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Evaluation of the Preventive Effect of Isoflavone Extract on Bone Loss in Ovariectomized Rats

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To examine a potential role for soybean phytoestrogens in postmenopausal bone loss, twenty-four 12-week-old Sprague-Dawley rats were divided randomly into 4 groups and given controlled diets for 16 weeks. The treatment groups were as followed: sham operated, ovariectomized (OVX) control, OVX + isoflavone extract (6.25 g/kg), and OVX + 17β-estradiol (4 mg/kg). OVX treatments reduced femoral and fourth lumbar vertebral bone density and mineral content ($p < 0.01$), decreased uterine weight ($p < 0.01$), accelerated body weight increases ($p < 0.05$), and increased the activities ($p < 0.01$) of both serum alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP). Supplementation with isoflavone prevented the losses of bone density and mineral content caused by OVX ($p < 0.01$). Although both isoflavone and 17β-estradiol exhibited similar bone-sparing ability on the OVX-induced bone loss, the effect of isoflavone was not the same as that of 17β-estradiol on the serum ALP and TRAP, body weight increase, and uterine weight change. We concluded that dietary supplementation with soybean isoflavone can prevent postmenopausal bone loss via a different mechanism of estrogen in OVX rats.

Key words: isoflavone; estrogen; ovariectomy; bone; uterus

Osteoporosis is characterized by decrease in bone mass and is widely recognized as a major public health problem.[1)] Nutritional and pharmacological strategies are necessary to prevent age-related bone loss. Ovarian hormone deficiency is a major risk factor for osteoporosis. A sharp decrease in ovarian estrogen production is the predominant cause of rapid bone loss during the first decade after menopause. Traditional therapies for postmenopausal osteoporosis have emphasized the use of antiresorptive agents such as estrogen, calcitonin, and bisphosphonates.[2–4)] Although these agents may prevent further bone loss in established osteoporosis, they cannot restore bone mass that has been lost already. Furthermore, some agents that stimulate bone formation (*e.g.*, sodium fluoride, growth hormone, and anabolic steroids) have been reported to have undesirable side effects and do not produce bone mass of a desirable quality.[5–7)] Thus it is necessary to develop naturally occurring compounds or synthetic substances with less undesirable side effects that can substitute or reduce the need for drugs used currently.[8,9)]

Epidemiological studies suggest that the low incidence of osteoporosis and heart disease in postmenopausal Asian women compared to American women is attributable to their higher intake of soybean-based foods.[1,10)] Kalu *et al.*[11)] observed that feeding soy protein instead of casein to elderly rats prevented age-related bone loss. This positive effect of soy protein on bone was ascribed to its amino acid composition. Omi *et al.*[12)] reported that female rats fed soymilk had greater bone mineral density and mechanical bone strength than did rats fed casein. The authors speculated that this beneficial effect was due to enhanced intestinal absorption of calcium. Arjmandi *et al.*[13)] found that feeding soy protein to ovariectomized rats prevented bone loss. Goyal *et al.*[14)] showed that this positive effect was due to the isoflavone in the soy.

Soybeans are a rich source of the isoflavones genistein and daidzein. Soy isoflavones, which have both weak estrogenic and antiestrogenic effects, are structurally similar to tamoxifen, an agent that acts similarly to estrogen in reducing postmenopausal bone loss.[8,12,13)] The biological effects of genistein and daidzein have not been fully clarified. Daidzein has been shown to have a

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strong inhibitory effect on mitochondrial aldehyde dehydrogenase,[15] and genistein inhibits protein tyrosine kinases.[16] There are several reports that genistein inhibits osteoclastic activity and can reduce bone loss in ovariectomized rats.[9,17–19] Moreover, it has been found that genistein has an anabolic effect on bone formation and mineralization by cultured bone cells.[15,20] Genistein also inhibits bone resorption in femoral metaphyseal tissues obtained from elderly rats *in vitro*.[21,22] Genistein has been shown to stimulate osteoblastic bone formation and to inhibit osteoclastic bone resorption. Gao and Yamaguchi[15] have reported that daidzein had an anabolic effect on bone metabolism in tissue cultures, and that this effect was similar to that of genistein.

Thus isoflavones appear to have potential for maintaining the bone mass of human subjects when consumed at optimal dosages.[8,23] Moreover, the ovariectomized rat model is suitable for studying problems that are related to postmenopausal bone loss, since this model has been shown to be useful for investigating the cellular mechanisms underlying the action of estrogen on the skeleton.[24] Therefore, the present study was designed to investigate the effect of isoflavone, which was extracted from soybean hypocotyls, on the bone of ovariectomized rats.

Materials and Methods

Extraction of soybean isoflavone. Isoflavone was extracted from soybean hypocotyls with 10 volumes of 80% aqueous methanol by stirring for 4 h at room temperature. The methanol extract was condensed in a rotary evaporator at 50 °C, and the soybean isoflavone extract was obtained by freeze-drying the concentrated methanol extract. An HPLC analysis of 3 isoflavones and their 9 derivatives was performed using a diode array detector (HP1100 system, Agilent, USA) and an eclipse XDB C-18 column (Agilent). UV detection was performed at a wavelength of 260 nm, and the injection volume was 5 μl. The mobile phases used for analysis were 0.1% acetic acid in H_2O (solvent A) and 0.1% acetic acid in acetonitrile (solvent B). A flow rate of 1.2 ml/min under an initial condition of 93:7 (A:B) was held for 25 min, brought to 15% B in 25 min, to 20% B in 5 min, and to 25% B in 15 min, all with a linear gradient. Genistein, daidzein, and glycitein were purchased from Sigma (St. Louis, USA), and each isoflavone derivative, malonyl-, acetyl-, and glycoside form, was purchased from Fujico (Tokyo, Japan) for use as standards in HPLC analyses.

Animals and diets. Twenty-four 3-week-old female Sprague-Dawley rats were purchased from BioGenomics (Seoul, Korea). On arrival at our institution, the rats were housed in an environmentally controlled animal laboratory and acclimated on an AIN76a diet for 9 weeks. At 12 weeks of age, the animals were divided into 4 weight-matched groups using a randomized complete-block design. One group of rats was sham-operated (SHAM), and three groups were subjected to bilateral ovariectomy (OVX).

Table 1. Composition of Experimental Diets

Ingredients	Experimental group[a] (g/kg diet)			
	SHAM[b]	OVX-C	ISO	E2
Casein	227.00	227.00	227.00	227.00
Sucrose	417.59	417.59	417.59	417.59
Corn starch	200.00	200.00	200.00	200.00
Fiber (cellulose)	56.00	56.00	49.75	56.00
Corn oil	57.00	57.00	57.00	57.00
Vitamin mixture[c]	10.00	10.00	10.00	10.00
Mineral mixture[d]	13.37	13.37	13.37	13.37
Vitamin D3	0.0016	0.0016	0.0016	0.0016
Methionin	2.00	2.00	2.00	2.00
Calcium carbonate	9.88	9.88	9.88	9.88
Sodium phosphate monobasic	3.88	3.88	3.88	3.88
Potassium phosphate monobasic	2.38	2.38	2.38	2.38
Potassium citrate monohydrate	0.90	0.90	0.90	0.90
Isoflavone extract[e]			6.25	
17β-estradiol				0.0039

[a] SHAM, sham-operated; OVX-C, ovariectomized control; ISO, ovariectomized and fed isoflavone extract; E2, ovariectomized and fed 17β-estradiol. [b] TD88190, Harlan Teklad. [c] TD40060, Harlan Teklad. [d] TD79055, Halan Teklad. [e] Containing genistein, 1.79; daidzein, 3.06; glycitin, 8.36; genistin, 57.63; daidzin, 258.99; and glycitin, 171.31 mg/6.25 g isoflavone extract.

After SHAM or OVX treatment, the rats were fed an experimental diet for 16 weeks (Table 1). They had free access to de-ionized drinking water throughout. The SHAM group and one OVX control group (OVX-C) received the basal diet. The other two OVX groups received either isoflavone extract (ISO, 6.25 g/kg diet) or 17β-estradiol (E2, 3.9 mg/kg diet). All rats were fed an isonitrogeneous and isocaloric casein-based diet. During the experimental period, body weight was measured once per week and food intake was measured every day. All animals were treated in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*.[25]

Bone density. All the rats were sacrificed 16 weeks after starting their respective diets. The fourth lumbar vertebra and the right femur and tibia were dissected out and carefully cleaned of soft tissue. Bone volume and density were measured using Archimedes' principle.[26] Each bone was placed in an unstoppered vial filled with deionized water and placed in a desiccator under vacuum overnight for degassing. Each bone was then removed from its vial, blotted with gauze sponge, weighed, and returned to the vial. The bone was reweighed under water, from which the bone volume and density were calculated. The bone mineral density was determined from the total ash content, and bone calcium was determined by atomic absorption spectrophotometry.

Serum analysis. Serum alkaline phosphatase (ALP), which is an index of bone formation, and tartrate-resistant acid phosphatase (TRAP), an index of bone resorption, were measured using diagnostic kits at 4, 8, and 16 weeks during the experimental period. ALP and TRAP kits were purchased from Asan (Seoul, Korea) and Iatron (Tokyo, Japan), respectively.

Statistical analysis. The effect of the diets was assessed by ANOVA using the SPSS program (Version 10.0). Significant differences between groups were determined by Duncan's multiple-range test, with significance defined at either $p < 0.05$ or $p < 0.01$.

Results

Isoflavone content and intake

The methanol extract of soybean hypocotyls was analyzed by an HPLC system. The isoflavone contents are listed in Table 2. The glycoside and malonyl forms were dominant in the isoflavone extract. The experimental diet of the ISO group contained isoflavone at about 521.94 mg/kg. One rat in the ISO group daily consumed about 10.67 mg of isoflavone during the experimental period, comprising 1.27 mg of genistein derivatives, 5.58 mg of daidzein derivatives, 3.82 mg of glycitein derivatives.

Body weight and food intake

All treatment groups started with similar mean body weights, but at the end of the study the OVX-C group had a significantly higher mean body weight than the SHAM group ($p < 0.05$). This higher final mean body weight for OVX-C was not a result of increased energy intake, since energy intakes were similar among the groups (Table 3). The OVX-induced body weight increase was suppressed significantly by intake of 17β-estradiol ($p < 0.05$), but not by intake of isoflavone extract.

Uterine and abdominal fat weights

The uterine weights of the OVX-C, ISO, and E2 groups were significantly lower than that of the SHAM group, as shown in Table 3 ($p < 0.01$). This ovariectomy-induced atrophy of the uterus was partially prevented by 17β-estradiol treatment. The uterine weight of the E2 group was significantly higher than that of the OVX-C group ($p < 0.01$). This preventive effect was not evident in the ISO group. The abdominal fat weight was significantly lower than in the SHAM group only in the E2 group ($p < 0.01$).

Bone density, bone calcium, and bone ash content

The densities of the right femur and tibia and fourth lumbar vertebra of the rats are shown in Table 4. The rats in the OVX-C group had significantly lower femur and lumbar densities as compared with the SHAM group ($p < 0.01$). Decreases in bone density did not appear in the E2 group as compared with the SHAM group. The animals treated with isoflavone had significantly higher femoral and lumbar vertebral bone densities than those in the OVX-C group ($p < 0.01$), and showed the equivalent bone densities to those of rats in the E2 group. There was no significant difference in tibia density among groups. As Table 4 shows, the OVX-C group had a significantly lower ash content than the SHAM group and the E2 group did not except for the ash content of lumbar vertebral bone. The ISO group showed a significantly higher ash content than the OVX-C group in all bone tissues examined. The bone calcium contents in the OVX-C and ISO groups, but not in the E2 group, were significantly lower than in the SHAM group in lumbar vertebral bone, and there was no significant difference of Ca content in femur and tibia among groups.

Table 2. Content of Genistein, Daidzein, and Glycitein of Isoflavone Extracts from Soybean Hypocotyls

Residue	Content (mg/g extracts)		
	Genistein	Daidzein	Glycitein
Aglycone	0.30 ± 0.01	0.51 ± 0.01	1.39 ± 0.02
Glucoside	5.14 ± 0.11	18.65 ± 0.43	18.54 ± 0.43
Malonyl	4.26 ± 0.06	23.82 ± 0.26	9.71 ± 0.11
Acetyl	0.20 ± 0.01	0.69 ± 0.01	0.30 ± 0.01

Each value is the mean ± SD.

Table 3. Effects of Ovariectomy, Isoflavone, and 17β-Estradiol on Food Intake, Body Weight, and Relative Organ Weight

Measure	Experimental group			
	SHAM	OVX-C	ISO	E2
Food intake, g/day	20.2 ± 2.5	20.2 ± 1.3	20.4 ± 2.0	18.4 ± 2.3
Initial body weight, g	258.6 ± 20.1	257.2 ± 16.4	257.2 ± 14.2	257.6 ± 13.3
Final body weight, g	367.5 ± 38.7	420.5 ± 31.0[a]	403.0 ± 39.6	353.1 ± 48.7[c]
Organ weight, g/100 g body weight				
Uterus	0.209 ± 0.055	0.024 ± 0.007[b]	0.035 ± 0.008[b]	0.106 ± 0.022[b,d]
Abdominal fat	8.65 ± 1.28	9.52 ± 1.21	7.95 ± 1.51	6.62 ± 1.73[d]

Each value is the mean ± SD ($n = 6$). Values with a superscript are significantly different from the SHAM group ([a], $p < 0.05$; [b], $p < 0.01$) or from the OVX-C group ([c], $p < 0.05$; [d], $p < 0.01$). SHAM, sham-operated; OVX-C, ovariectomized control; ISO, ovariectomized and fed isoflavone extract; E2, ovariectomized and fed 17β-estradiol.

Table 4. Bone Ash and Calcium Contents of the Right Femur and Tibia and the Fourth-lumbar Vertebra in Rats

Measure	Experimental group			
	SHAM	OVX-C	ISO	E2
Bone density (g/cm^3)				
Femur	1.689 ± 0.032	1.539 ± 0.036^{b}	1.755 ± 0.083^{d}	1.625 ± 0.033^{d}
Tibia	1.619 ± 0.034	1.560 ± 0.042	1.677 ± 0.081	1.611 ± 0.032
Lumbar	1.381 ± 0.051	1.206 ± 0.017^{b}	1.330 ± 0.088^{d}	1.346 ± 0.050^{d}
Ash content (g/cm^3)				
Femur	1.122 ± 0.036	0.973 ± 0.056^{b}	1.096 ± 0.059^{d}	1.060 ± 0.027^{c}
Tibia	1.052 ± 0.030	0.986 ± 0.026^{a}	1.034 ± 0.031^{c}	1.030 ± 0.017
Lumbar	0.849 ± 0.039	0.660 ± 0.046^{b}	$0.756 \pm 0.074^{a,c}$	$0.767 \pm 0.049^{a,d}$
Bone Ca content (mg/cm^3)				
Femur	456.6 ± 82.5	391.6 ± 33.2	420.8 ± 52.9	422.6 ± 60.6
Tibia	402.3 ± 25.2	404.3 ± 21.0	409.7 ± 28.1	410.9 ± 27.2
Lumbar	357.5 ± 41.9	263.3 ± 37.3^{b}	263.5 ± 27.1^{b}	317.9 ± 27.7

Each value is the mean $\pm$ SD ($n = 6$). Values with a superscript are significantly different from the SHAM group (^{a}, $p < 0.05$; ^{b}, $p < 0.01$) or from the OVX-C group (^{c}, $p < 0.05$; ^{d}, $p < 0.01$). SHAM, sham-operated; OVX-C, ovariectomized control; ISO, ovariectomized and fed isoflavone extract; E2, ovariectomized and fed 17β-estradiol.

Table 5. Serum Alkaline Phosphatase (ALP) and Tartrate-resistant Acid Phosphatase (TRAP) Activities

	ALP (U/l)			TRAP (U/l)		
	4 weeks	8 weeks	16 weeks	4 weeks	8 weeks	16 weeks
SHAM	73.3 ± 26.8	64.0 ± 20.1	37.2 ± 15.2	10.1 ± 1.8	8.5 ± 1.9	10.2 ± 3.2
OVX-C	135.5 ± 11.9^{b}	105.0 ± 26.5^{b}	81.0 ± 21.2^{b}	15.7 ± 3.9^{a}	14.2 ± 1.4^{b}	17.9 ± 3.0^{b}
ISO	123.8 ± 28.7^{a}	87.4 ± 9.8	77.8 ± 8.5^{b}	16.0 ± 3.1^{a}	13.3 ± 2.2^{b}	13.4 ± 2.7
E2	91.5 ± 6.6^{c}	79.5 ± 12.9	49.5 ± 13.8^{d}	12.0 ± 0.8	11.9 ± 1.9	9.6 ± 2.2^{d}

Each value is the mean $\pm$ SD ($n = 6$). Values with a superscript are significantly different from the SHAM group (^{a}, $p < 0.05$; ^{b}, $p < 0.01$) or from the OVX-C group (^{c}, $p < 0.05$; ^{d}, $p < 0.01$). SHAM, sham-operated; OVX-C, ovariectomized control; ISO, ovariectomized and fed isoflavone extract; E2, ovariectomized and fed 17β-estradiol.

Serum analysis

The activities of serum ALP and TRAP are given in Table 5. Both ALP and TRAP activities showed a tendency to decrease during the experimental period. At the end of experiment (after 16 weeks), the ALP activities for the OVX-C and ISO groups were higher than that for the SHAM group, and the TRAP activity for the OVX-C group was also higher than that for the SHAM group. The OVX-induced ALP and TRAP increases were significantly suppressed by intake of 17β-estradiol ($p < 0.05$), but not by intake of isoflavone extract.

Discussion

The main purpose of this study was to evaluate whether isoflavone extract from soybean hypocotyls prevents bone loss due to ovariectomy. In this study, we used the E2 group as a positive control, because the bone-conserving effects of estrogen are well established in the OVX rat model of osteoporosis.[24] Our data on uterine weight, bone density, bone calcium, and bone ash weights confirm the observations of other investigators that bone loss in the OVX rat is prevented by estrogen administration, and that estrogen can also suppress the OVX-induced rise in two biochemical makers of bone turnover: serum ALP and TRAP.[9,13]

As expected, the femurs and fourth-lumbar vertebral bones of rats in the OVX-C group had lower values for bone density and mineral content than those of in the SHAM group. Treatment with isoflavone extract at 6.25 g/kg appeared to prevent bone loss in the femur and lumbar vertebra. These results are supported by previous observations of the positive effects of soybean products — such as soybean protein and soy milk powder — on the bone density of female rats,[8,12,13] because these products contained genistein and daidzein. But, the effects of isoflavone on ALP and TRAP activities are not the same as that of estrogen. Serum concentrations of both ALP and TRAP were significantly greater in the OVX-C and ISO groups than in the SHAM and E2 groups, and this suggests that, unlike estrogen, isoflavone does not inhibit bone turnover. Thus the mechanism by which soybean isoflavone prevents OVX-induced bone loss appears to be different from that of estrogen.

In this study, isoflavone extract had greater proportions of glycitein and its derivatives than of genistein and its derivatives. In general, genistein and daidzein account for the major portion of soy isoflavone and have been the focus of numerous studies, and few studies of glycitein have been reported to date. Glycitein and its derivatives account for 5–10% of the total isoflavone in most soy foods. Zhang *et al.*[27] showed that the urinary

disposition of three main isoflavones was different in humans, with more glycitein excreted than daidzein and more daidzein than genistein. Song *et al.*[28] reported that glycitein has weak estrogenic activity — comparable to that of the other soy isoflavones but much lower than that of 17β-estradiol. Further studies are needed to examine the individual biological effects of glycitein on bone metabolism.

Ishida *et al.*[9] reported that OVX animals had uterine atrophy, which could be prevented by the administration of estrogen but not by genistin and daidzin at a dosage of 50 mg/kg/day. Ishimi *et al.*[17] also reported that genistein, a typical soybean isoflavone, prevents bone loss caused by estrogen deficiency without exhibiting estrogenic action in the uterus. But, phytoestrogens, including soybean isoflavones, have received considerable attention due to their possible effects as endocrine disrupters.[29] Santell *et al.*[30] have reported that the administration of large amounts of genistein caused uterine hypertrophy in immature rats. The estrogenic activity of isoflavones, however, is very weak compared to that of estrogen. Genistein and daidzein are converted to *p*-ethylphenol and eqoul respectively by intestinal bacteria, which are rapidly absorbed in the gut and conjugated in the liver and readily excreted in the urine.[31,32] Both *in vitro* and *in vivo* studies have shown that genistein exerts a weak estrogenic effect: 10^{-3} to 10^{-5}-fold that of estrogen.[1,33] It was recently reported also that genistein induced uterine hypertrophy only at doses more than 10-fold higher than the dose needed to prevent bone loss in OVX mice.[34] According to our results, isoflavone extract from soybean hypocotyls was able to prevent OVX-induced bone loss without exhibiting estrogenic action in the uterus of OVX rats at a dosage of 10.67 mg/day (comprising 1.27 mg of genistein derivatives, 5.58 mg of daidzein derivatives, and 3.82 mg of glycitein derivatives).

In conclusion, this study demonstrates that isoflavone extract from soybean hypocotyl may be effective in suppressing bone loss due to OVX, and may represent an effective alternative to estrogen replacement therapy for the maintenance of postmenopausal bone mass.

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