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Effects of Phytoestrogenic Isoflavones from Red Clover (*Trifolium pratense* L.) on Experimental Osteoporosis

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The most common type of osteoporosis is bone loss associated with ovarian hormone deficiency at menopause. There is evidence that diets which contain high levels of phytoestrogenic isoflavones are associated with a low incidence of osteoporosis and menopausal symptoms. Plant extracts, which contain high levels of isoflavones, such as Red clover (*Trifolium pratense* L.), have been used to reduce menopausal symptoms.

The objective of this study was to evaluate the preventive effects of Red clover total isoflavones on the progression of bone loss induced by estrogens deficiency (ovariectomy) in rats. Bilateral ovariectomy was performed on female Wistar rats. One week after the operation the rats were treated with an oral dose of 20 and 40 mg of total isoflavones daily for 14 weeks. The results from this study showed that the ovariectomy reduced bone mineral content, femoral weight, femoral density, mechanical strength of the tibia and increased the levels of bone specific alkaline phosphatase in the serum and the number of osteoclasts in the femur sections compared with sham operated controls. Treatment with isoflavones significantly increased bone mineral content, mechanical strength of the tibia, femoral weight, femoral density and prevented the rise of serum alkaline phosphatase levels. In addition, the treatment with isoflavones significantly reduced the number of osteoclasts compared with the ovariectomized control rats. These findings suggest that Red clover isoflavones are effective in reducing bone loss induced by ovariectomy, probably by reducing of the bone turnover via inhibition of bone resorption. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords: Trifolium pratense L.; Fabaceae; isoflavone phytoestrogens; osteoporosis; menopause.

INTRODUCTION

Osteoporosis is a group of syndromes characterized by a net loss of bone mass. The decline in estrogens in postmenopausal women is at least in part responsible for the increase in bone fragility and thereby in the incidence of skeletal fractures. Epidemiological data show that a diet rich in phytoestrogenic isoflavones is associated with a low incidence of menopausal symptoms, osteoporosis, dementia from Alzheimer's disease, cardiovascular disease and cancer in Oriental women (Kurzer and Xu, 1997). Since the side-effects of estrogen replacement therapy include a slight but significant increase in the risk of developing breast and endometrial cancer (Colditz *et al.*, 1995), women are increasingly using herbal remedies as an alternative therapy (Murkies *et al.*, 1998). Plant extracts such as Red clover (*Trifolium pratense* L.) which contain high levels of isoflavones (genistein, daidzein, biochanin A and formononetin) (Fig. 1) have been used in Western countries to reduce menopausal symptoms (Stolze, 1982; Tham *et al.*, 1998). Genistein and daidzein are structurally similar to tamoxifen, an agent that acts similarly to estrogen in reducing postmenopausal bone loss (Arjmandi *et al.*, 1998). However, the biological effects of red clover isoflavones have not been clarified fully. In addition, the results from *in vitro* and *in vivo* studies and the dosage used, are not consistent. Nevertheless, phytoestrogens, including Red clover isoflavones, have received considerable attention due to their possible effects as endocrine disrupters. Genistein has been shown to have an anabolic effect on bone formation and mineralization in cultured bone cells and an inhibitory effect on bone resorption in femoral metaphyseal tissues obtained from elderly rats *in vitro* (Yamaguchi and Gao, 1998; Yamaguchi *et al.*, 2000; Gao and Yamaguchi, 1999). Ishida *et al.* (1998) reported that ovariectomized (OVX) animals had uterine atrophy which could be prevented by the administration of estrogen but not by genistein and daidzein at a dose of 50 mg/kg/day. Ishimi *et al.* (1999) also reported that genistein prevents bone loss caused by estrogen deficiency without estrogenic action in the uterus. Santell *et al.* (1997) reported that the administration of large amounts of genistein caused uterine hypertrophy in immature rats. Moreover, the ovariectomized rat is the most appropriate model for studying the efficacy of different drugs to prevent bone loss (Wronski and Yen, 1991; Miller, 1997). There are several similarities between the adult rat model of ovarian hormone deficiency bone loss and postmenopausal osteoporosis. Bone remodeling involving a phase of activation, osteoclastic resorption, and a phase of

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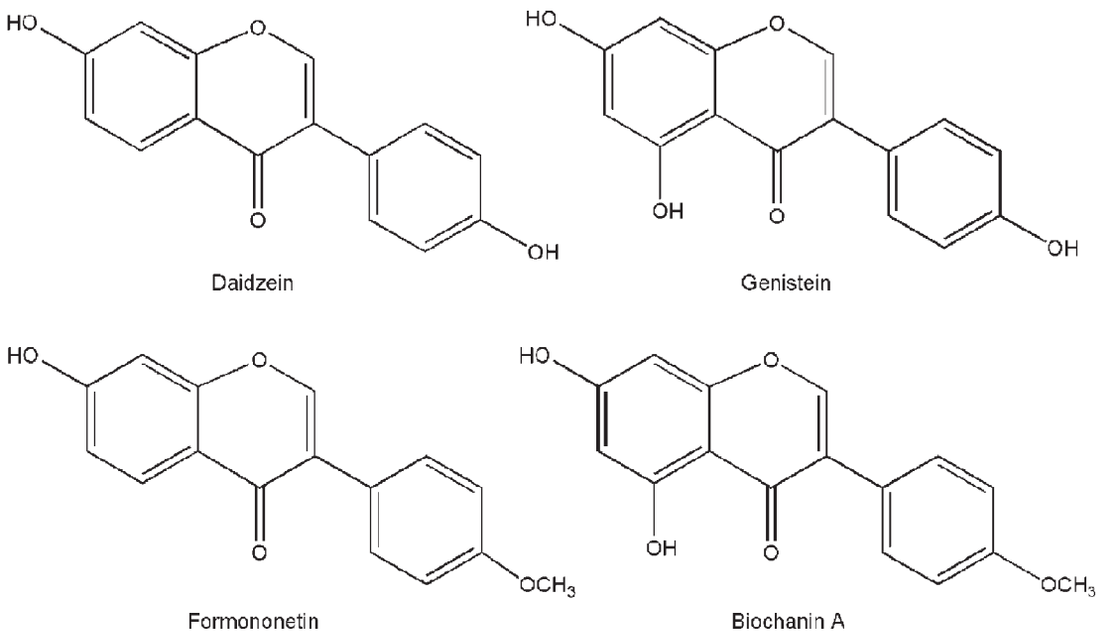

Figure 1. Chemical structure of the four isoflavones from Red clover extract.

osteoblastic bone formation occurs in trabecular bone in rats (Baron *et al.*, 1984) as well as in humans (Frost, 1964). In rats, bone loss from estrogen deficiency results from at least two defects: a decrease in intestinal absorption of calcium and a net increase in bone resorption (Kalu *et al.*, 1989). Ovariectomy in rats is thus followed by an increase in bone turnover (with resorption exceeding formation) associated with bone mass loss at several sites rich in trabecular bone and in a decrease in bone density, mineralization and strength (Einhorn, 1992). Similarly, in women the bone loss immediately after menopause is associated with high levels of biochemical markers of bone turnover such as alkaline phosphatase (Garn, 1975; Riis *et al.*, 1986).

The present study was therefore designed to evaluate the preventive effect of total isoflavones from Red clover on the rapid bone loss occurring after ovariectomy in female rats.

MATERIALS AND METHODS

Plant material. The Red clover extract was standardized to a minimum of 11% isoflavone content by weight (11 g of total isoflavones per 100 g of total extract) of four isoflavones: genistein, 0.18%; daidzein, 0.62%; biochanin A, 2.62% and formononetin, 7.53% (present as hydrolysed aglycones). Red clover extract was supplied by Named s.r.l., Sesmo (MI). Our phytochemical analyses carried out on the commercial extract of Red clover by TLC and HPLC confirmed the presence and the relative amounts of isoflavones.

Animals. Female Sprague-Dawley rats 90 days of age were allowed a few weeks to acclimatize to housing conditions before being used for the study. On arrival at our institution the rats were housed in standard laboratory conditions at a temperature of 22 ± 3 °C, relative humidity 50%–55% and a 12 h light/dark cycle. Drinking water and a nutritionally balanced synthetic pelleted diet were supplied *ad libitum*. The food intake per day of all animals was 20 g throughout the study period. The reason for pair-feeding the rats and carefully monitoring their food intake was to prevent postovariectomy hyperphagia that would complicate interpretation of the effects of ovarian hormone deficiency on bone and calcium metabolism. Animal care, environmental conditions and use followed the EC Council guidelines. The experimental procedures were approved by the Bioethical Committee of the Italian National Health Institute.

Experimental design. Twenty rats were selected and randomized into four groups of five animals each. The rats from all groups were bilaterally ovariectomized except for the rats in group I, that served as sham operated controls (Sham). Ovariectomy was performed under general anaesthesia with diethyl ether, by ligation and excision of the ovaries along the upper horns, via (Edgren and Calhoun, 1957) aseptic incisions of the dorsal skin and muscle layers. Rats from group II received the vehicle and served as ovariectomized controls (OVX). Groups III and IV were treated daily with an oral dose of 20 and 40 mg, respectively, of total isoflavones (ISO) from Red clover. The doses of isoflavones used were based on typical clinical doses for humans. Treatment of the respective groups commenced 7 days after the ovariectomy and continued for 14 weeks. The body weight and the vaginal smear of all animals were examined at the beginning and at weekly intervals throughout the 14 week experimental period. At the end of the study period, the blood samples of all rats were collected and serum samples were obtained in order to estimate bone specific alkaline phosphatase, a marker of bone formation (Horn, 1972). All the animals were killed under anaesthesia and necropsied. The right and left femora along with tibias were dissected out. The left femurs were thawed, autoclaved for 15 min at 110 °C for the measurement of thickness, weight, length, volume, density and bone mineral content by determining total ash weight and calcium content. The cleaned

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right femurs were immediately fixed in 10% neutral buffered formalin for histological examination. The right tibias were carefully separated from the femurs and stored in 50% saline–ethanol to determine the mechanical properties of the bone using a traction test apparatus.

Physical and biochemical parameters measured. The thickness and the length of the left femur were measured with calipers. They were then dried in an evacuated oven at 110 °C for 48 h and weighed. Femur bone volume was measured using the plethysmometric method (Archimedes' principle) and bone density (g/mL bone vol) was calculated. For the bone mineral content estimation, the femurs were ashed in a muffle furnace at 700 °C for 8 h until the ash reached constant weight and their calcium content was determined by atomic absorption spectrophotometry after extraction with 3 M HCl. In order to measure the mechanical strength of the tibia, tests of simple traction were carried out at a constant speed using traction test apparatus. The tibias were pulled at the two extremities until they broke and the estimated breaking strength was subsequently calculated in proximity of the breakage. Serum alkaline phosphatase was measured using the colorimetric method with a kit from Sigma Chemical Co.

Bone histology. After fixation in 10% neutral buffered formalin for 24 h at 4 °C, the right femurs were decalcified in 5% ethylenediamine tetraacetic acid for 7 days, embedded in paraffin and cut into longitudinal sections of 5 μm thickness. The sections were stained with hematoxylin–eosin and tartrate-resistant acid phosphatase, a cytochemical marker for osteoclasts and finally counterstained with hematoxylin (Bancroft and Cook, 1998; Drury, 1980). Osteoclasts were identified as multinucleated cells. The number of positively stained osteoclasts in the sections of the median portion of the whole femora was recorded for the four groups.

Evaluation of estrogenic activity. Estrogenic activity was evaluated using vaginal cytology. Weekly vaginal smears were taken using an eye dropper containing saline, placed on slides and observed under a light microscope after staining with hematoxylin–eosin in order to monitor cellular differentiation. Cells were identified as either leukocytes (indicating a diestrous stage), or nucleated or cornified epithelial cells (indicating an estrous stage).

Statistical analysis. The results were expressed as the mean with the standard error. One-way ANOVA was first performed to test for any significant difference between the groups. When significant ($p < 0.05$), a post test, Dunnett's multiple comparison test was applied to determine the specific difference between the groups. The Student's *t*-test was used when only two experimental groups were compared. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Effect of isoflavones on body weight

Table 1 shows the effect of isoflavones on the body weight of the animals. All the experimental groups showed a progressive increase in body weight during the 14 week experimental period. But no significant difference was observed between the Sham (group I), the OVX (group II) and the isoflavone treated groups (III and IV).

Effect of isoflavones on physical parameters of the femur

The effect of isoflavones on femoral thickness, length, dry weight, volume, density, total bone mineral content and calcium content are presented in Table 2. The results show that there were significant differences between the groups in some of the parameters examined.

Table 1. Effect of Red clover isoflavones on body weight changes

Group	Initial body weight (g)	Week 4 (g)	Week 8 (g)	Week 12 (g)	Week 14 (g)
Sham	205 ± 5.00	230 ± 4.20	242 ± 5.50	250 ± 4.10	265 ± 7.30
OVX	200 ± 4.40	235 ± 6.00	238 ± 4.85	245 ± 5.25	250 ± 5.15
ISO 20 mg	208 ± 5.10	238 ± 5.70	240 ± 6.70	250 ± 5.60	258 ± 5.40
ISO 40 mg	198 ± 4.85	230 ± 6.40	235 ± 6.20	247 ± 4.15	262 ± 4.25

All values are expressed as mean ± SEM.

Table 2. Effect of Red clover isoflavones on physical parameters of the femur

Parameter	Sham	OVX	ISO 20 mg	ISO 40 mg
Thickness (mm)	5.70 ± 0.15	5.65 ± 0.12	5.75 ± 0.10	5.72 ± 0.18
Length (mm)	27.55 ± 0.24	27.35 ± 0.25	27.60 ± 0.18	27.45 ± 0.16
Weight (g)	0.58 ± 0.015	0.52 ± 0.020[a]	0.56 ± 0.010[b]	0.57 ± 0.012[b]
Volume (mL)	0.40 ± 0.011	0.41 ± 0.013	0.39 ± 0.012	0.40 ± 0.010
Density (g/mL)	1.45 ± 0.018	1.26 ± 0.015[a]	1.43 ± 0.014[b]	1.42 ± 0.012[b]
Total ash (g)	0.35 ± 0.010	0.32 ± 0.016[a]	0.33 ± 0.018	0.34 ± 0.011[b]
Calcium content (mg/g)	198.20 ± 5.30	181.40 ± 3.70[a]	193.45 ± 4.65[b]	195.38 ± 3.50[b]

All values are expressed as mean ± SEM.
[a] $p < 0.01$ compared with Sham group.
[b] $p < 0.01$ compared with OVX group.

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Table 3. Effect of Red clover isoflavones on mechanical strength of the tibia

Group	Breaking strength (N)	Breaking section (mm)
Sham	90.00 ± 0.50	3.90 ± 0.18
OVX	73.00 ± 0.85[a]	3.60 ± 0.25
ISO 20 mg	90.10 ± 0.40[b]	3.00 ± 0.15
ISO 40 mg	88.00 ± 0.75[b]	3.90 ± 0.20

All values are expressed as mean ± SEM.
[a] $p < 0.05$ compared with Sham group.
[b] $p < 0.01$ compared with OVX group.

Ovariectomy (group II) caused a significant decrease in femoral weight, density, ash content and calcium content without a change in femoral thickness and volume compared with the Sham control animals (group I). Treatment with an oral dose of 20 and 40 mg of total isoflavones from Red clover daily for 14 weeks in groups III and IV significantly increased femoral weight, density and bone mineral content compared with ovariectomized control rats (group II).

Effect of isoflavones on bone specific phosphatase alkaline

Ovariectomized control rats (group II) showed significantly increased levels of serum alkaline phosphatase (154.54 ± 3.22) compared with Sham operated controls (117.58 ± 2.85) (group I). Increased serum alkaline phosphatase levels was significantly prevented in the groups treated with isoflavones (III and IV) compared with OVX controls (Fig. 2). No significant difference was observed between the Sham and ISO treated groups.

Effect of isoflavones on mechanical strength of the tibia

The results of the traction test (Table 3) indicate that the breaking strength of the tibia was lower in the OVX control group (73.00 ± 0.85 N) compared with the Sham group (90.00 ± 0.50 N). Treatment with isoflavones greatly enhanced the biomechanical properties, as evident from the increase of breaking strength in groups III and IV (ISO 20 mg 90.10 ± 0.40 N; ISO 40 mg 88.00 ± 0.75 N) compared with the OVX controls.

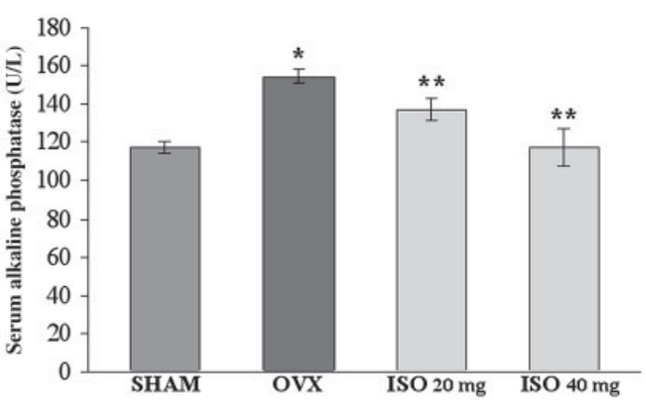

Figure 2. Effect of isoflavones (ISO) on serum phosphatase alkaline. * $p < 0.05$ compared with Sham control. ** $p < 0.05$ compared with OVX control.

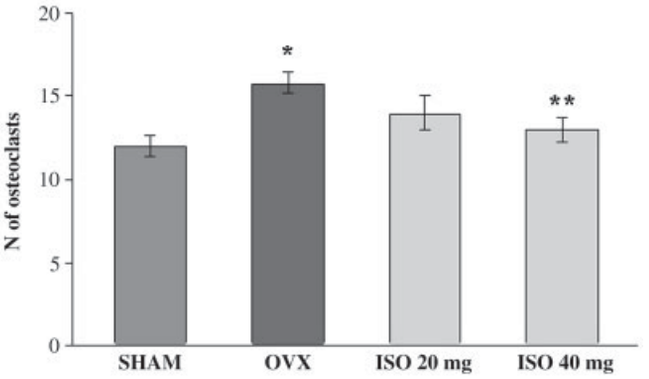

Figure 3. Effect of isoflavones (ISO) on number of osteoclasts in the sections of the median portion of whole femora in rats. ($n = 4$). * $p < 0.05$ compared with Sham operated control. ** $p < 0.05$ compared with OVX control.

Histological examination

The histological examination of the femur sections in the region proximal to the epiphyseal growth plate showed an increased number of positively stained osteoclasts in the OVX control rats compared with the Sham control rats. In the isoflavone treated groups (III and IV) the number of red stained positive osteoclasts was lower than the OVX group (Fig. 3).

Effects on vaginal cytology

The vaginal smears of ovariectomized control rats (100%) showed a typical vaginal cytology caused by hypoestrogenism with permanently undifferentiated small cells compared with Sham rats. The administration of Red clover isoflavones to ovariectomized rats at both the doses used resulted in a significant increase in vaginal cell differentiation after 1 week. Vaginal cells exhibited a distinct pattern of maturation in response to isoflavone extract, beginning as a population of leukocytes, advancing to neucleocytes and terminating as fully cornified cells. All treated animals (100%) showed complete estrogenic cornification in the vaginal epithelium within 4 weeks. In earlier studies, it was observed that the OVX rats treated with 17-β estradiol (0.1 μg/rat/day s.c.) showed full vaginal cornification within 8 days (Circosta *et al.*, 2006).

DISCUSSION

Menopause involves a dramatic decrease in the production of estrogens and progesterone and tends to accelerate bone aging, just as it accelerates skin and vascular aging. Estrogen-deficiency in postmenopausal women is at least in part responsible for the decrease in bone mass and the increase in the incidence of fractures. In the present study, ovariectomized rats developed bone changes similar to those seen in osteoporotic women as indicated by a decrease in femur weight, density, bone mineral content, mechanical strength of

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the tibia and by an increase of bone specific alkaline phosphatase and of the number of osteoclasts in the femur sections. Treatment with isoflavones significantly increased bone mineral content, biomechanical strength of the tibia, femoral weight, femoral density and prevented the rise of serum alkaline phosphatase levels compared with ovariectomized control rats. These results suggest that Red clover isoflavones are effective in reducing bone loss induced by ovariectomy, probably due to their inhibition of bone resorption as evidenced by the lower number of osteoclasts compared with OVX controls.

Red clover has recently been used due to its high content of estrogenic isoflavones and this plant is hypothesized to be of potential use in menopause as a natural form of hormone replacement therapy. Red clover extracts showed consistent estrogenic effects in different *in vivo* and *in vitro* assays. It is reported that Red clover extract increases uterine weight and vaginal cell cornification in ovariectomized rats. Genistein, daidzein, biochanin A and formononetin have been implicated as the causes of the estrogenic activity of Red clover and have been shown to competitively bind to ER_α and ER_β with affinities that are approximately one thousandth that of 17-β estradiol. Genistein probably plays the most important role in terms of the estrogenic activity of Red clover followed by daidzein and biochanin A (Liu *et al.*, 2001; Burdette *et al.*, 2002). According to our results, isoflavone extract from Red clover was able to prevent ovariectomy-induced bone loss by manifesting estrogenic action in the vaginal epithelium of ovariectomized rats at a dose of 20 and 40 mg/day. In addition, these findings confirm the observations of other investigators that bone loss in ovariectomized rats is prevented by estrogen administration, and that estrogen can also suppress the OVX-induced increase in biochemical markers of bone turnover such as serum phosphatase alkaline. In fact, the bone-conserving effects of estrogen are well established in the OVX rat model of osteoporosis. Several studies have shown that 17-β estradiol (used as a positive control) is able to prevent the decrease of bone density, bone ash and calcium content, bone and uterine weights induced by ovariectomy in rats (Lee *et al.*, 2004).

In conclusion, this study demonstrates that isoflavone extract from Red clover may be effective in maintaining the bone mass of OVX rats when administered at optimal dosages.

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