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INVESTIGATION OF THE ULTRASTRUCTURE OF THE EPIDIDYMIS AFTER APPLICATION OF OESTROGEN-FREE AND OESTROGEN-RICH DIETS

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ABSTRACT

Purpose: Phytoestrogens are oestrogens that are non-steroidal and are naturally found in plants. This study examined the effects of a phytoestrogen-containing diet on the thin layer of epididymis at electron microscopic level.

Materials and Methods: This study was carried out to investigate the effects of a non-phytoestrogen diet (Phyto-0 group), a standard diet containing the same amount of phytoestrogen (Phyto-500 or control group), and a diet with a high dose of phytoestrogen (Phyto-1000 or phyto-rich group). Three different diets were prepared. Eighteen Swiss Albino type male mice 21 days old were obtained soon after weaning before they received any supplementary food and they were fed until they were 63 days old (9 weeks). For electron microscopy, tissue sections were processed and stained using the usual methods.

Results: Basal cells and chief cells built-in in the basal epithelium of the epididymis were examined and found to have their normal structure in the Phyto-0 group. It was interesting that chief cells showed two different structural characteristics and basal cells preserved their general structure in the Phyto-500 group, while light and dark coloured chief cells and basal cells were examined and found to have diffuse degeneration in the Phyto-1000 group.

Conclusion: Histological changes seen in the epididymis due to a high dose of phytoestrogen in the diet may cause infertility in male mice.

Key Words: Phytoestrogens, Epididymis, Electron Microscopy.

ÖSTROJENDEN YOKSUN VE ZENGIN DIYET UYGULAMASINDA EPIDIDIMIS'IN INCE YAPISININ INCELENMESI

ÖZ

Amaç: Fitoöstrojenler, bitkilerde doğal olarak bulunan steroid yapıda olmayan östrojenlerdir. Bizim bu çalısmamızdaki amacımız, değisik yoğunluklarda fitoöstrojen içeren diyetin epididimis ince yapısı üzerindeki etkilerini elektron mikroskop düzeyinde arastırmaktır.

Gereç ve Yöntem: Bu çalısmada fitoöstrojen içermeyen diyet (Fito-0 grubu), standart diyet ile aynı miktarda fitoöstrojen içeren (Fito-500 yada kontrol grubu) ve yüksek dozda fitoöstrojen içeren (Fito-1000 yada fito zengin grubu) diyetin etkilerinin arastırılması planlandığı için üç ayrı diyet hazırlandı. 18 adet 21 günlük Swiss Albino cinsi erkek fare sütten kesilir kesilmez hiçbir ek gıda almadan temin edildi ve rastgele her bir grup 6 fare içerecek sekilde 3 gruba ayrıldılar. Fareler 63 günlük (9 hafta) oluncaya değin bu ortamda üç ayrı diyetle beslenecek sekilde bakıldılar. Carl Zeiss EM 900 elektron mikroskobunda epididimisin ince yapısı incelendi.

Bulgular: Fito-0 grubunda; epididimise ait bazal hücreler (B) ve esas hücreler (E) normal yapılarıyla izlendiler. Fito-500 grubunda; bazal hücreler genel yapılarını korurken , esas hücrelerin iki farklı tipte yapısal özellik gösterdiği ilgiyi çekti. Hücreler açık (A) ve koyu (K) renk sitoplazmalıydı. Fito-1000 grubunda; koyu ve açık renk esas hücreler ve bazal hücreler yaygın dejenerasyon ile izleniyordu.

Sonuç: Sonuç olarak, diyetle yüksek miktarda fitoöstrojen alınması epididimis hücrelerinde dejeneratif değisikliklere yol açmıstır ve bu erkek farelerde infertiliteye neden olabilir.

Anahtar Kelimeler: Fitoöstrojen, Epididimis, Elektron Mikroskop.

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INTRODUCTION

Phytoestrogens are oestrogens that are non-steroidal and are naturally found in plants. They are activated in a similar way to oestrogens and imitate the effects of oestrogen. At least about 20 phytoestrogen components are detected in more than 300 plants of 16 different families.[1] Basic phytoestrogens are isoflavones (genistein, daidzein, biochanin A, formononetin), lignans (matairesinol, secoisolaricisinol, enterolactone, enterodiol), coumestans, and equol.[2] The most significant characteristics that distinguish phytoestrogens from synthetic oestrogens are that they can easily undergo fission, can be stored in the tissue, and spend a short time in the body. Phytoestrogens attach to α and β receptors in the body and display their effects through these receptors, similar to estradiol.[3, 4] The efficiency of phytoestrogens is significantly lower than that of steroid-derived oestrogens. They have both oestrogen and antioestrogen efficiency in terms of target tissue. This difference is due to their greater attachment to β oestrogen receptors than to α receptors. It is also known that these receptors exist in different amounts in different tissues. The situation explains the desirable and undesirable effects of phytoestrogens on the body. α and β receptors attach to estradiol with similar efficiency but display selectivity for different phytoestrogen.[5] In two different studies, it was observed that some phytoestrogens such as genistein and coumestrol show 20 times more affinity for β receptors than for α receptors, and phytoestrogens are basically efficient with β receptors.[3, 5, 6] After the studies performed in the 1970s, the roles of oestrogens in the male reproductive system have begun to be understood. It was observed in many studies that oestrogen receptors (α and β) and aromatase enzyme were highly abundant in the male reproductive system.[6-14] Nutrition with a diet rich in phytoestrogens has various useful effects. These effects are connected with the anti-oestrogenic and oestrogen-resembling effects of phytoestrogens.[3] A diet rich in phytoestrogens also has negative (harmful) effects. Its negative effect on the reproductive systems of some animals has long been evident.[15] Various studies in subsequent years have shown that the positive and negative effects of phytoestrogens depend on many factors such as dose, target body, and animal species. Our goal in this study was to examine the effects of a phytoestrogen-containing diet on the thin layer of epididymis at electron microscopic level.

MATERIALS AND METHODS

2.1 Diet: The oestrogen content and the oestrogenic activity of the diet used in mice and rats differ. Many animal laboratories use high phytoestrogen diets (500 μg in each gram of the diet) such as Purina 5001, which is a standard diet for nutrition of mice.[16, 17] Since the phytoestrogen amount in the diet is not standard, in studies like ours, which only investigated the effects caused by differences

in phytoestrogen levels, it is essential to know the exact amount of phytoestrogen in the diet. That is why we used a purified diet. This study was carried out to investigate the effects of a non-phytoestrogen diet (Phyto-0 group), a standard diet containing the same amount of phytoestrogen (Phyto-500 or control group), and a diet with a high dose of phytoestrogen (Phyto-1000 or phyto-rich group). Three different diets were prepared (Table 1).

Table 1: Phytoestrogens and their amounts in the diet groups.

Group	Diet	Phytoestrogen content
1. Group	AIN-76A	(-)
2. Group	AIN-76A+350 µg/g genistein+150 µg/g daidzein	500 µg/g
3. Group	AIN-76A+700 µg/g genistein+300 µg/g daidzein	1000 µg/g

The 3 different diets and their phytoestrogen contents used in the study were determined as follows:

1. Diet (Phyto-0 group): Non- phytoestrogen, kazein radical AIN-76A diet.

2. Diet (Phyto-500 group): Similar to the 214 µg/g genistein and 277 µg/g daidzein content in the standard mouse diet Purina 5001, a diet of a total of 500µg/g of phytoestrogen, which contained 350 µg/g genistein and 150 µg/g daidzein. This diet was prepared by adding 0.35 gr genistein and 0.15 gr daidzein to 1 kg of AIN-76A.

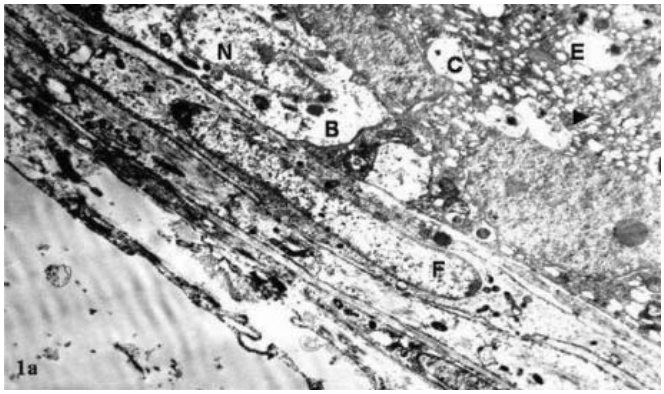
3. Diet (Phyto-1000 group): A diet of a total of 1000µg/g phytoestrogen which contained 700 µg/g genistein and 300 µg/g daidzein. This diet was prepared by adding 0.7 gr genistein and 0.3 gr daidzein to1 kg of AIN-76A

2.2 Test Animals: Mice of 3 weeks of age were selected for our study. They were weaned in the third week and put on solid food, and this provided their distribution to three separate diet groups and thus the precise determination of the diets' efficiency. Eighteen Swiss Albino male mice of 21 days of age were obtained soon after they were weaned before they received any supplementary food. They were separated into three groups, each containing at random 6 mice, and, after weighing, they were placed into polycarbonate cages. The laboratory conditions were designed for a cycle of 12 hours light and

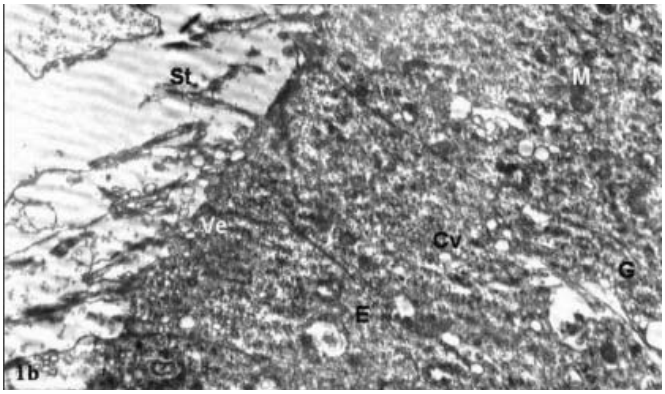
12 hours dark, 21 °C (22 ± 2 °C) temperature, and 50% humidity. Three different diets were placed into each cage and unlimited water and feed were provided for the mice. The mice were kept in these conditions, fed three different diets, until they were 63 days (9 weeks) of age. At the end of 63 days, mice that had completed their sexual adolescence were weighed and anaesthetized by intramuscular Ketalar and Rompun (0.2 ml). Then the fixing solution, 2.5% of phosphate buffered glutaraldehyde, was carefully injected into the left ventricle. The fixation procedure continued until the end of contractions. After the completion of fixation, epididymis tissues were excised and placed separately in small bottles containing 2.5% of phosphate-buffered glutaraldehyde solution. The tissues were washed in the phosphate buffer 2-3 times and fixed again in 1% of osmium tetroxide (OsO4) with 1/15 M of phosphate buffer at +4 °C. After fixing, tissue segments were rewashed with the phosphate buffer, passed through ethyl alcohol series, and removed from the water. The tissue segments removed from the water were then embedded in Araldite. The polymerization procedure was achieved by leaving them in the sterilizer for 24 hours at 40 °C and 48 hours in the sterilizer at 60 °C. Half-thin slices taken from the blocks were dyed in toluidine blue and the thin slices obtained from the marked zone were dyed in lead citrate and uranyl acetate. The thin structure of the epididymis was then examined under a Carl Zeiss EM 900 electron microscope.

RESULTS

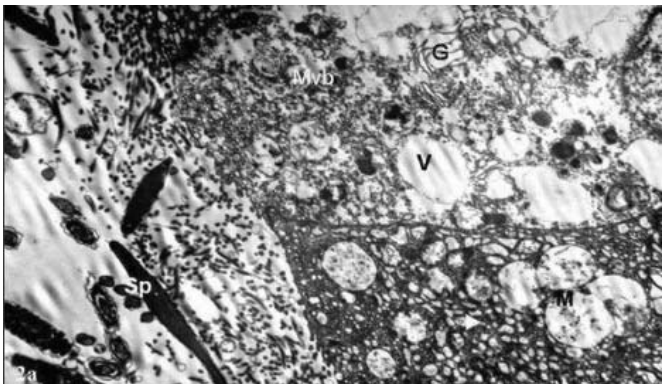
This study began with 6 mice in each diet group; however, following the death of one of the mice in the Phyto-0 group a day after the diet application, the study was completed with 5 mice in this group. On day 21 of the study, there was no dif-



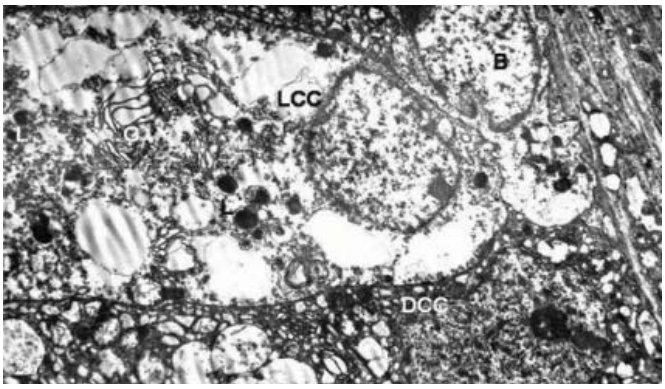
Resim 1a: Electron microscopic photo of Phyto-0 group. Basal cells (B), nuclei (N) of the basal cell and chief cell(C) are seen. Diffuse distribution of non-granulated ER (►)was interesting. Fibroblasts (F) are visible in sub-epithelial connective tissue. Endosomes (E) were distinguished in the lumen-facing parts of chief cells (Uranyl acetate, Lead citrate X3000).



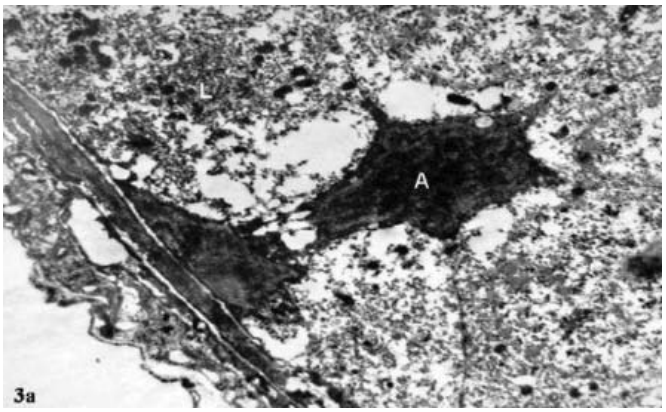
Resim 1b: Electron microscopic photo of Phyto-0 group. Density of mitochondrial matrices (M) in the cells was remarkable. Golgi complex (G), vesicles (Ve) and coated vesicles (Cv) were distinguished. Stereocilia (St) were fairly developed (Uranyl acetate, Lead citrate X3000).



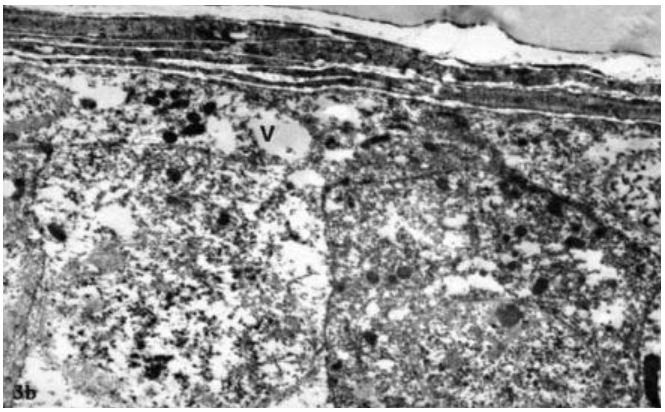
Resim 2a: Electron microscopic photo of Phyto-500 group. Lipid-like vacuolization (V),developed Golgi complex (G), enlarged non-granulated ER tubules (►), multivesicular bodies (mvb) and mitochondria (M), degenerated in patches are visible. Spermium (Sp) slices were seen in the lumen (Uranyl acetate, Lead citrate X3000).



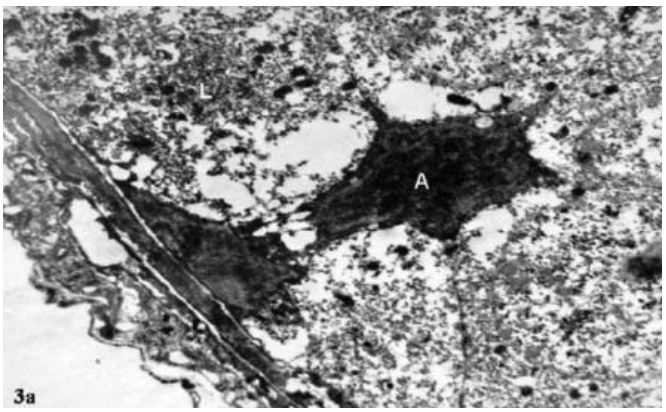
Resim 2b: Electron microscopic photo of Phyto-500 group. Basal cell (B), light-coloured cytoplasmic chief cell (LCC) and dark-coloured cytoplasmic chief cell (DCC) are visible. Lysosomes (L) besides Golgi complex (G) was remarkable (Uranyl acetate, Lead citrate X3000).



Resim 3a: Electron microscobic photo of Phyto-1000 group. Primary lisosomes (L) are diffuse. Apoptosis in basal and chief cells (A) were remarkable (Uranyl acetate, Lead citrate X3000).



Resim 3b: Electron microscopic photo of Phyto-1000 group. Fairly large vacuolar zones (V) and less homogenous zones in patches in the cytoplasm were interesting (Uranyl acetate, Lead citrate X3000).



Resim 3c: Electron microscopic photo of Phyto-1000 group. Shortage of stereocilia (St) and mitochondria (M) in normal structure were visible (Uranyl acetate, Lead citrate X3000).

ference in the average weights of mice in the three diet groups. During the 6-week period, average weights increased regularly in all groups. Although no significant statistical difference was observed between the groups each week, the average weight increase in the Phyto-0 group was higher than that in the Phyto-500 group, and the increase in the Phyto-500 group was higher than that in the Phyto-1000 group. In the Phyto-0 group, basal cells (B) and chief cells (C) built-in in the basal epithelium of the epididymis were observed to maintain their normal structure. The deficiency of organelle distribution in the basal cells was remarkable. It was observed in the chief cells that the nucleus was built into the basal cytoplasm and it was oval. The distribution of agranular ER tubules (►) diffuse in the subnucleus and upper nucleus was interesting. Fibroblasts (F) and connective tissue fibers were seen in the sub-epithelial connective tissue. Horizontal slices of non-granule ER tubules, endosome (E), Golgi complex (G), and Golgi complex-related vacuoles and vesicles (Ve) were distinguished in the lumen-facing parts of basal cells. Coated vesicles were seen as well. Pinocytotic vesicles were diffuse under the apical plasmalemma. Dense mitochondrial matrices (M) in the cells were remarkable. Stereocilia (St) were fairly well developed (Figure 1a-1b).

In the Phyto-500 group, it was interesting that chief cells showed two different structural characteristics, while the basal cells preserved their general structure. The cells had light-coloured cytoplasm (LCC) and dark-coloured cytoplasm (DCC). In the light-coloured cytoplasmic cells, lipid-like diffuse vacuolization (V), developed Golgi complexes (G), lyso-

somes (L) and endosomes near the Golgi complex, myelin-like formations, multi-vesicular bodies (Mvb), and narrow non-granule ER tubules were diffuse. In the dark-coloured cytoplasmic cells, mitochondria degenerated in patches (M), enlarged non-granule ER tubules (►) and Golgi complex were remarkable. The nuclear chromatin was fairly diffuse in these cells. Coated vesicles (Cv) were observed in the apical cytoplasm. Stereocilia (St) were developed and spermium (Sp) slices were visible in the lumen (Figure 2a-2b).

In the Phyto-1000 group, light- and dark-coloured chief cells and basal cells were seen to be in diffuse degeneration. GER and non-granule ER tubules were fairly ambiguous. Dense zones in short homogeneous patches in the cytoplasm were interesting. Shortage of stereocilia (St) in the apical plasmalemma was remarkable. Mitochondria (M) had a normal structure. There were fairly large vacuolar zones (V) in the cytoplasm. Primary lysosomes (L) were abundant. The cytoplasmic structure of the cell was not clearly distinguished. Apoptosis in basal and chief cells (A) was remarkable (Figure 3a-3b-3c).

DISCUSSION

It is known that oestrogens, either during pregnancy or in the neonatal period, have many negative effects on the male reproductive system, such as undescended testes, epididymal defects, and infertility.[18] In the last 50 years, it has been considered that synthetic oestrogens or those of herbal origin, taken as medicine or in the diet, damage sperm production and cause infertility.[6] It was not until the beginning of the 1990s that it came to light that oestrogen may also have a positive effect on the male reproductive system. Fertility is negatively affected in male mice that lack oestrogen. α-receptor or aromatase enzyme, and β-receptor, which are present in the male reproductive system at a high rate are evidence of oestrogen requirement for the male reproductive system.[19,20] Showing the negative and positive effects of oestrogen on the male reproductive system raises the question of the possible effects of oestrogens present around and in the diet. Diets rich or poor in phytoestrogens, which have oestrogen-like efficiency, may affect the male reproductive system positively or negatively. Phytoestrogens can display their own oestrogenic effects similarly as estradiol, by connecting to oestrogen receptors in the body. After Bennets and colleagues' study in 1946 in West Australia, which showed the development of permanent infertility in sheep fed rich clover, it was evident that nutrition with a diet rich in phytoestrogens had negative effects on both the male and the female reproductive systems.[15,21]

In their study in which pregnancy, birth, and consequently the newborns were monitored, Declos and colleagues examined the effects of different doses of genistein application on male and female genital systems and on other organs at macroscopic and microscopic level.[22] Starting with the non-genistein diet group, no macroscopic change was observed in male rats to which 7 different doses of genistein were applied. In a similar study, Fritz and colleagues did not determine any histomorphological changes in the reproduction organs of the male rats either, to which genistein was applied.[23] In our study, no macroscopic anomaly was observed in the epididymis or in other genital organs such as the testes or prostate of male rats fed non-phytoestrogen, controlled and high-dose phytoestrogen diets. Fielden and colleagues stated that a high phytoestrogen diet does not cause a change in sperm number, active sperm rate, or the in vitro fertilization ability of mice.[24]

In the histological examination of the testes and epididymis, Declos and colleagues determined some changes due to phyoestrogen content in the diet. Hypospermatogenesis in the testes and at the head of the epididymis was found to be highest in the high phytoestrogen group. However, there was no difference in the number of spermatid heads in the testes or the number of epipidymal spermatozoa between the groups.[22] In their study on the genistein effect on spermium function, Kumi-Diaka and colleagues showed that a low dose does not affect the sperm activity or sperm shape, but that a high dose may cause a decrease in active sperm percentage and deficiency in fertilization ability and consequently infertility.[25]

In conclusion, histological changes seen in the epididymis due to a high dose of phytoestrogen in the diet may cause infertility in male mice. Moreover, it should be kept in mind that in animal studies in which oestrogen effects are examined, the amount of phytoestrogen in the diet may affect the results of the study.

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