Effect of Ethanol Extract of Safflower Seed on Bone Loss in Ovariectomized Rat

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Abstract We investigated whether ethanol extracts of the safflower seeds containing phenolic compounds were responsible for the bone-protecting effects. Crude ethanol extract (CEE) of the safflower seeds was fed for 4 weeks at the level of 1% in diet to female Sprague-Dawley rats that had been subjected to bilateral ovariectomy (OVX). The CEE effects (OVX+CEE) were evaluated by comparing results obtained from OVX, Sham, and OVX injected with 17β-estradiol (OVX+E$_2$) groups. OVX resulted in a dramatic reduction in the trabecular bone mass of the proximal tibia (approximately 40% of the Sham group) and an increase in fat deposition in bone marrow. In OVX+E$_2$ group, the bone loss was completely prevented as well as marrow adiposity. In OVX+CEE group, approximately 80% of the bone mass was maintained compared with Sham group and fat deposition in the bone marrow was prevented. Meanwhile, the partially purified ethanol extract containing the phenolic compounds stimulated proliferation of the ROS 17/2.8 osteoblast-like cells in a dose-dependent manner, as potently as positive controls of E$_2$ and genistein. The present data demonstrate that the ethanol extracts of safflower seeds reduced bone loss caused by estrogen deficiency. The bone-protecting effect of safflower seeds seems to be mediated, at least partly, by the stimulating effect of the phenolic compounds on the growth of osteoblasts.

Keywords: safflower (*Carthamus tinctorius* L.) seed extract, phenolic compound, bone loss, osteoblast

Introduction

Estrogen deficiency in postmenopausal women has been recognized as one of the major factor of osteoporosis, cardiovascular disease, and dementia (1). Of these, osteoporosis is the most common disease, and hormone replacement therapy (HRT) is very effective in the prevention of the bone loss, and decreases the incidence of bone fracture over 50% (2). However, the use of HRT in postmenopausal women has come into question due to the possible risks of breast and endometrial cancers with long-term use (3, 4). Consequently, alternatives to conventional HRT, such as synthetic and natural selective estrogen receptor modulators (SERM), have attracted considerable attention (5, 6). SERMs bind to estrogen receptors and exert estrogenic action on bone and the cardiovasecular system, but have antiestrogenic action on the breasts and uterus (7, 8).

Recently, synthetic SERMs such as raloxifen and ipriflavone have been widely used clinically (9). In addition, potential health benefits of phytoestrogens, natural SERMs, have been suggested (10, 11). All phytoestrogens are phenolic compounds and are found in a wide variety of plant products (12). Their main classes are isoflavones, lignans, and coumestans (13). Soy beans and soy foods are the most significant dietary sources of isoflavones, and are effective in reducing bone loss in experimental animals (14-17) as well as in postmenopausal women (18-20). Besides soy isoflavones, limited data are available on the bone-protecting effects of phytoestrogens isolated from other plants.

Safflower (*Cathamus tinctorius* L.) seed has long been used clinically in Korea as an herbal medicine to promote bone formation, but its effects have not been scientifically proven. Recent reports, however, have suggested that safflower seed powder or extract can promote recovery from bone fractures and stimulate osteoblast differentiation in rats (21-24); it also increased bone density in three osteoporotic patients (25). Recently, Bae *et al.* (26) and Kim *et al.* (27) isolated and identified phenolic compounds from the safflower seeds, and found that safflower seeds are rich sources of not only phytoestrogen-like flavones and lignans, but also serotonin derivatives. Our previous study showed that defatted safflower seeds markedly attenuated bone loss in ovariectomized rats (28).

In the present study, we investigated whether crude ethanol extract (CEE) of defatted safflower seeds rich in phenolic compounds reduce bone loss in ovariectomized rats. In addition, the CEE was further fractionated with ethylacetate and then purified by Diaion HP-20 column chromatography to obtain a partially purified ethanol extract (PPEE) of safflower seed. The growth promoting effects of the PPEE were also examined in bone forming osteoblasts.

Materials and Methods

Preparation of crude ethanol and partially purified ethanol extracts from safflower seed Defatted safflower seed powder was prepared by eliminating the lipids of

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roasted seeds via an *n*-hexane treatment, and grinding to 30 mesh. The seed powder was extracted twice with 80% hot ethanol (EtOH), and the resultant extract was filtered and evaporated under reduced pressure to obtain CEE yielding approximately 10% of the seed powder. CEE was dispersed with water and further partitioned with ethylacetate. The ethylacetate fractions were collected, dehydrated with anhydrous sodium sulphate, filtered, and concentrated under vacuum to obtain an ethylacetate (EtOAc) extract yielding approxi-mately 1% of the safflower seed powder. The EtOAC fraction was further suspended in 40% aq. EtOH and subjected onto a Diaion HP-20 column. The column was washed with 40% EtOH to remove lower molecular phenolic acids and color pigments, and then eluted with 80% EtOH to yield PPEE yielding approximately 0.2% of the seed powder.

Animals and diets Female Sprague-Dawley rats were obtained from the Korean Center for Experimental Animals and maintained with standard chow in a facility equipped with stainless steel cages having wire mesh bottoms and constant temperature (25±1°C) and lighting (light on, 08:00-20:00 hr) for a week. Thirty rats weighing approximately 200 g were bilaterally ovariectomized and 10 rats were sham operated under anesthesia with pento-barbital (40 mg/kg, ip) and urethane (230 mg/mL). After the surgery, ovariectomized rats were then randomly allocated to 3 groups (each n=10) ovariectomized control (OVX), safflower seed extract (OVX+CEE), and 17-β-estradiol (OVX+E₂) treated groups.

All 4 groups of rats including the Sham group were fed experimental diets (Table 1) prepared according to AIN-76 composition (29) and had free access to tap water for 4 weeks. In the experimental diet, the CEE of defatted safflower seed powder added to diets at a level of 1%. The E₂ group was subcutaneously injected with 17β-estradiol (Sigma Chemical Co., St. Louis, MO, USA) dissolved in

soybean oil (50 µg/0.1 mL) twice per week. Food intake was measured daily and body weight every 3 days. All animal procedures were reviewed and approved by the Catholic University of Daegu Intramural Animal Care and Use Committee.

Scanning electron microscopy (SEM) To evaluate three dimensional changes in the amount and structure of the trabecular bone, the right tibiae of three rats in each experimental group were processed for SEM. The proximal tibiae were trimmed in the frontal plane to expose the epiphyseal and metaphyseal trabecular bone and treated with 5% sodium hypochlorite to dissolve organic material. They were then dehydrated in a graded series of ethanols. The critical point was dried, coated with platinum gold, and examined with a scanning electron microscope (Hitachi S 2300; Hitachi Ltd., Tokyo, Japan) at 20 kV.

Tissue preparation and histomorphometry For the histomorphometrical analyses of trabecular bone amount and adipocyte content, the proximal portion of right tibiae of 7 rats in each experimental group was removed, fixed in 10% buffered formalin for 3 days, and demineralized in 8 % formic acid for 1 week. The specimens were dehydrated in a graded series of alcohols and embedded in paraffin. Longitudinal sections of 5 µm thickness were cut and stained with hematoxylin and eosin.

Two sections 50 µm apart from each other and showing full thickness cortex and intact marrow space, were chosen in each specimen. Measurement was performed in the area (1.5-2.0 mm²/section) of the metaphysis with exclusion of the areas 1 mm from the epiphyseal plate, as well as one cortical thickness from the endocortical surfaces to exclude the primary spongiosa. The parameters were measured on a digitizing tablet at 100× magnification using a light microscope connected to a Macintosh personal computer with NIH image software (public domain, NIH, Bethesda, MD, USA). The measured parameters included 1) trabecular bone volume, percentage of trabecular bone area in the tissue space of secondary spongiosa, and 2) adipocyte area, percentage of the adipocyte area to tissue area.

Table 1. Diet compositions and estrogen treatments for experimental groups

Ingredident (g/kg) \ Group	Sham	OVX	OVX+E₂	OVX+CEE
Casein	150	150	150	150
Corn starch	485	485	485	475
Sucrose	150	150	150	150
Lard	40	40	40	40
Soybean oil	75	75	75	75
Mineral mix[1]	40	40	40	40
Vitamin mix[2]	10	10	10	10
Cellulose	50	50	50	50
CEE[3]	0	0	0	10
E2 treatment[4]	-	-	+	-

[1] AIN-76 mineral mix.
[2] AIN-76 vitamin mix.
[3] Crude ethanol extract of defatted safflower seeds.
[4] 50 µg of 17 β-estradiol in soybean oil was injected subcutaneously twice a week for 4 weeks.

Cell culture and MTT assay ROS 17/2.8 osteoblast-like cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in Dulbecco's Modified Eagles Medium (DMEM) (Gibco BRL, Gland Island, NY, USA) containing 10% FBS (HyClone Lab Inc., Logan, UT, USA) and 1% antibiotic-antimycotic (Gibco BRL). Cell growth was assessed by MTT assay, which stain viable cells with 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (30).

ROS 17/2.8 cells were seeded at a density of 4×10³/well in 96-well plates. Sub-confluent cells were growth arrested to reach a quiescent state for 24 hr in 0.5% FBS. Then, cells were treated with 5-100 µg/mL of the safflower seed PPEE, or with positive controls such as genistein, a soy isoflavone, and E₂ in DMEM containing 2.5% FBS. After 48 hr, 50 µL of MTT solution (1.1 mg/mL) was added to each well and then incubated for an additional 4 hr. After centrifugation, the supernatant was removed from each

well. The colored formazan crystal produced from MTT was dissolved in 150 µL of DMSO and then the optical density value was measured at 540 nm by a plate reader (Multiscan@ MCC/340, Miami, FL, USA).

Statistical analysis All data were represented as mean±SE. The statistical significance of the differences in the *in vivo* experiment was assessed using one-way analysis of variance (ANOVA). When significant differences were indicated by ANOVA, group means were compared using Duncan's multiple range tests. The significance of the differences in the proliferative responses of osteoblastic cells to different doses of each compound was determined using Student's *t*-test. A probability of less than 5% was required for significance.

Results and Discussion

Body and uterine weight Food intake and body weight changes during the 4 weeks of the experimental period are shown in Table 2. Initial body weight was similar among the four groups, but the OVX rats fed with or without CEE ingested more food and gained more weight than the sham-operated rats. However, OVX rats treated with E_2 showed similar food intake and weight gain to the Sham group. Food efficiency ratios were not significantly different among the four groups, although those of the OVX and OVX+CEE groups appeared higher than the other two groups. Therefore, more greater weight gains in the OVX and OVX+CEE groups were mainly due to higher food intakes. Ovariectomy produced a marked atrophy of the uterus, and feeding CEE of safflower seeds did not affect uterine weight in the ovariectomized rats. However, E_2 treatment to the ovariectomized rats increased uterine weight similar to the Sham level.

The present results demonstrate different effects of CEE and E_2 on food intake and the uterus. E_2 treatment in the OVX rats decreased food intake, but increased uterine weight, whereas CEE affected neither food intake nor the uterine weight of OVX rats. Estrogen has been known to decrease body weight gain by affecting regulation of food intake and by suppressing activity of lipoprotein lipase activity (31) and the accumulation of fat in adipose tissue (32). The uterotrophic activity of estrogen may increase the risk of endometrial cancer. In contrast, no uterotrophic activity of CEE corresponds to the reported beneficial effect of phytoestrogens in lowering the risk of endometrial cancer. Isoflavone, a soy phytoestrogen, seems to prevent tumor growth by binding estrogen receptors on the tumor and hence competitively inhibiting estrogen uptake (33).

Histromorphometry of proximal tibia Morphology of the proximal tibia was examined both by scanning electron micrographs and H-E staining and is showed in Fig. 1 and 2, respectively. The Sham-operated group (A) showed dense and well-formed trabecular bone filling the metaphyseal space of proximal tibiae. OVX (B) caused dramatic changes in the structure and amount of secondary spongiosa area of metaphysis compared with the Sham group. In contrast, OVX+CEE group (C) showed significantly reduced bone loss in the secondary spongiosa compared to the OVX group. E_2 (D) produced a greater bone-protecting effect than CEE, as well as structural changes of the trabecular bones consisting of rod- and plate-like structures with numerous connections.

The histomorphometrical parameters were analyzed with photomicrographs of the proximal tibia metaphysis and are presented in Fig 3. Ovariectomy markedly decreased trabecular bone volume (A), but increased adipose tissue content (B) in bone marrow, 41 and 162% of the Sham levels, respectively. A 4-week feeding of CEE in OVX rats significantly prevented trabecular bone loss by restoring bone mass up to 81% of the sham level, and maintained almost the same level of fat deposition as the Sham group. E_2 treatment increased bone volume to 115 %, but decreased the marrow adipose content to 72% of the Sham level.

Fat deposition in bone marrow has been reported to be increased in conditions that lead to bone loss, such as ovariectomy (34, 35), treatment with glucocorticoids (36) or immobilization (37). In the present study, feeding CEE almost completely prevented an increase in fat deposition in bone marrow after OVX. Both osteocytes and adipocytes are derived from common progenitor cells in bone marrow (38), and an *in vitro* study has shown an inverse relationship between the differentiation of osteogenic and adipocytic cells (39). Recently, Dang *et al.* (40) reported that E_2 directly stimulated the differentiation of progenitor cells into osteoblasts and concurrently inhibited adipocyte formation in an estrogen receptor-dependent way. Whether phenolic compounds in safflower seeds also affect the differentiation of bone marrow stromal cells requires further clarification.

Table 2. Body and uterus weights, and food efficiency ratios (FER) of Sham and ovariectomized rats administered with and without CEE of defatted safflower seed[1]

Group	Initial body wt (g)	Body wt gain (g)	Food intake (g/day)	Food efficiency ratio	Uterus wt (mg/100 g)
Sham	198.5±9.4[3)NS]	70.8±1.5[b2)]	15.7±1.3[b]	0.16±0.06	150±5.2[a]
OVX	198.7±5.8	105.7±5.2[a]	19.8±1.2[a]	0.21±0.03	83±6.4[b]
OVX+CEE	204.3±10.2	98.1±9.1[a]	18.3±1.4[a]	0.18±0.08	62±4.3[b]
OVX+E₂	195.5±7.4	64.0±2.6[c]	15.2±1.9[b]	0.15±0.03	154±5.7[a]

[1)]Values are mean±SE (*n*=10).
[2)]Values in the same column not sharing common superscript letters are significantly different at *p*<0.05 by Duncan's test.
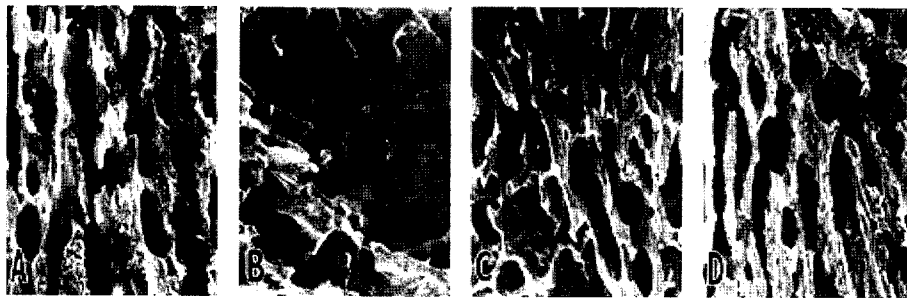[3)]Not significant.

Fig. 1. Scanning electron micrographs of the proximal tibial hemisections from Sham operated rats (A), ovariectomized rats (OVX) untreated (B), or treated with CEE from defatted safflower seeds (OVX+CEE) (C), and 17β-estradiol (OVX+E₂) (D) for 4 weeks.
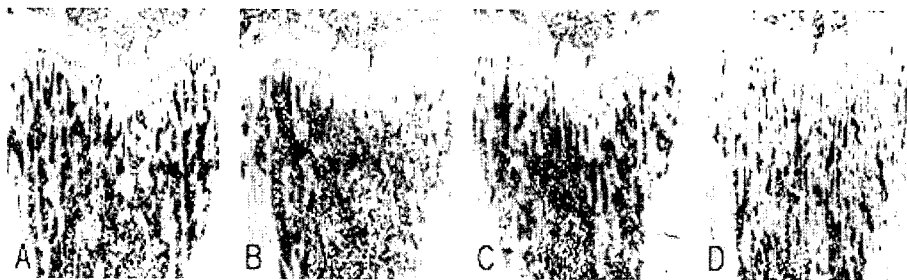

Fig. 2. Longitudinal sections of the proximal tibia from rats that were Sham-operated (A), ovariectomized rats (OVX) untreated (B), or treated with CEE from defatted safflower seeds (OVX+CEE) (C), and 17β-estradiol (OVX+E₂) (D) for 4 weeks. Hematoxylin-eosin stain. Original magnification, 20×.
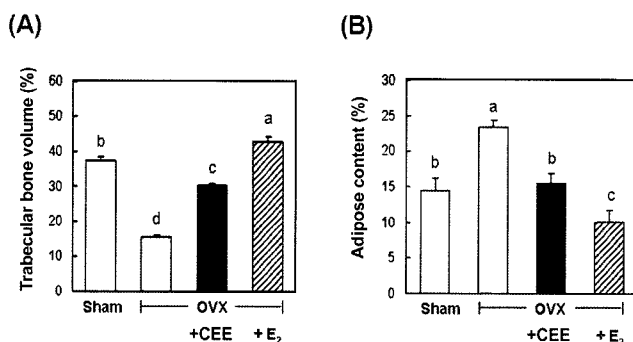

Fig. 3. Histomorphometrical analysis of trabecular bone density (A) and adipocyte density (B) in the proximal metaphysis of the tibia of Sham-operated and ovariectomized rats untreated (OVX) or treated with CEE from defatted safflower seeds (OVX+CEE) and 17β-estradiol (OVX+ E₂) for 4 weeks. Values represent mean±SE of 10 rats in each group. Different superscript letters show significant differences at $p < 0.05$ by Duncan's test.
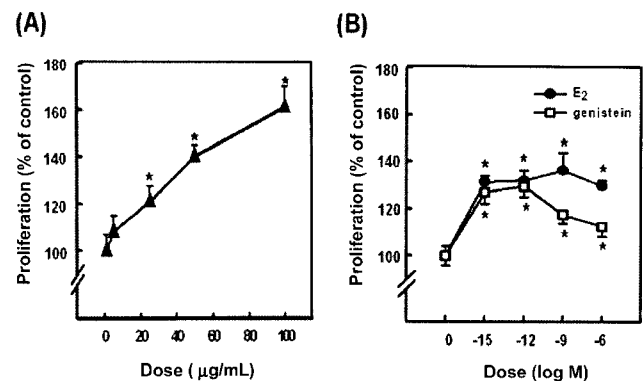

Fig. 4. Dose-dependent effects of the partially PPEE obtained from defatted ethanol extract of safflower seeds (A), and positive controls (B) of 17β-estradiol (E₂) and genistein on proliferation of ROS 17/2.8 osteoblast-like cells. Results are expressed as percentage of the control and values represent mean±SE from 6 separate experiments.

Effect of PPEE of safflower seed on growth of osteoblasts In an attempt to investigate underlying mechanisms, the effects of PPEE were assessed in ROS 17/2.8 osteoblastic cells. PPEE stimulated growth of the osteoblastic cells in a dose-dependent manner (5-100 µg/mL) as shown in Fig. 4A. When genistein, a soy isoflavone, and E₂ were tested to compare the effect of PPEE, they also increased proliferation of the osteoblastic cells in a dose-dependent fashion (Fig. 4B), as reported previously (41, 42).

The present results suggest that the beneficial effect of

CEE on bone loss may be mediated, at least in part, by stimulating proliferation of osteoblasts. We previously isolated and quantified three different types of phenolic compounds in CEE and PPEE of defatted safflower seed (26, 27). CEE contained approximately 6.10% (dry base of extract) four serotonin derivatives (*N*-feruloylserotonin, *N*-feruloyl-serotonin 5-glucoside, *N*-(*p*-coumaroyl)serotonin, and *N*-(*p*-coumaroyl)serotonin 5-glucoside), 5.01% 4 flavonoids (luteolin, luteolin 7-glucoside, acacetin, and acacetin 7-glucuronide), and 2.22% 4 lignans (matairesinol, matairesinol 4'-glucoside, 8'-hydroxyarctigenin, and 8'-hydroxyarctigenin

4'-glucoside), whereas PPEE contained approximately 22.85% serotonin derivatives, 19.62% flavonoids, and 10.73% lignans. Since the PPEE contain the phenolic compounds about five-fold greater than the CEE, and more than half of PPEE is composed of various phenolic compounds, the growth promoting effect of CEE seems to be due to phenolic compounds. Among the phenolic compounds in PPEE, flavonoids and lignans are classified as phytoestrogen-like compounds, but not serotonin derivatives. Soy isoflavones have been known to prevent bone loss in ovariectomized rats (16, 17) and postmenopausal women (18-20). However, there are limited data available on the bone-protective effects of lignans (43, 44) and serotonin derivatives. Serotonin derivatives, the most abundant phenolic compounds in safflower seeds, are not classified as a phytoestrogen, but instead serotonin is well known as an important neurotransmitter. Recently, Gustafsson *et al.* (45) reported that long-term administration of serotonin increased bone mineral density in rats. To investigate possible bone-protecting effect of each phenolic compound in safflower seeds, further *in vivo* and *in vitro* experiments are required.

In the process of osteoporosis, activated osteoclasts increase bone resorption (46). Estrogen and various SERMs may reduce bone loss by suppressing bone resorption and stimulating bone formation (47). Many studies have reported osteoblastic cell growth-promoting effects of phytoestrogens such as genistein (28), coumestrol (48), as well as those in synthetic SERMs such as ipriflavon (49) and raloxifen (46). However, further studies are necessary to discern the effects of natural or synthetic SERMs on osteoclast metabolism.

In summary, feeding ethanol extracts of safflower seeds markedly prevented OVX-induced bone loss without uterotrophic effects. The beneficial effects of safflower seeds on bone may be mediated by growth-stimulating effects in the osteobalsts. These effects appeared to be mediated by the phenolic compounds in the ethanol extracts of the seeds, which consisted of phytoestrogens and serotonin derivatives. Ultimately, the CEE and PPEE of safflower seeds may be utilized in preventing bone loss caused by estrogen deficiency.

Acknowledgments

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