**Trifolium pratense** (Red Clover) Exhibits Estrogenic Effects In Vivo in Ovariectomized Sprague-Dawley Rats

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ABSTRACT Studies were conducted using an ovariectomized rat model to determine the estrogenic and antiestrogenic activity of **Trifolium pratense** L. (red clover) extracts. A red clover extract, standardized to contain 15% isoflavones was administered by gavage [250, 500 and 750 mg/kg·d] to virgin, ovariectomized 50-d-old Sprague-Dawley rats, for 21 d in the presence and absence of 17β-estradiol [50 μg/kg·d]. Estrogenic effects included an increase in uterine weight, vaginal cell cornification and mammary gland duct branching. Red clover produced a dose-dependent increase in uterine weight and differentiated vaginal cells at the two higher doses, but it did not stimulate cell proliferation in the mammary glands. Neither antiestrogenic nor additive estrogenic properties were observed in any of the tissues studied. These data suggest that red clover extract is weakly estrogenic in the ovariectomized rat model. J. Nutr. 132: 27–30, 2002.

KEY WORDS: • **Trifolium pratense** • rats • mammary gland • uterus • estrogenic

A decline in the level of endogenous circulating estrogens indicates the onset of menopause. Primary health concerns resulting from menopause include hot flashes, vaginal atrophy, reductions in cardiovascular health and enhanced risk for developing osteoporosis and Alzheimer's disease. Hormone replacement therapy (HRT) helps to prevent the development of these pathologies in postmenopausal women. However, because of a greater incidence of breast and endometrial cancer has been linked to some forms of HRT (1), increased attention has been placed on finding viable and safe alternatives (2). Also, due to the fear of developing cancer and discomfort, many users of HRT exhibit poor compliance (3). As a result, natural estrogenic alternatives for the treatment of menopausal pathologies and symptoms are frequently considered, because this offers the hope of improved safety and greater compliance.

The estrogenic compounds isolated from red clover are isoflavones, including genistein, daidzein, biochanin A and formononetin (4,5). Two of these compounds, genistein and daidzein, are also found in soy (6) and have been reported to cause an increase in uterine weight (7–9). However, studies performed with soy protein isolate have not detected estrogenic activity (10–12). Investigations to determine whether red clover extracts can induce estrogenic effects similar to genistein and daidzein in ovariectomized rats have not previously been reported.

The biological effects associated with ingesting isoflavones indicate that dietary supplements rich in these compounds might be useful for alleviating menopausal health concerns. Epidemiological data have shown that a diet high in isoflavones, such as those in red clover and soy, may reduce the risk of cardiovascular disease and breast and endometrial cancer (2,3). Human clinical studies have suggested benefits of consuming red clover isoflavones at a dose of 40 mg/d followed by 80 mg/d, such as increasing arterial elasticity and improving cardiovascular health (14). Isoflavones might also help to prevent the onset of hormonal carcinogenesis by decreasing genotoxic estrogen metabolites (15). Genistein and daidzein have been shown to help reverse bone loss in ovariectomized rats, although this may lack clinical relevance in humans (16–18). Previous studies have indicated that red clover extracts induce estrogen-responsive proteins, up-regulate the expression of the estrogen-inducible genes progesterone receptor and prenenilin 2 and contain ligands, which compete for 17β-estradiol with both estrogen receptors (ERα and ERβ) (5).

Because estrogens have typically been used for the treatment of menopausal symptoms and because isoflavones in red clover have been shown to improve some symptoms associated with menopause, we investigated the potential estrogenic effects of red clover. Suggested doses of commercially available red clover extracts for human intake are ~40 mg of isoflavones per day. In the present study, we evaluated the potential of a red clover extract to exhibit estrogenic or antiestrogenic effects in the uterus, vaginal cells and mammary glands of ovariectomized rats.

**MATERIALS AND METHODS**

**Animals.** Guidelines established by our institutional Animal Care and Use Committee and state and federal regulations were followed for all procedures. The protocol complied with the Guide for the Care and Use of Laboratory Animals and the facilities are Association for the Assessment and Accreditation of Laboratory Animal Care approved. Female ovariectomized Sprague-Dawley rats weighing ~230 g were received at 7 wk of age from Harlan (Indianapolis, IN). Following 3 d acclimation, the rats were weighed and housed in groups of three. Rat cages were arranged randomly to limit variation based on temperature.

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and light. They were maintained in barrier rooms under a 12:12-h light-dark cycle, with a temperature of 22 ± 1°C and a relative humidity of 50%.

**Diets and treatment.** All rats consumed a Harlan Teklad Global 16% protein rodent diet (Indianapolis, IN), which contains no alfalfa or soybean meal. A diet containing minimal amounts of phytoestrogens was necessary to avoid confounding experimental results with red clover isoflavones. Access to food was unrestricted and water was administered through an automatic watering system. The red clover extract was standardized to a minimum of 15% isoflavone content by weight (15 g of total isoflavones per 100 g of total extract) of four isoflavones: genistein, 0.88%; daidzein, 0.34%; biochanin A, 6.57%; and formononetin, 8.56%, present as hydrolyzed aglycones. Red clover extracts and the negative control, carboxymethylcellulose (CMC) at 0.1% purchased from Sigma-Aldrich (St. Louis, MO), were administered by intestinal gavage. 17β-Estradiol suspended in sesame oil or the oil alone was administered subcutaneously.

**Estrogenic and antiestrogenic activity of red clover.** Red clover powdered extract was dissolved in ± 1 g/L CMC solution to yield concentrations of 125, 250, and 750 g/L. 17β-Estradiol was dissolved in a minimum amount of ethanol and suspended in sesame oil to a concentration of 100 mg/L. The treatment groups and number of rats per group were arranged as follows: group 1, n = 5 (sesame oil and 0.1% CMC); group 2, n = 6 (50 mg/kg·d 17β-estradiol and 0.1% CMC); group 3, n = 6 (sesame oil and 250 mg/kg·d red clover); group 4, n = 6 (sesame oil and 500 mg/kg·d red clover); group 5, n = 6 (sesame oil and 750 mg/kg·d red clover); group 6, n = 6 (50 mg/kg·d 17β-estradiol and 250 mg/kg·d red clover); group 7, n = 4 (50 mg/kg·d 17β-estradiol and 500 mg/kg·d red clover); and group 8, n = 6 (50 mg/kg·d 17β-estradiol and 750 mg/kg·d red clover). Each rat was treated for 21 d. The doses of red clover were based on typical clinical doses for humans (40 mg isoflavones/d). Estrogenic and antiestrogenic effects were evaluated based on vaginal cytology, uterine weight and mammary gland alveolar and ductal structure. Rats were weighed biweekly to monitor toxicity and to adjust the dose accordingly.

**Vaginal cellular differentiation analysis.** Vaginal cytology smears were obtained daily to monitor cellular differentiation. A smear was performed on all rats before dosing to establish a baseline and to confirm that all ovariectomized animal smears showed no cornification. Vaginal smears were taken daily using an eyepopper containing 8.5 mL of saline, placed on ringed slides, and observed under a light microscope using a 10X eyepiece and a 10X objective. The same technician, unblinded, read smears immediately and cells were identified as leukocytes, nucleated, or cornified epithelial cells. A raw score on a scale of 1 through 5 was assigned for cell populations ranging from entirely leukocytes (indicating a pro-estrous stage) to entirely cornified (indicating a diestrous stage).

**Determination of uterine weight.** Twenty-four h after the final treatment, the rats were killed by CO2 asphyxiation. At necropsy, the uteri were collected, trimmed of fat and connective tissue, cut open, and drained of intrauterine fluid, weighed, frozen in 1.5-mL cryogenic vials on dry ice, and stored at −80°C. Adrenals, kidneys, liver, pancreas and spleen were removed, weighed and stored in formalin.

**Analysis of mammary gland whole mounts.** The abdominal inguinal mammary gland was removed as a strip of tissue containing the primary duct, the edge of the gland and part of the mammary tree. Whole mount slides were prepared from the mammary glands as described previously (19). Whole mount pictures were taken using a Kodak camera attached to a dissecting microscope and magnified under a 5X lens with TMX 200 speed Kodak black and white film (Rochester, NY).

**Statistical analyses.** Uterine and organ weights were analyzed by a multiple comparison analysis using a one-way ANOVA and the follow-up analysis was performed using Tukey's test. For estrogenic analysis, the three doses of red clover were compared with the vehicle and estradiol treatment groups. For the antiestrogenic analysis, the three groups treated with red clover plus estradiol were compared with the vehicle and estradiol treatment groups. Body weights were analyzed as a two-way ANOVA based on different days and different weights. Tukey's test was used as the follow-up test. Data are reported as the mean ± so. Difference was considered significant at P < 0.05.

## Results

**Uterine and organ weights.** Red clover extracts administered to ovariectomized rats at 250, 500 and 750 mg/kg·d tended to dose-dependently increase uterine weight [P < 0.05 (Table 1) and thickness (Fig. 1). However, the response was significantly less than that observed in 17β-estradiol-treated rats, indicating lower potency. The ability of red clover to antagonize

### Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Uterine weight, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>17β-Estradiol, 50 μg/kg·d</td>
<td>6</td>
<td>0.39 ± 0.02</td>
</tr>
<tr>
<td>Red clover, 250 mg/kg·d</td>
<td>6</td>
<td>0.10 ± 0.03</td>
</tr>
<tr>
<td>Red clover, 500 mg/kg·d</td>
<td>6</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>Red clover, 750 mg/kg·d</td>
<td>6</td>
<td>0.13 ± 0.03</td>
</tr>
<tr>
<td>Red clover, 750 mg/kg·d + 17β-estradiol, 50 μg/kg·d</td>
<td>6</td>
<td>0.38 ± 0.04</td>
</tr>
</tbody>
</table>

1 Values are the means ± so.
2 Rats demonstrated a significant increase in uterine weight compared with vehicle.
3 Rats demonstrated a significant difference in uterine weight compared with the 17β-estradiol treatment groups.

**Figure 1** Photographs of uteri excised from rats treated with red clover, 17β-estradiol or vehicle. The photograph demonstrates the dose-dependent increase in uterine thickness in response to red clover. (A) Red clover [750 mg/kg·d], (B) red clover [500 mg/kg·d], (C) red clover [250 mg/kg·d], (D) 17β-estradiol positive control, and (E) vehicle control.
uterotrophic effects of 17β-estradiol was evaluated by comparing the uterine weights of groups treated with 17β-estradiol and groups receiving 17β-estradiol plus red clover. When given 17β-estradiol, red clover extract neither stimulated nor antagonized the 17β-estradiol-induced change in uterine weight. In estradiol-treated rats, spleen weight differed between the control group and each of the three groups receiving red clover. Weights of the other organs examined, including adrenals, kidneys, liver and pancreas, were not affected (data not shown).

Vaginal cellular cornification. Vaginal cells exhibited a distinct pattern of maturation in response to 17β-estradiol, beginning as a population of leukocytes, advancing to nucleocytes and terminating as fully cornified cells. Rats fed 17β-estradiol showed full vaginal cornification within 3 d. The administration of red clover to ovariecotomized female rats resulted in a dose-dependent increase in vaginal cell differentiation after 14 d (Fig. 2). Red clover, when administered with 17β-estradiol, exhibited no antagonistic or additive effects on vaginal cell maturation (data not shown). Only the lowest dose of red clover [250 mg/(kg · d)] did not stimulate differentiation relative to controls.

Body weight. Over the period of treatment, the body weight of rats treated with the lowest dose of red clover or vehicle increased 30% relative to d 0. Rats treated with 17β-estradiol or red clover plus 17β-estradiol maintained a similar body weight throughout the experiment relative to their body weight at the outset of the experiment. The weight gains of the control and red clover-treated groups were significantly greater than the 17β-estradiol-treated rats or the rats treated with 17β-estradiol plus red clover. Rats fed red clover showed a dose-dependent decrease in body weight gain with increasing concentrations of red clover. The decreased weight gains in the red clover-treated rats were significant in the 750 mg/(kg · d) group compared with the vehicle control group. The trend for decreased body weight gain with increasing red clover concentration suggests that red clover has biological effects similar to those of 17β-estradiol-treated rats (data not shown).

Mammary glands. The mammatrophic effects of red clover were evaluated in ovariecotomized rats by examination of duct branching and alveolar structure. Glands from rats treated with vehicle only displayed thin branches and little alveolar budding. The mammary glands of 17β-estradiol-treated rats demonstrated extensive ductal branching and defined buds. Administration of the red clover extract for 21 d at all three tested doses did not stimulate the mammary glands. Rats that were treated with red clover plus 17β-estradiol were comparable to the 17β-estradiol treatment group; no obvious attenuation of alveolar budding or ductal branching resulted from the addition of red clover (data not shown).

DISCUSSION

Uterotrophic effects of red clover. Administration of red clover to ovariecotomized rats tended to dose-dependently increase uterine weight, indicating that red clover contains estrogen-like compounds. Isoflavones have shown mixed estrogenic activity, acting as agonists when 17β-estradiol is not present, but competing for the ERα and ERβ ligand binding sites when 17β-estradiol is present. The four isoflavones in red clover have been shown to competitively bind to ERα and ERβ (genistein > daidzein > biochanin A > formononetin) with affinities that are approximately one thousandth that of 17β-estradiol (5,20). The uterine weight increase was significantly lower than that of the 17β-estradiol-treated group, indicating that red clover is only weakly estrogenic at high doses. Other studies with genistein and daidzein have also demonstrated weak estrogenic effects as reflected by changes in uterine weight (21–23). However, at doses that do not increase uterine weight, compounds from red clover have been shown to improve bone density and protect against cardiovascular disease in the ovariecotomized rat model (16,17, 24–27). These data suggest that the dose of isoflavones in the diet determines the extent to which ovariecotomized rats have improved cardiovascular health, reduced bone loss and larger uteri.

Previous studies have shown that isoflavones can act as ER antagonists in the uterus (12,28,29). In contrast, red clover did not antagonize the effects of 17β-estradiol, as judged by changes in uterine weight gain in this study. These differences were probably due to variations in experimental conditions. For example, the amount of genistein present in the highest dose of red clover extract [6.375 mg/(kg · d)] in our experiment would not reach the level (1600 mg/kg) used by Folman and Pope (29), and the extract was administered by gavage rather than subcutaneously. In the study by Forh and Cline (28), primates were used rather than rats, and soy (on an energy basis of 1 and 148 mg/d per animal) was administered rather than red clover. Lastly, Tarsley et al. (12) showed that soy isoflavones (117.8 mg, isoflavones/7.53 MJ) antagonized conjugated equine estrogen found in HRT but not 17β-estradiol. Our results suggest that red clover induces dose-dependent uterine weight gain without antagonizing the effects of 17β-estradiol.

Vaginal cellular cornification. Another indication of estrogenic activity is the cornification of vaginal cells. In this study, red clover induced partial cornification of vaginal cells at the two highest doses tested [500 and 750 mg/(kg · d)]. These results are consistent with the trend for uterine weight gain in these rats. Cornification was not obvious until 2 wk after beginning treatment, which indicates that the estrogenic effects of red clover may not result from short-term consumption. Other studies conducted with animals receiving isoflavone-supplemented diets have not shown estrogenic stimulation based on the cornification of vaginal cells (12,30), possibly due to shorter treatment periods and the use of different sources of isoflavones (i.e., soy or coumestrol).

Body weight gain. Rats given the red clover diet gained significantly more weight than rats fed 17β-estradiol alone, or
Effects of red clover on the mammary gland. Mammary glands usually exhibit a pattern of ductal branching and alveolar budding in response to estrogenic compounds. We found no increased expression of these typical patterns as a result of exposure to red clover, similar to previous studies (28). In another rat study, using genistein alone at a relatively high dose (750 µg · kg⁻¹ · d⁻¹), inhibition of regression was observed (33). Therefore, the lack of ductal branching observed in the current study with red clover might be due to the dose that was used. Further analyses of the effect of the isoflavones found in red clover on breast tissue are necessary to fully define the dose capable of exerting estrogenic effects.

17β-Estradiol given subcutaneously effectively reversed ovario-ectomy-induced mammary gland regression (23). Because 17β-estradiol induced mammary gland ductal branching, we evaluated the potential of simultaneous dosing of red clover to mitigate this effect; however, no activity was observed. These data suggest that red clover had no effect on the mammary gland either as an estrogen or an antiestrogen at the three doses tested.

In this study, red clover extract affected the uterus but not the breast. One explanation for this difference might lie in the selective affinity for ERα and ERβ by red clover isoflavones (32). Estrogenic stimuli are highly complex and a variety of promoters regions upstream from estrogen responsive genes confer specificity of tissue activation (33). In vitro studies have confirmed that isoflavones tend to have a higher affinity for ERβ than for ERα (5), and tissue analyses have shown that higher amounts of ERα found in the breast and uterine relative to ERβ (34). However, red clover extract affected the uterus and not the breast. Thus, a simple interpretation of ER specificity as the key determinant of tissue-specific properties of red clover is insufficient (20). Studying isoflavones mixtures such as found in red clover might help uncover potential mechanisms whereby exogenous isoflavones confer tissue selectivity. In addition, using a standardized extract to study red clover will help eliminate questions concerning the dose of the active compounds and their mixed estrogenic behavior.

In conclusion, we have shown that red clover has in vivo estrogenic effects in the rat uterus and vaginal cells but not in the mammary gland. In addition, red clover did not produce any additive estrogenic or antiestrogenic activity when administered together with 17β-estradiol.

LITERATURE CITED


