including spermatagonia, spermatocytes, round spermatids and elongated spermatids.

Table 1. Dietary concentrations and calculated metabolizable energy values in each study group

<table>
<thead>
<tr>
<th>Product #</th>
<th>Group 1 phyto-free diet D10001</th>
<th>Group 2 phyto-500 diet D03140101</th>
<th>Group 3 phyto-1000 diet D03140102</th>
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<td></td>
<td>gm% kcal%</td>
<td>gm% kcal%</td>
<td>gm% kcal%</td>
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<td>20.3 20.8</td>
<td>20.3 20.8</td>
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<tr>
<td>Carbohydrate</td>
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<td>66.0 67.7</td>
<td>65.9 67.7</td>
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<tr>
<td>Fat</td>
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<td>5.0 11.5</td>
<td>5.0 11.5</td>
</tr>
<tr>
<td>Total</td>
<td>100.0 100.0</td>
<td>100.0 100.0</td>
<td>100.0 100.0</td>
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<tr>
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<tr>
<td>Ingredient</td>
<td>gm kcal</td>
<td>gm kcal</td>
<td>gm kcal</td>
</tr>
<tr>
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<td>200 800</td>
<td>200 800</td>
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<td>3 12</td>
<td>3 12</td>
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<td>150 600</td>
<td>150 600</td>
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<tr>
<td>Sucrose</td>
<td>500 2000</td>
<td>500 2000</td>
<td>500 2000</td>
</tr>
<tr>
<td>Cellulose, BW</td>
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<td>0 50</td>
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<td>35 0</td>
<td>35 0</td>
</tr>
<tr>
<td>Vitamin mix V10001</td>
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<td>10 40</td>
<td>10 40</td>
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<tr>
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<td>2 0</td>
<td>2 0</td>
</tr>
<tr>
<td>Genistein</td>
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<td>0.7 0</td>
</tr>
<tr>
<td>Daidzein</td>
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<td>0.3 0</td>
</tr>
<tr>
<td>Total</td>
<td>1000 3902</td>
<td>1000.5 3902</td>
<td>1001 3902</td>
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</table>

Formulated by Research Diets Inc., 1/14/03.

Figure 1. Mean weights of mice in each group according to the weeks. There was no statistically significant difference among groups (One-way ANOVA).
staining in three groups were compared by one-way ANOVA, with statistical significance assigned at \( p < 0.05 \). When a significant \( p \) value was obtained, Scheffe’s test was used in the post hoc analysis. SPSS 11.0 was used for analysis.

**Results**

At the beginning of the study at postnatal day 21, the mean weight of mice was not different among three groups. During the 6 weeks of study, all mice in three diet groups increased their weights regularly, but there was no statistically significant difference among groups for each week (Figure 1). Although it was not significant, feeding by phytoestrogen-rich diet caused a decrease in body weight. Mean body weight of mice was higher in the phyto-0 group than in the phyto-500 and phyto-1000 groups and it was lowest in the phyto-1000 group.

No gross lesions were observed at necroscopy in both genital organs like penis, testis, epididymis, prostate and also other abdominal organs in three different diet groups. Testis weights were similar among the groups. Histological examination showed normal testicular morphology by light and electron microscopy in the phyto-0 dietary group. The seminiferous tubules, spermatogenetic cells, Sertoli and Leydig cells and other interstitial structures were normal (Figure 2). The increased amount of phytoestrogens in the diet caused a series of changes in seminiferous tubules, spermatogenetic cells, Sertoli and Leydig cells along with the other interstitial structures. Increased apoptosis in the cells nearby the seminiferous tubule lumen was detected in the phyto-500 diet group by light and electron microscopy (Figure 3). These changes were even more obvious in the phyto-1000 group and included increased apoptosis in the germ cells of seminiferous tubules, acceleration of germ cell maturation, increased edema in interstitial area, deposition of hyaline-like substance and increased lipid deposition in Leydig cells (Figure 4).

Immunostaining by caspase-3 was carried out to demonstrate the apoptosis. Mean number of apoptotic cells in the phyto-free, phyto-500 and phyto-1000 groups were 7.87±3.8, 18.6±4.8 and 19.5±4.3, respectively. The results showed that there was no increase in apoptosis in germ cells in the phyto-free group compared to other two groups, where as it was significantly increased in primary spermatocytes in both the phyto-500 group and the phyto-1000 group (\( p=0.000 \)) (Figure 5). But, although the mean number of apoptotic cells was higher in the phyto-1000 group compared to the phyto-500 group, this was not statistically significant (\( p=0.86 \)).
Phytoestrogens are naturally occurring non-steroidal plant chemicals that are found in both animal and human diet. Commonly used rodent diets differ significantly in estrogenic activity and many institutions use a chow-based diet such as Purina 5001 to maintain and breed their mice. Purina 5001, a standard rodent diet used particularly in the USA, contains a high level of phytoestrogens, approximately 214 µg/g genistein and 277 µg/g daidzein, totally about 500 µg/g (15-18). Weber et al. (19) used a phytoestrogen-rich diet in their study and described it containing 600 µg/g isoflavones. But, quantities of phytoestrogens in these diets are not constant from batch to batch and variation in the reported levels may result from differences in the analytic methods. Therefore, in order to establish diets that only differed in the phytoestrogen content, we preferred to use a purified diet rather than a chow-based diet.

Phytoestrogens in the animal and human diets cause increases in plasma isoflavonoid concentrations. The circulating plasma phytoestrogen concentration of animals fed by a phytoestrogen rich diet including 600 µg/g, was approximately 35 times higher when compared to animals fed by phytoestrogen free diet (19). The plasma concentration of genistein in rats fed by a diet including 750 µg/g genistein was 2.2 micromole/L (conjugated plus free) and this concentration was sufficient to elicit estrogenic effects in ovariectomized rodents (20). ArKO animals raised on a soy positive diet showed normal testicular morphology with a normal spermatocyte, round and elongated spermatid number whereas ArKO mice fed by soy negative diet showed evidence of spermatogenetic disruption. The most likely explanation for marked improvement of germ cell development and testicular morphology in the soy positive ArKO mice was that dietary soy could have direct effects on receptors within the male reproductive tract because LH and FSH levels were not different between soy negative and soy positive ArKO mice. Robertson et al. (5) also demonstrated that dietary soy was able to produce changes in testicular histology in normal mice highlighting the fact that soy found in commercial rodent chow has an action on the testis of normal healthy mice. Their explanation for this situation was that dietary phytoestrogens might antagonize the action of endogenous estrogens (24,25) or prevent estrogen biosynthesis through the inhibition of enzymes such as aromatase (26,27).

Previous studies have also suggested that dietary soy can effect male reproductive system and these effects of phytoestrogens may be concentration-dependent. Odum et al. (17) evaluated the five rodent diets including different amount of phytoestrogens and they found that phytoestrogen content in diet could affect the timing of both male and female sexual development in rats. Delclos et al. (28) investigated the effect of dietary phytoestrogens in different concentrations. Body weight and feed consumption of the treated dams prior to parturition showed a decreasing trend with a significant reduction at highest dose. No gross abnormality including retained or small testes, retention of Müllerian duct remnants and hypospadias were detected in study groups. But there was evidence of treatment-related effects on testes and epididymides. Semineferous tubules showed retention of elon-
gated spermatids in Stages X-XII and depletion of spermatids and degeneration of spermatocytes at earlier stages in the highest (1250 ppm) dose group. Fritz et al. (29) investigated the effect of dietary genistein on sex steroid receptor expression including androgen receptor (AR) and estrogen receptor-alpha and beta in the dorso-lateral prostate, and circulating androgens including testosterone and DHT and the potential for toxicity in the male rat reproductive tract. There were no significant differences in the body or reproductive tract weights and male reproductive tract histomorphology in animals. They found that life time and short-term exposure to dietary genistein reduced sex steroid expression in the dorso-lateral prostate, and increased circulating testosterone levels in a dose dependent manner without evidence of toxicity to male reproductive tract. They suggested that down-regulated sex steroid receptor expression might be responsible for the lower incidence of prostate cancer in populations on a diet containing high levels of phytoestrogens. Weber et al. (19) compared the effect of phytoestrogen-rich diet containing 600 µg/g isoflavones to phytoestrogen-free diet in adult male Sprague-Dawley (70-day-old) rats. After 12 and 29 days at diets, the phyto-600 group displayed higher locomotor levels suggesting the potential influence of dietary phytoestrogens on locomotor activity. When testicular characteristics were examined, there were no significant differences in testes weight, Sertoli or Leydig cell number or morphology between these groups. For animals fed the phyto-600 diet, the body and ventral prostate weight was significantly lower compared with the phyto-free group. They concluded that consumption of dietary phytoestrogens over a relatively short period, results in very high plasma isoflavone level and can significantly alter body and prostate weight and plasma androgen levels without affecting gonadotropin levels.

Although it was not statistically significant, feeding by phytoestrogen-rich diet caused a decrease in body weight in our study. These results were in agreement with the studies reported by others (5,19,28). Estrogens are known to alter feeding behavior, locomotor activity and body weight composition in rats (30). This was consistent with the estrogenic hormonal action of these molecules (31) and may be related with alterations in leptin and adipose tissue deposition. No gross lesions were observed at necropsy in neither genital organs nor other abdominal organs in three different dietary groups in our study. Histological examination showed no difference in testicular morphology in phyto-free dietary group, whereas the increased amount of phytoestrogens in the diet caused a series of changes in seminiferous tubules, spermatogenetic cells, Sertoli and Leydig cells along with the other interstitial structures. The critical role of apoptosis in normal sperm production is a well known event and the removal of androgens and gonadotropins or estrogen administration induces germ cell apoptosis (32). Assinder et al. (32) investigated the effects of high dietary phytoestrogens on adult male rats and TUNEL analysis demonstrated an increased apoptosis in spermatocytes and round spermatids. They concluded that exposure of the adult male rats to high phytoestrogen diet disrupts spermatogenesis and increases germ cell apoptosis. This effect is independent of the hypothalamo-pituitary-testicular axis and is likely to be due to disruption of estrogen’s actions in the testis.

In conclusion, the data presented in the literature suggest that estrogen production is important for the maintenance of germ cell development and Sertoli cell function and shows that dietary phytoestrogens can mimic the action of endogenous estrogen within the seminiferous tubules via estrogen receptors, particularly estrogen receptor-β. As demonstrated by ArKO mouse, dietary phytoestrogens in combination with endogenous estrogens may have antagonistic effects, but agonistic effects in the absence of endogenous estrogen. These antagonistic effects of dietary phytoestrogens in the presence of endogenous estrogen may cause fertility impairment and may be concentration dependent. Our study results together with others have suggested this idea, but the most important limitation of this study was the small sample size. It should also be kept in mind that different concentrations of phytoestrogens in rodent diets can alter the results of studies that investigate the estrogenic and reproductive activity of different compounds.

References

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