

Original Article

Evaluation of the estrogenic effects of dietary perinatal *Trifolium pratense*

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This study was designed to investigate the potential estrogenic effects of perinatal dietary phytoestrogens on the rat uterus. Pregnant rats were divided to three groups provided the following diets: (1) rat chow, (2) rat chow with 7.5% *Trifolium (T.) pratense*, or (3) rat chow supplemented with 17 β -estradiol (0.5 mg/kg). The dams in each group were kept on the same diet during pregnancy and lactation. Female offspring were euthanized on day 21 at which time body and organ weights were recorded and tissue samples were taken for histology. Immunohistochemistry was performed to detect estrogen receptor alpha (ER α) and progesterone receptor (PR) levels. Our results revealed estrogen-like biological effects of perinatal *T. pratense* exposure. Relative uterus and ovary weights in the experimental groups were increased compared to control. The number of uterine glands and luminal epithelium heights were also increased. However, there were no statistically significant changes detected in the immunostaining intensity of ER α and PR between the groups.

Keywords: estrogen receptor alpha, progesterone receptor, rat uterus, red clover, *Trifolium pratense*

Introduction

Trifolium (T.) pratense, a fodder plant, contains phytoestrogens that possess putative weak estrogenic or anti-estrogenic activity. *T. pratense* contains four important estrogenic isoflavones (biochanin A, formononetin, genistein, and daidzein) and coumestans [20]. Early studies demonstrated that isoflavone intake was associated with impaired fertility in sheep and silage-fed cows exposed to isoflavone-rich *T. pratense* [1,12]. Reportedly, even low concentrations of phytoestrogens may have substantial deleterious effects on the reproductive rate of sheep, and

possibly cattle, or may manifest as subclinical infertility in the absence of any visible clinical signs [1].

T. pratense is important not only as animal feed but also as an alternative to conventional hormone replacement therapy due to its high isoflavone content [3]. Although phytoestrogens were shown to be responsible for fertility-related disorders in animals, they have also been found to be beneficial for certain diseases. Extracts obtained from *T. pratense* have been shown to inhibit aromatase activity, angiogenesis, and inflammation, and have been tested for treatment of breast and prostate cancers [11,15,19,24]. A number of clinical trials have been performed to address the effects of *T. pratense* on menopause, cognitive function, and bone and cardiovascular health with no agreement on their effectiveness [5,8,16]. Further studies are necessary to satisfy this controversy and evaluate the risks of long-term use of phytoestrogens. Phytoestrogens have generated considerable interest because of their potential cancer preventive, cardioprotective, and anti-osteoporotic effects, as well as their ability to relieve menopausal symptoms. There have been contradictory results published on the effects of isoflavones on a variety of estrogen-regulating organs postulating both beneficial and adverse effects on human health [25].

Animal studies have indicated that neonatal exposure to phytoestrogens and environmental estrogens may affect male and female reproductive development [2]. A soy isoflavone, genistein, was reported to modulate sex steroid receptor expression in prepubertal rat uteri when given at considerably high pharmacologic doses [9]. Moreover, extracts of *T. pratense* tested in an adult ovariectomized rat model were suggested to be weakly estrogenic [7] or to have no effect on rat uterus [18].

Compounds with estrogenic activity, which naturally occur in food products or fodder plants, may alter the reproductive performance of animals and may also have an impact on animal and/or human health. Therefore, experimental studies based on physiologic dose levels evaluating exposure at different periods of life are needed. In this study, we investigated whether perinatal exposure to *T. pratense* through dietary intake would alter the uterine health of prepubertal rats.

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Materials and Methods

Adult, female Wistar albino rats (180 ~ 250 g; n = 15) and their female littermates (n = 40) were used for this study. The experimental protocol was approved by Istanbul University's Animal Care and Use Committee (Approval number: 18/13072001) consistent with the NIH Guidelines for the Care and Use of Laboratory Animals. The rats were maintained under standard laboratory conditions with a 12:12 light/dark cycle and free access to pellet feed and tap water.

Experimental diets and groups

T. pratense was gathered from local fields in the Marmara region of Turkey. The plant was identified by Associate Professor Dr. Tuna Ekim (Faculty of Pharmacy, University of Istanbul, Turkey) as *T. pratense* L. var. *Pratense*. A dried specimen was deposited in the Herbarium of Istanbul University, Faculty of Pharmacy, with registration number 39935. The areal part of *T. pratense* was dried in a non-humid room at 25°C and then grinded with a blender. The pulverized plant was thoroughly mixed and stored in the dark until the time of use. The concentrations of different isoflavones in *T. pratense* were analyzed by Krenn *et al.* [14]. The extract was prepared by maceration with water and extraction of the free genins was accomplished with methanol under reflux. The concentrations of the isoflavones in our material were as follows: biochanin 0.3%, formononetin 0.27%, daidzein 0.01%, and genistein 0.01%.

The study consisted of three groups receiving different diets as follows: the control group received standard rat diet (Eris Yem, Turkey); the *T. pratense* group included pregnant rats fed the control diet mixed with pulverized *T. pratense* (75 g/kg diet); and the estradiol group had 0.5 mg estradiol [(17 β)-estra-1,3,5(10)-triene-3,17-diol; Sigma, USA] dissolved in corn oil (Sigma, USA) added to 1 kg of the control diet (12.5 μ g/rat/day). The dams were exposed to different diets from the seventh day of gestation throughout lactation until postpartum day 21 at which time the female littermates were euthanized. Daily diet consumption was recorded as ~25 g. Only female littermates were used in the experiment.

Histology and histomorphometric methods

The uteri samples were fixed in Bouin's solution for 18 ~ 20 h. After fixation, the samples were dehydrated in ethanol, cleared in xylene, and embedded in paraffin. Tissue sections were cut at a thickness of 5 μ m and stained with hematoxylin and eosin. Images were taken with a Microphot FX light microscope (Nikon, Japan) and digitized with a CCD camera (F10; Panasonic, Japan). Body and uteri weights were determined in all groups. Relative weights were calculated by dividing organ weights by body weights and multiplying by 10,000.

Histometric methods were used to measure uterine layer areas and uteri luminal epithelium heights. Each uterine horn was cut into four equal pieces and the number of uterine glands was counted from these different segments. For measurement of uterine layer area, digitalized cross sections of the uteri were transferred to the AutoCad 2002 program (Autodesk, USA) and a line was drawn around each uterine layer using the polyline tool. After transferring the same sections to the Corel Draw 12 program (Corel, USA), the freehand tool was used to measure the epithelial height at 4 ~ 6 different points in each section. Calibration was performed with a milimetric ruler (1 mm, 0.01 Div; Graticules Pysersgi, UK), which was photographed with the same magnification as all other images. Measurements of luminal epithelial cell height were completed for 10 animals per group.

Immunocytochemistry

Progesterone receptor (PR) antibody (rabbit anti-human PR, recognizes both forms of PR; Dako, Denmark), and estrogen receptor alpha (ER α) antibody (monoclonal mouse anti-human ER; Dako, Denmark) were used for immunohistochemistry. Paraffin sections were dewaxed and rehydrated. After washing in water, the sections underwent antigen retrieval in a microwave oven in 10 mM sodium citrate buffer, pH 6.0, for 15 min. The sections were allowed to cool and then rinsed with phosphate buffered saline (PBS). Endogenous peroxidase activity was blocked with 1% H₂O₂ in PBS for 20 min. PR (1:400) and ER α (1:200) antibodies were diluted in PBS with 3% bovine serum albumin containing 0.05% Tween. The sections were incubated with primary antibodies overnight at +4°C, washed with PBS, and incubated with horseradish peroxidase conjugated Dako EnVision™ systems (Dako, Denmark) for 30 min. The slides were rinsed twice with PBS and color was developed with diaminobenzidine substrate (Dako, Denmark). The sections were then counterstained with Mayer's hematoxylin, dehydrated, and mounted.

Slides were analyzed using light microscopy. The number and distribution of ER α and PR stainings were determined from uteri sections of five animals in each group at \times 200 magnification. Uterine structures, namely luminal/glandular epithelium, circular/longitudinal muscle, and stroma, were analyzed. The proportional score of positively stained cells was evaluated and given a score of zero for no staining, 1 for weak staining, 2 for moderate staining, 3 for strong staining, and 4 for intense staining. The proportional score reflected the number of nuclear ER α and PR positive epithelial cells in the layers of the uteri.

Statistics

Statistical analyses were performed using Windows SPSS (ver. 10.0; SPSS, USA) with Duncan's one-way ANOVA test. *P* value < 0.05 was considered significant.

Results

Body, ovaria, and uteri weights

Treatment of rats from gestation day 7 to postnatal day 21 with *T. pratense* or with estradiol, slightly decreased body weights, but this decrease was not statistically significant (Table 1). Relative ovaria weights were significantly increased only in the *T. pratense* group. *T. pratense* caused a statistically significant increase in relative uterine weights with a similar increase also found in the estradiol group (Table 1).

Morphometric analysis

The number of uterine glands was significantly increased in animals treated with *T. pratense* or estradiol (Table 2). Luminal epithelial cell height was significantly increased in both *T. pratense* and estradiol treated animals compared to control animals (Table 2) and endometrium volumes were significantly increased in both *T. pratense* and estradiol groups as well (Table 3).

ER α and PR immunostaining

ER α and PR positive nuclei were localized in the epithelial, stromal, and muscular cells of the uteri (Fig. 1). Uterine sections not receiving primary antibody showed no staining.

Table 1. Body weights and relative ovaria and uteri weights

	Control	<i>T. pratense</i>	Estradiol
Body weights (g)	23.89 \pm 2.41	21.4 \pm 1.66	19.87 \pm 0.43
Relative ovaria weights	5.98 \pm 0.19	6.87 \pm 0.2*	5.68 \pm 0.27
Relative uterus weights	5.85 \pm 0.24	7.23 \pm 0.21*	7.14 \pm 0.42*

Body and uteri weights from control, *Trifolium (T.) pratense*, and estradiol groups. Relative weights were calculated by dividing organ weights by body weights and multiplying by 10,000 (n = 10 per group). * $p < 0.01$ significantly different compared to control group. All data represent mean \pm SD.

Table 2. Number of uterine glands and luminal epithelial cell height

	Control	<i>T. pratense</i>	Estradiol
Number of uterine glands	2.19 \pm 0.10 (81)	2.49 \pm 0.12* (50)	2.93 \pm 0.09* (52)
Luminal epithelial cell height (μ m)	11.34 \pm 0.11 (189)	11.99 \pm 0.07* (228)	12.22 \pm 0.08* (184)

* $p < 0.001$ significantly different compared to control group. All data represent mean \pm SD (n).

Table 3. Endometrium, myometrium, and total area of uterine layers

	Control	<i>T. pratense</i>	Estradiol
Myometrium (mm ²)	0.190 \pm 0.006 (81)	0.198 \pm 0.006 (50)	0.198 \pm 0.006 (52)
Endometrium (mm ²)*	0.120 \pm 0.004 (81)	0.132 \pm 0.039* (50)	0.134 \pm 0.005* (52)
Total uterine area (mm ²)	0.310 \pm 0.009 (81)	0.330 \pm 0.009 (50)	0.332 \pm 0.010 (52)

* $p < 0.05$ shows significant differences compared to control group. Endometrium area is significantly bigger in *T. pratense* and estradiol treated groups. All data represent mean \pm SD (n).

Strong PR immunoactivity was observed in the luminal epithelium of uterine tubes but only faint staining was observed in *T. pratense* and estradiol treated groups (Fig. 1). The staining intensity of glandular, luminal, stromal, and muscular compartments for ER α in the uteri of rats was reduced in both *T. pratense* and estradiol treated animals compared to controls (Fig. 2A); however, this decrease was not statistically significant. PR staining intensity decreased in luminal and glandular compartments in *T. pratense* and estradiol treatment groups. No changes were observed in the staining intensity of muscular and stromal layers between the control and estradiol groups. Decreased stromal staining intensity was observed in the *T. pratense* group compared to the control group. No PR staining was observed in the muscular layer of *T. pratense* treated animals (Fig. 2B).

Discussion

In the present study, the effects of dietary perinatal exposure to *T. pratense* were investigated in female rats. Dietary estradiol was used as a reference compound for estrogenicity. In addition to isoflavones, *T. pratense* contains other potentially important components including phenolic acids and flavonoids that may affect uterine health in combination with isoflavones. We administered *T. pratense* by adding it to the diet rather than giving it as an extract to simulate the consumption of isoflavones through diet.

In agreement with earlier data [14], HPLC analysis showed that biochanin A and formononetin were the most abundant isoflavones detected in *T. pratense*, followed by genistein and daidzein. Genistein is the major isoflavone found in soy. *T. pratense* differs from soy not only in its lower genistein content but also in its higher phytoestrogen content. The total amount of isoflavones found in *T. pratense* extract used in the present study (0.61%) was lower than the amount reported by Saloniemi *et al.* [21] (1 ~ 2.5%). The low isoflavone content of *T. pratense* used in the present study may be due to the extraction method, seasonal changes, temperature (*e.g.* cool weather during the growing season), phosphorus content of the soil, changes in ozone exposure,

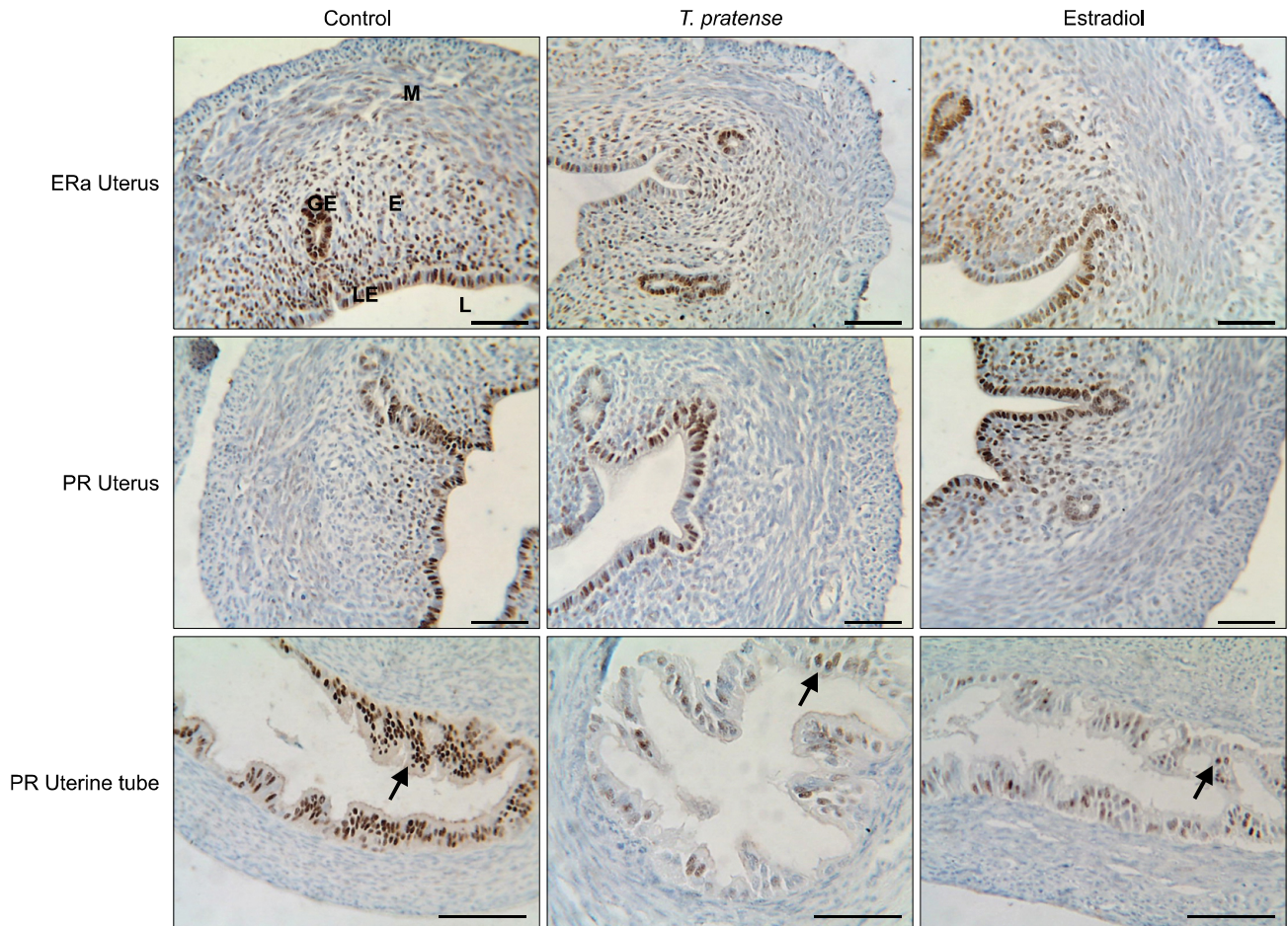


Fig. 1. Immunohistochemical expression of estrogen receptor alpha ($ER\alpha$) in the uterus and uterine tube. M: myometrium, GE: glandular epithelium, E: endometrium, LE: luminal epithelium, L: lumen. Arrows: nuclear staining of $ER\alpha$ and PR. Counterstain with Mayer's hematoxylin. Scale bars = 50 μ m.

or differences among *T. pratense* varieties [4,21-23].

Both estradiol and *T. pratense* treatments slowed the growth of pups. Exposure to either estradiol or *T. pratense* throughout pregnancy until day 21 increased relative uterine weights, suggesting estrogenic activity. *In vitro* studies demonstrated that *T. pratense* extracts exhibit estrogenic activity in an estrogen-dependent MCF-7 cell proliferation assay and had preferential agonist activity towards $ER\beta$ [6]. In accordance with our results, 2~3 g dry matter per day was found to increase the weight of immature rat uteri [18]. In an ovariectomized uterotrophic adult rat model, *T. pratense* extract was reported to be weakly estrogenic; however, this effect was based mainly on the semi-quantitative results of the Allen-Doisy vaginal cell cornification assay. Although, the highest dose (750 mg/kg per day) of *T. pratense* extract slightly increased uterine weight, it is not clear whether this difference was statistically significant compared to the control group [7]. In another study, ovariectomized adult rats were treated for 21 days with 4, 40, or 400 mg/kg per day of *T. pratense* extract. Neither uterine weight nor vaginal

cornification assay results were significantly altered [18]. It is possible that rats at this perinatal period are more sensitive to estrogenic stimuli than adults. Thus, the timing and/or route of administration of *T. pratense* may account for differences in results.

In the current study, morphometric analysis showed that the number of uterine glands found in the endometrium was significantly increased in both the *T. pratense* and estradiol groups. In accordance with our study, Foth and Cline [10] reported that diet containing soy protein isolates (SPI) given together with estradiol increased endometrial thickness, gland area, and epithelial proliferation in macaque monkeys. Interestingly, in their study, the effect of estradiol on proliferation was partially antagonized by SPI. In our study, the area of endometrium showed an increase in treated groups, but the total area (epithelium plus myometrium) of the uterus was not significantly different than that of the control group. Furthermore, the luminal epithelial cell height increased in both *T. pratense* and estradiol treated groups. In a study by Overk *et al.* [18], no data on luminal

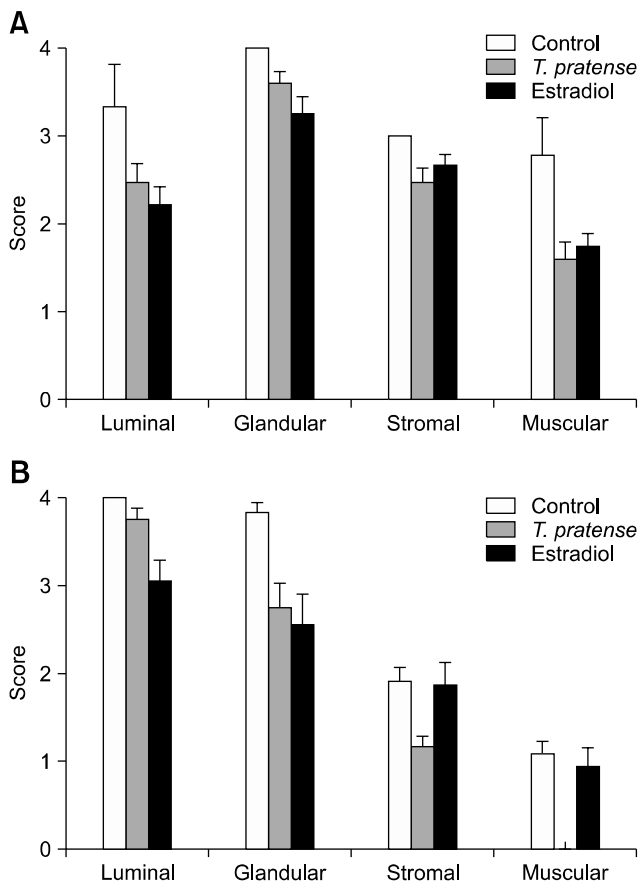


Fig. 2. ER α (A) and PR (B) immunostaining score in the uterus of 21-day-old rats. The proportion of positively stained cells was scored as follows: 0, none; 1, weak; 2, moderate; 3, strong; 4, intense staining.

epithelial cells were presented in rats treated with *T. pratense*. Increased epithelial height indicates hypertrophy of the cells. Water imbibition, mainly in the endometrium, may be responsible for uterine weight increase even though histological observation indicated no evidence of edema in the uterine wall. Although we did not measure the proliferation index, it is also possible that increased cell proliferation in epithelial as well as stromal cells may be responsible for the increased thickness of the endometrium.

The expression of PR is under the control of estrogen and progesterone [13]. PR is a classic gene marker for estrogen action containing estrogen response elements in the 5' region. Estrogen increases PR expression while progesterone decreases its expression in most target tissues. Progesterone treatment can oppose the estrogen induction of PR and progesterone has been shown to decrease total PR expression in the uterus [17]. In the current study ER α and PR expression was detected by immunohistochemistry. Our results revealed that neither *T. pratense* nor estradiol significantly altered the expression of ER α or PR in the luminal and glandular epithelium, which could be due to the

fact that only five animals from each group were analyzed. In a study by Cotroneo *et al.* [9], no significant alteration was detected in ER α expression by Western blot analysis or immunohistochemistry after dietary administration of genistein and estradiol to Sprague Dawley rats. However, a decrease in staining intensity was evident when pharmacological doses were given by injection. In line with our results, they demonstrated that PR expression was not altered by dietary genistein administration, but the injection of pharmacological doses of both genistein and estradiol increased PR expression as shown by Western blot analysis.

Our results revealed that consumption of *T. pratense* via the oral maternal route caused estrogen-like biological effects on the genital organs of rats, namely increased uterine weight and luminal epithelial cell height. However, no significant changes were detected during immunohistochemical evaluation of ER α and PR levels.

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