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The Effect of *Trifolium pratense* L. (Red Clover) on Rat Testes [1]

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Summary

Trifolium pratense L. is a legume containing large amounts of phytoestrogen. It contains four important estrogenic isoflavones (biochanin A, formonentin, genistein, daidzein) and coumestans. The effect of *Trifolium pratense* L. on the testes of rats was investigated in terms of the following parameters: (a) testis weights, (b) volume densities of seminiferous tubule, seminiferous tubule epithelium, spermatogonium nucleus and Sertoli cell nucleus and (c) Sertoli cell number. For comparative purposes, the effect of the well characterized exogenous estrogen, 17-β estradiol was also examined. Female Wistar rats (n = 80) and their male offspring (n = 54) were divided into 3 diet groups. The first group were given a basic diet with 7.5% *Trifolium pretense* L. added, the second group were given the basic diet with 0.5mg/kg dose of 17-β estradiol added and the control group fed with the basic diet alone. The male offspring were sacrificed at postnatal days 18 and 90. *Trifolium pretense* L. in the diet was found to have a short term positive effect on pupertal spermatogenesis but a negative effect on it in the long term.

Keywords: *Trifolium pratense L., Estrogen, Spermatogenesis, Testis*

Trifolium pratense L. (Kırmızı yonca)'nın Rat Testislerine Etkisi

Özet

Legimünöz ailesine ait olan *Trifolium pratense* L. fitoöstrojen içeren bir mera bitkisidir. Dört önemli isoflavon (biochanin A, formonentin, genistein, daidzein) ve koumestanları taşıdığı bilinmektedir. Bu çalışmada, *Trifolium pratense* L.'nin sıçan testisleri üzerine olan etkileri araştırıldı. Bu bağlamda testis ağırlığı, seminifer tübül ve seminifer tübül epitelinin hacim yoğunluğu ile spermatogonyum hücrelerinin hacim yoğunluğu ve Sertoli hücrelerinin çekirdekleri hacim yoğunluğu yönünden incelendi. Ayrıca *Trifolium pratense* L.'nin Sertoli hücresi sayısı üzerine olan etkileri saptandı. Karşılaştırma yapabilmek için 17-β estradiol'un etkisi, yukarıda bildirilen parametreler yönünden incelendi. Bu araştırmada, dişi Wistar sıçanlar (n=80) ve bu dişilerden doğan erkek yavrular (n=54) üç gruba ayrıldı. Birinci grubun temel diyetine %7.5 oranında Trifolium pratense L., ikinci grubun temel diyetine 0.5 mg/kg 17-β estradiol eklendi. Üçüncü grup ise kontrol grubu olarak ayrıldı. Dişi sıçanlar, gebelik ve laktasyon periyodları boyunca ait oldukları grubun diyetleri ile beslendi. Bu annelerden doğan erkek yavruların sütten kesildikten sonra 12 hafta boyunca kendi annelerinin diyetleriyle beslenmesi yoluna gidildi. Her grupta, erkek yavruların yarısı 18 günlük olduklarında, diğer yarısı da 90 günlük olduklarında CO_2 anestezisi altında sakrifiye edildiler. Sonuçta; diyetteki *Trifolium pratense* L.'nin spermatogenezis üzerinde kısa süreli olumlu bir etkiye sahip olduğu, uzun dönemde ise bu etkinin olumsuz yönde olduğu saptandı.

Anahtar sözcükler: *Trifolium pratense L., Östrojen, Spermatogenezis, Testis*

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INTRODUCTION

Several studies have shown that estrogens play a significant physiological role in male reproductive development and function [1]. It is clear that exposure of developing males to exogenous estrogen either in utero or neonatally can result in a range of abnormalities of reproductive development and function. Phytoestrogens are plant compounds which have estrogen-like biological activity. They are present in a wide variety of food sources. Phytoestrogens are similar to endogenous estrogens, in both structure and function [2,3]. There is growing evidence that neonatal exposure to phytoestrogens and environmental estrogens affects spermatogenesis in males [4]. Red clover (*Trifolium pratense* L.; family *Leguminoceae*) (Tp), a legume containing large amounts of the phytoestrogen, is used as a source of feed. It contains four important estrogenic isoflavones (biochanin A, formonentin, genistein, and daidzein) and coumestans. Isoflavones are the most potent estrogenic compounds in Tp [5].

The aim of this study was to investigate the effects of Tp on the testes of the rats in terms of the following parameters: (a) testis weights, (b) volume densities of seminiferous tubule, seminiferous tubule epithelium, spermatogonium nucleus and Sertoli cell nucleus and (c) Sertoli cell number. For comparative purposes, the effect of the well characterized exogenous estrogen, 17-β estradiol (E2), which has adverse effect on testis weight [4,6,7], was also examined.

MATERIAL and METHODS

Plant materials

Trifolium pratense was gathered from local fields in the Marmara region of Turkey, between the month of May and July 2005. The plant was dried in a non-humid room at 25°C and the dried leaves of the plant were grounded with a blender. The powdered part was kept in nylon bags and stored in the dark until the time of use. The plant was identified by Assoc. Prof. Dr. Tuna Ekim. A dried specimen was deposited in the Herbarium of Istanbul University, Faculty of Pharmacy (ISTE-Herbarium), with registration number 39935.

The isoflavone content of 200 mg of the dry matter was determined with RP-HPLC by the Institute of Pharmacognasy, Pharmacenter, Vienna 8. The mean isoflavone contents (%) of Tp were 0.0132, 0.0189, 0.2739, 0.3030 for daidzein, genistein, formononetin and biochanin A, respectively. E2 was purchased from Sigma Chemical Co. (St. Louis, USA).

Female Wistar rats were fed on a commercially available diet (basic diet) for 2 weeks before being mated. The diet consisted of bran 35%, wheat 7,8%, barley 10%, soy bagasse 25%, sugar beet pulp meal 2%, limestone 2%, ether extract 2%, middlings 10%, maize 5%, salt 1% and vitamin premix 0.2%.

They were then divided into 3 diet groups. The first group, Tp were given the basic diet with 7.5% Tp added, the second group E2 were given the basic diet with 0.5mg/kg dose of E2 added and the control group were fed with the basic diet alone. The female rats (n=80) and their male offspring (n=54), were given these diets throughout the gestation and lactation periods and their male offspring continued on their mother's diets for 12 weeks after weaning. The male offsprings were sacrificed at age 18 and age 90 days , since 18 days has been determined to be the time by which Sertoli cell number per testis has reached its final quantity, and 90 days assumed the time by which rats have gained fertility and the capability of mating [9,10].

The animals were killed by cervical dislocation after being anesthetized with CO_2. The body and testis weights of all animals were recorded at death and the gonado-somato indices (testis weight/ body weight) were calculated. The testes were fixed in Bouin's solution for 24 h. Sections of tissue, 4 μm thick, embedded in paraffin, were sampled in a random systematic manner and stained with Harris' haematoxylin and eosin.

The phytoestrogen content of the Tp used in our study was different from that of the Tp found in other regions reported in literature. However the phytoestrogen content of Tp has been found to vary with climate, the conditions at which it is dried and the method of silage production [11,12]. The feed used in this study consisted of 25% soybean bagasse, which was estimated to have an average phytoestrogen content of 500 ppm, according to Degen et al. [13]. The 7.5% Tp added diet, according to our estimation, contributed a further 500 ppm of phytoestrogen, making the total phytoestrogen content of this group about 1000 ppm. This level was high but non toxic as the toxic dose for genistein has been reported to be 1250 ppm [14]. Furthermore, in a preliminary study we found that

adding 7.5% Tp did not cause a toxic effect. The Animal Experiments and Ethic committee of İstanbul University Faculty of Veterinary Medicine approved this experiment.

Histological evaluation

The volume densities of the seminiferous tubule, seminiferous tubule epithelium were determined at age 18 and 90 days and those of the spermatogonium nucleus and Sertoli cell nucleus were determined only at 18 days with the standard point counting method [15]. Four sections from each testis were randomly selected. The images were transferred from a Nikon Fx microscope to a computer monitor. Using a systematic clock-face sampling pattern from a random starting point, 20 fields were evaluated. A 130-point grid printed on a transparency was placed over each field. The number of grid points over the Sertoli cell nucleus, spermatogonium nucleus, seminiferous tubule epithelium and seminiferous tubule were counted and expressed as percentage.

For determining Sertoli cell numbers the percentage volume (volume density) was converted to absolute volume per testis by multiplying it by the testis volume, which was taken to be a measure of testis weight. This was justified as shrinkage was minimal, i.e. testis weights before and after fixation were comparable. The Sertoli cell number was determined by the nucleator method as described previously [9,16]. In brief, this involved obtaining the Sertoli cell nuclear volume for each animal by measuring 4 separate nucleolus to nuclear membrane radii for each of between 50 and 70 Sertoli cell nuclei, and then determining the mean value of the nuclear volume with the aid of a standard equation. The Sertoli cell number was then obtained by dividing the absolute Sertoli cell nuclear volume by the mean Sertoli cell nuclei volume in each testis.

Statistical Analysis

Data were analysed with one-way analysis of variance (ANOVA) and followed by a Duncan test using the SPSS 11.0 program. The significance level was ascertained at $P<0.05$.

RESULTS

Testis weight

Among the 18 day old rats the Tp group had the heaviest body weight although it was only significantly heavier than that of the E2 group in *Table1*.

Among the 90 day old rats, the body weights of both the Tp and E2 groups were significantly lower than controls *(Table 1)*.

Among the 18 day old rats, the left testis weights of E2 group were significantly lower than those of the other groups. Among the 90 day rats, the left testis weights of both the Tp and E2 groups were significantly lower than controls. The gonado-somato indices did not significantly differ between diet groups for either the 18 day or 90 day rats *(Table 1)*.

Histological evaluations

In the 18 day old animals, the seminiferous tubule volume density did not significantly differ among groups in *Table 2*. However, the seminiferous tubule epithelium volume density was significantly higher in the Tp group than in the others *(Table 2)*. Yet in the 90 day animals, in both experimental groups, the seminiferous tubule epithelium volume density was less than the controls in *Table 3*. Although the differences were not statistically significant, in the 18 day animals, Sertoli cell number and spermatogonium nucleus volume density were highest in Tp group *(Table 2)*.

Table 1. *Body weights (g), left testis weights (g), gonado-somato indices (testis weight/body weight) of 18 and 90 day old rats*
Tablo 1. *18 ve 90 günlük sıçanların, vücut ağırlığı (g), sol testis ağırlığ (g), gonad-vücut ağırlığı indeksi (testis ağırlığı/vücut ağırlığı)*

GROUPS (n)	Body Weight (g)		Left Testis Weight (g)		Gonado-Somato Index	
	18 day	90 day	18 day	90 day	18 day	90 day
Control (10)	20.9±0.59 [ab]	187.89±3.61[a, ***]	0.050±0.001 [a]	1.31±0.04 [a, ***]	0.0023±0.00005	0.0069±0.0001
Tp (10)	23.4±2.24 [a]	118.58±5.96 [b]	0.057±0.005 [a]	0.66±0.08 [b]	0.0024±0.00007	0.0054±0.0005
E2 (10)	16.9±0.10 [b, *]	123.77±5.29 [b]	0.038±0.0004 [b, **]	0.79±0.04 [b]	0.0022±0.00002	0.0056±0.0007

a, b: *The differences between the means of groups with different superscripts (in the same column) are significant*
** P<0.05, ** P<0.01, *** P<0.001 are in comparison with the respective control group. Data are mean ± SE*

Table 2. *Volume densities of Seminiferous tubule and epithelium, spermatogonium, Sertoli cell nucleus and Sertoli cell number of 18 day old rats*
Tablo 2. *18 günlük sıçanların, seminifer tubulunun ve epitelinin, spermatogonyum ve Sertoli hücresi çekirdeğinin hacim yoğunluğu ve Sertoli hücresi sayısı*

GROUP (n) 18 day	S. tubule volume density (%)	S. tubule epithelium volume density (%)	Sertoli cell nucleus volume density (%)	Spermatogonium volume density (%)	Sertoli cell number
Control (10)	79.69±1.23	10.25±0.40 [b]	10.00±0.53	3.01±0.18 [ab]	45084881±5382871 [a]
Tp (10)	79.39±1.31	13.01±0.54 [a, ***]	9.46±0.93	3.56±0.37 [a]	47292129±4713108 [a]
E2 (10)	77.79±1.25	11.12±2.32 [b]	7.75±0.46	2.28±0.34 [b, *]	25727856±1645717 [b, **]

a, b: *The differences between the means of groups with different superscripts (in the same column) are significant*
** P<0.05, ** P<0.01, *** P<0.001 are in comparison with the respective control group. Data are mean ± SE (n=10)*

Table 3. *Seminiferous tubule and epithelium volume density of 90 day old rats*
Tablo 3. *90 günlük sıçanların seminifer tubulunun ve epitelinin hacim yoğunluğu*

GROUP (n) 90 day	S. tubule volume density (%)	S. tubule epithelium volume density (%)
Control (10)	79.37±1.03	8.78±0.33 [a, *]
Tp (10)	82.52±0.86	6.81±0.36 [b]
E2 (10)	81.58±1.13	7.09±0.36 [b]

a, b: *The differences between the means of groups with different superscripts (in the same column) are significant.*
** P<0.001 is in comparison with the respective control group. Data are mean ± SE (n=10)*

DISCUSSION

Testis weight

Many studies have also reported a decrease in testis weights of animals administered estradiol [4,6,7]. Soybean and its products which contain isoflavones, i.e. genistein and daidzein have been the subject of much research. Genistein and soy-containing diets have been reported to have no significant effect on testis weights of animals [17-19]. However, Atanassaova et al.[4] reported that for both 18 day old and adult rats, those on a soybean free diet had significantly higher testis weights than those on a diet containing soybean. On the other hand, Kang et al.[20] reported that testis weights increased during the neonatal period and did not change during the adult period in animals receiving genistein. The observed increases in testis weights in the 18 day Tp group are consistent with those of Kang et al.[20]. However the decrease in the adult differs from the the findings of the studies above and may be due to the higher Tp dose used in our study. However, lower testis weight in the Tp, could simply have been due to the 7.5% Tp, lowering total energy and protein intake over an extended period leading to lower body weights thus testis weight. No significant differences in terms of gonado-somato indices between the groups supported this assumption. In other studies the active substances have been either injected or added to the feed in very low percentages. However, it has been also reported that soy-containing diets resulted in reduction of testis weight of 68 day old rats [21].

Histological evaluations

High doses of Diethylstilbestrol (DES; potent and nonstreoidal synthetic estrogen) have been reported to decrease Sertoli cell number in the neonatal period [1,4,9,10,22,23]. Yet reported findings for estradiol have been contradictory. Atanassaova et al. found Estradiol to decrease Sertoli cell number [4], which was compatible with our finding. Yet Wistuba et al.[7] reported estradiol treatment to have no effect on Sertoli cell number. Atanassaova et al.[1] showed that the absolute volumes of Sertoli cell increased after eliminating soybean from the diet. However we found no significant difference in either Sertoli cell nucleus volume density or Sertoli cell number between the 18 day Tp rats and control.

For the 90 day old rats, in both the Tp and E2 groups, all parameters except seminiferous tubule density were lower than those of controls indicating that estradiol and Tp had adverse affects on testis in the long term.

In summary, Tp in diet had a short term positive effect on pupertal spermatogenesis but displayed a negative effect in the long term.

Further studies of the relative phytoestrogenic effects of various extracts of Tp would appear to be warranted.

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