CLINICAL STUDY

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 Fitoöstrojenlerin Fare Testisi Üzerindeki Etkileri: Elektron Mikroskopisi ve Kaspaz-3 İmmün Boyama ile Yapılan Değerlendirme

Amaç: Bu çalışmanın amacı, diyette bulunan fitoöstrojenlerin fare testisi üzerindeki etkilerini ışık ve elektron mikroskobu ve kaspaz-3 immün boyama ile araştırmaktır.
Materyal ve Metot: On sekiz Swiss Albino erkek fare 21 günlük iken sütten kesilerek üç gruba ayrıldılar. Bu üç gruptaki fareler fitoöstrojen içermeyen (fito-0 grubu) diyet, 500 µg/g fitoöstrojen içeren diyet (fito-500 grubu) ve 1000 µg/g fitoöstrojen içeren diyet (fito-1000 grubu) olmak üzere 3 farklı diyet ile 6 hafta süreyle beslendiler. Altmış üçüncü günde cinsel olgunluklarını tamamladıktan sonra tüm farelerin anestezi altında iken çıkarılan testisleri, ışık ve elektron mikroskobu ile incelenmek ve apoptozisi göstermek için kaspaz-3 immün boyama yapmak üzere hazırlandılar.
Sonuçlar: Her üç diyet grubundaki farelerin çalışma süresince ağırlıkları düzenli olarak artış gösterdi, ancak her bir hafta için gruplar arasında istatistiksel olarak anlamlı bir fark gözlenmedi. Fito-0 grubunda ışık ve elektron mikroskobu bulguları normaldi ve kaspaz-3 ile artmış apoptozis gözlenmedi. Fito-500 ve fito-1000 diyet ise testislerde germ hücrelerinde artmış apop-

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tozis, interstisiyel alanda artmış ödem, hiyalin benzeri madde birikimi ve Leydig hücrelerinde artmış lipid birikimi olmak üzere bir seri değişikliğe neden oldu. Bu değişiklikler, fito-1000 grubunda daha belirgindi. Kaspaz-3 ile yapılan immün boyama, primer spermatositlerde hem fito-500 hem de fito-1000 grubunda fito-0 grubuna oranla istatistiksel olarak anlamlı düzeyde artmış apoptozis gösterdi (p=0.000).

Tartışma: Bulgularımız, fitoöstrojenlerin doza bağlı olacak şekilde erkek üreme sistemi fonksiyonları üzerinde olumsuz etkilerinin olabileceğini düşündürmektedir.

Anahtar sözcükler: fitoöstrojenler, diyet, fare, testis, erkek üremesi

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 spermatogenetic 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 xenoestrogens 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 contains phytoestrogens. These compounds are able to bind to both estrogen receptors, particularly the estrogen receptor-β isoform, in agonistic fashion with high affinity and are thought to exert their estrogenic effects through mechanism similar to estradiol (9,10). Because the relative potency of phytoestrogens is significantly lower than that of steroidal estrogens, exposure to them has been regarded as no harmful, even beneficial. The consumption of diets with high levels of soy has been related to multiple beneficial effects including chemopreventive activities against various hormone-dependent cancers including breast and prostate and alleviation of some of the adverse consequences of menopause (11). They are also known to be protective in the prevention of cardiovascular disease and osteoporosis. However, the consumption of certain 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 phtoestrogen 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 Hıfzısıhha Institute of Ankara after weaning at postnatal 21st day. These 18 mice were separated into three groups, each

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

Product #	Group 1 phyto-free diet D10001		Group 2 phyto-500 diet D03140101		Group 3 phyto-1000 diet D03140102	
	gm%	**kcal%**	**gm%**	**kcal%**	**gm%**	**kcal%**
Protein	20.3	20.8	20.3	20.8	20.3	20.8
Carbonhydrate	66.0	67.7	66.0	67.7	65.9	67.7
Fat	5.0	11.5	5.0	11.5	5.0	11.5
Total		100.0		100.0		100.0
kcal/gm		3.90		3.90		3.90
Ingredient	**gm**	**kcal**	**gm**	**kcal**	**gm**	**kcal**
Casein	200	800	200	800	200	800
DL-Methionine	3	12	3	12	3	12
Corn starch	150	600	150	600	150	600
Sucrose	500	2000	500	2000	500	2000
Cellulose, BW	200	50	0	50	0	500
Corn oil	50	450	50	450	50	450
Mineral mix S10001	35	0	35	0	35	0
Vitamin mix V10001	10	40	10	40	10	40
Choline bitartrate	2	0	2	0	2	0
Genistein	0	0	0.35	0	0.7 0	0
Daidzein	0	0	0.15	0	0.3 0	0
Total	1000	3902	1000.5	3902	1001	3902

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

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 Xylazin 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 spermatogonia, 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 immuno-

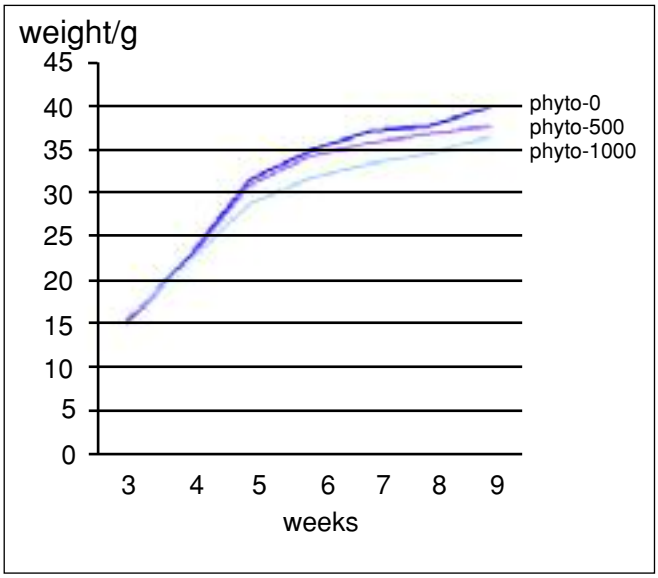

Figure 1. Mean weights of mice in each group according to the weeks. There was no statistically significant difference among groups (One-way ANOVA).

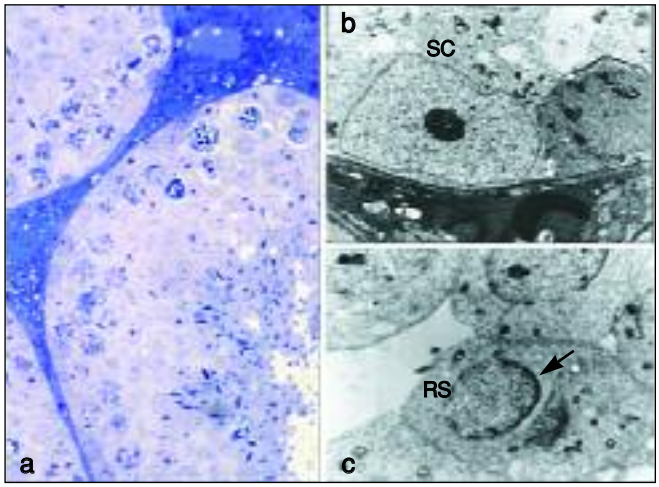

Figure 2. Findings in phyto-free diet group; (a) seminiferous tubules and interstitium in normal appearance by light microscopy (Toluidin blue x400). (b-c) normal Sertoli cell (SC) and round spermatid (RS) with acrosomal cap (arrow) by electron microscopy (Lead citrate, uranyl acetate x3000).

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.

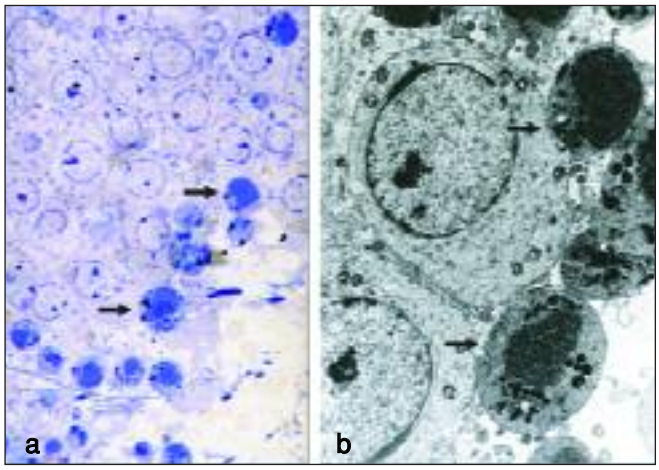

Figure 3. Findings in phyto-500 diet group; (a) arrows show apoptotic germ cells in seminiferous tubules by light microscopy (Toluidin blue, x400). (b-c) arrows shows apoptotic germ cells by electron microscopy (Lead citrate, uranyl acetate, x3000).

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).

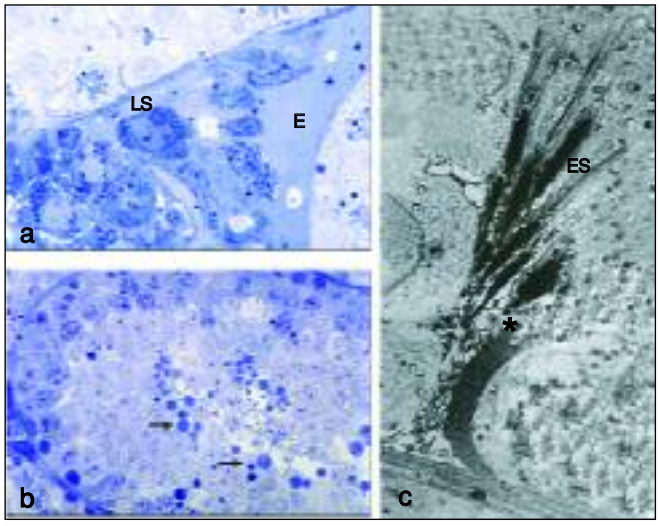

Figure 4. Findings in phyto-1000 diet group; (a-b) apoptotic germ cells in seminiferous tubules (arrows), increased lipid granules in Leydig cells (LS) and edema (E) in interstitial area by light microscopy (Toluidin blue x400, x1000). (c) elongated spermatids (ES) and apoptotic Sertoli (*) cells by electron microscopy (Lead citrate, uranyl acetate, x4400).

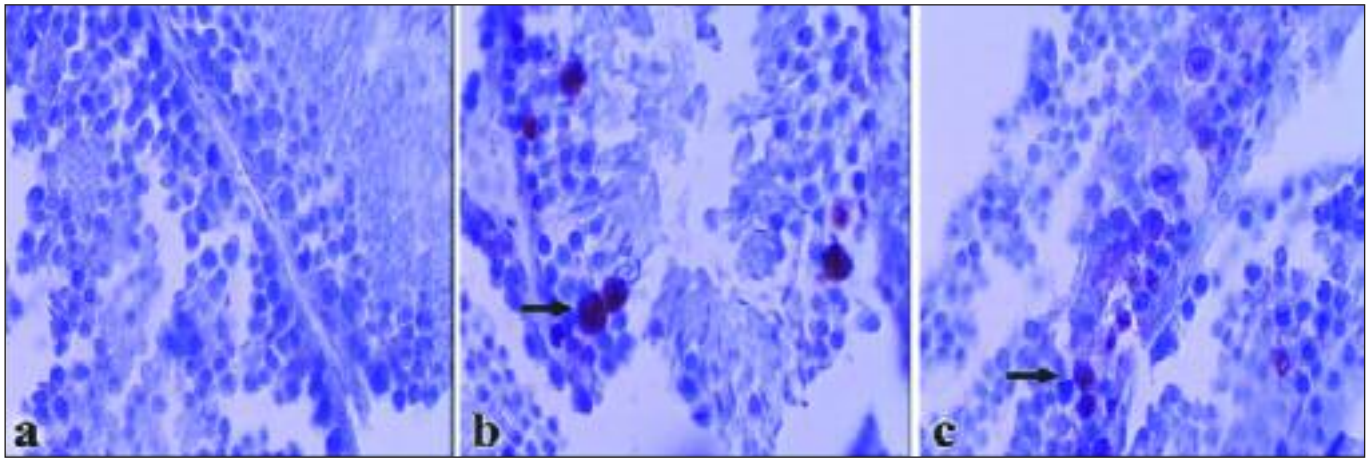

Figure 5. Findings by caspase-3 imunostaning. (a) There is no immunoreactivity in phyto-free diet group. (b-c) arrows show significantly increased apoptotic germ cells, particularly in spermatocytes in phyto-500 and phyto-1000 diet groups, respectively.

Discussion

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). Consumption of a standard phytoestrogen-containing diet (210 µg/g) also elicited a high estrogenic response in the uterus of immature rats (21). Therefore, it is reasonable to speculate that these ligands may have direct effect on male reproductive function and these effects can be changed by pyhtoestrogen concentration in the diet (22,23).

The ArKO mouse, which lacks aromatase products, is an ideal model to test whether the phytoestrogens in the diet have an agonistic estrogenic effect on the male reproductive system. Robertson et al. (5) used the ArKO mouse and normal mice to test the hypothesis that estrogenic substances in dietary soy meal have a biological effect on the testis. They showed that soy consumption including approximately 146 mg/g isoflavones clearly had a beneficial agonistic effect on the testis in the absence of endogenous estrogens, particularly in terms of the maintenance of testis weight, germ cell development and seminiferous tubule epithelial and luminal volume. 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 spermotocytes 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, on 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 dorsolateral 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 necroscopy 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.

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