Preventive Effect of Dietary Fermented Soybean on Bone Loss in Ovariectomized Rats: Enhancement with Isoflavone and Zinc Supplementation

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The effect of experimental diets with fermented soybean (natto) containing isoflavone and zinc on ovariectomy (OVX)-induced bone loss was investigated. Sham-operated rats or OVX rats were given experimental diets containing soybean protein for 3 months, and other OVX rats were fed dietary natto, with or without calcium, calcium plus zinc, calcium plus isoflavone or calcium plus zinc and isoflavone for 3 months. Experimental diets contained 2.1 to 9.7 mg zinc per 100 g and 44.6 to 92.4 mg isoflavone (including genistin, genistein, daidzin, and daidzein) per 100 g. OVX caused a significant reduction in the dry weight and mineral density of the femur. Also, the calcium content, zinc content and alkaline phosphatase activity in femoral-diaphyseal and metaphyseal tissues was significantly reduced by OVX. These reductions were largely prevented by feeding natto diets. This prevention was significantly enhanced in OVX rats fed natto diets supplemented with isoflavone and zinc. This study demonstrates that the prolonged intake of dietary natto supplemented with isoflavone and zinc has a preventive effect on OVX-induced bone loss, suggesting that it may have a role in the prevention of osteoporosis.

Key words —— isoflavone, zinc, fermented soybean, bone metabolism, osteoporosis

INTRODUCTION

Osteoporosis is widely recognized as a major public health problem. The most dramatic expression of this disease is represented by fractures of the proximal femur the incidence of which increases as the population ages.[1,2] Bone mass decreases with increasing age and this decrease is due to increased bone resorption and reduced bone formation. Nutritional factors can help prevent bone loss with increasing age[3] but these factors are poorly understood.

Recent studies have shown that isoflavone found in Leguminosae has an anabolic effect on bone metabolism in rats.[4,5] Soybean contains large quantities of isoflavone including genistin, genistein, daidzin, and daidzein. Genistin and daidzin are hydrolyzed to genistein and daidzein by β-glucosidase in gastric juice. Genistein and daidzein have been shown to stimulate osteoblastic bone formation,[6,7] and to inhibit osteoclastic bone resorption.[8,9] Isoflavone can prevent bone loss in ovariectomized (OVX) rats,[10–12] and it may be an important nutritional factor in preventing osteoporosis.

Zinc, an essential trace element, has been shown to have a potent stimulatory effect on bone formation.[13,14] Zinc can stimulate protein synthesis in osteoblastic cells and bone tissue culture systems *in vitro* by means of activating aminoacyl-*t*RNA synthetase.[13,14] Zinc has been also shown to inhibit osteoclastic bone resorption.[15] Oral administration of zinc compounds prevent bone loss in OVX rats.[16]

Whether the combination of nutritional factors exhibits an additive or synergistic effect on bone components has not been fully clarified. This knowledge may be important in preventing bone loss with increasing age. More recently, it has been reported that the combination of genistein and zinc can produce a synergistic effect on bone components using

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Table 1. Composition of Experimental Diets

	Control	OVX	OVX + natto				
			None	Ca	Ca+Zn	Ca+ISF	Ca+ISF+Zn
Ingredients (%)							
Soybean protein	21	21	–	–	–	–	–
Natto powder	–	–	50	50	50	50	50
Corn starch	16	16	–	–	–	–	–
USP-vitamin mixture[a]	1	1	1	1	1	1	1
USP-mineral mixture[b]	4	4	4	4	4	4	4
Cellulose	3	3	–	–	–	–	–
Glucose	45	45	45	45	45	45	45
Soybean oil	10	10	–	–	–	–	–

a) USP-vitamin mixture (100 g diet): vitamin A, 2000 IU; vitamin E, 101 U; vitamin K, 1.0 mg; choline, 200 mg; *p*-aminobenzoic acid, 10 mg; inositol, 10 mg; niacin 4 mg; calcium pantothenate, 4 mg; vitamin B_2, 0.8 mg; vitamin B_1, 0.5 mg; vitamin B_6, 0.5 mg; folic acid, 0.2 mg; vitamin H, 0.04 mg; vitamin B_{12}, 0.003 mg. *b*) UPS-mineral mixture (100 g diet): NaCl, 557.2 mg; KI, 3.16 mg; KCl, 852.9 mg; $MgSO_4$, 229.2 mg; $FeSO_4$, $7H_2O$, 108.0 mg; $MnSO_4 \cdot H_2O$, 16.04 mg; $ZnSO_4 \cdot 7H_2O$, 2.192 mg; $CuSO_4 \cdot 6H_2O$, 1.908 mg; $CoCl_2 \cdot 5H_2O$, 0.092 mg.

femoral tissues from female rats.[17–19]

This study was undertaken to determine the preventive effect of a soybean diet on bone loss in OVX rats. Experimental diets containing the fermented soybean (natto) supplemented with isoflavone and zinc were given to OVX rats for 3 months. We found that natto diets can prevent OVX-induced bone loss, and this preventive effect is enhanced by supplementation with isoflavone and zinc.

MATERIALS AND METHODS

Animals —— Female Wistar rats (conventional) weighing 110–120 g (6 weeks old) were obtained from Japan SLC (Hamamatsu). The animals were fed commericial laboratory chow (solid) containing 1.1% calcium and phosphorus at a room temperature of 25°C, with free access to distilled water. Rats were divided into seven groups of six rats each. Some animals were given a sham-ovariectomy, and other animals had a bilateral OVX under ether anesthesia. In the sham-operated animals, both ovaries were handled, but not removed.

Experimental Diets —— The experimental diets contained either soybean protein or freeze-dried natto powder (the natto content was 50%) with calcium, calcium plus zinc, calcium plus isoflavone, or calcium plus zinc and isoflavone. The composition of experimental diets is shown in Table 1 and the nutritional analysis of experimental diets is shown in Table 2.

Animal Experiments —— Animals in group 1 (sham-operated control) and group 2 (OVX) were allowed free access to experimental diets without natto supplementation. OVX animals in groups 3, 4, 5, 6 and 7 were given free access to natto diets supplemented with either nothing extra (group 3), calcium (group 4), calcium plus zinc (group 5), calcium plus isoflavone (group 6) or calcium plus isoflavone and zinc (group 7), respectively. The feeding of experimental diets was started 14 d after sham operation or OVX. All animals were fed matched amounts of the chow previously described for 3 months (90 d), but no pair feeding was carried out.

Analytical Procedures —— Rats were killed by cardiac puncture under light ether anesthesia, and blood samples and femur were removed immediately. Blood samples were centrifuged for 30 min after collection, and the serum was separated. The serum calcium concentration was determined by the method of Willis,[20] serum inorganic phosphorus was measured by the method of Taussky and Shon[21] and serum zinc was determined by atomic absorption spectrophotometry.[19]

The femur was removed after bleeding and soaked in ice-cold 0.25 M sucrose solution. It was cleaned of soft tissue, and then dried for 16 h at 100°C to measure the mineral density and dry bone weight. The mineral density was measured in total sections of the femur and a section of the femoral metaphysis, using a dual X-ray bone densitometer (XR-26; Norland Co., Ltd.).[16] After this measurement, the femurs were separated into diaphysis and metaphysis (not containing epiphyseal tissues) and weighed to determine the calcium and zinc content. The femoral-metaphyseal and diaphyseal tissues were digested with nitric acid, and calcium and zinc were determined by atomic absorption spectrophotometry.

Table 2. Analytical Values of Experimental Diets

| | Control | OVX | OVX + natto | | | | |
			None	Ca	Ca+Zn	Ca+ISF	Ca+ISF+Zn
Analytical value (%)							
Protein	18.5	18.5	20.3	20.8	19.8	19.6	19.8
Fat	9.9	9.9	12.5	12.4	12.3	11.2	11.1
Carbohydrate	60.5	60.5	55.0	54.3	55.2	56.3	55.6
Analytical value (per 100 g diets)							
Calcium (Ca; mg)	0.81	0.81	0.91	1.01	0.99	0.93	0.93
Phosphorus (g)	0.61	0.61	0.73	0.73	0.74	0.75	0.75
Zinc (Zn; mg)	2.1	2.1	3.4	3.4	9.7	3.2	9.2
Vitamin K_2 (mg)	–	–	0.11	0.11	0.12	0.11	0.10
Isoflavone (ISF; mg)	44.6	44.6	67.3	65.8	66.5	88.9	92.4
Daidzin (mg)	2.6	2.6	12.5	11.8	12.3	30	31.9
Daidzein (mg)	2.1	2.1	6.6	6.5	6.5	3.8	4.3
Genistin (mg)	5.8	5.8	43.5	42.8	42.2	36.4	37.3
Genistein (mg)	2.4	2.4	1.8	1.8	2.3	5.8	6.4

To assay bone alkaline phosphatase activity, the diaphyseal and metaphyseal tissues were immersed in 3.0 ml ice-cold 6.5 mM barbital buffer (pH 7.4), cut into small pieces, homogenized with a Potter-Elvehjem homogenizer, and disrupted for 60 s using an ultrasonic device. The supernatant centrifuged at $600 \times g$ for 5 min was used to measure enzyme activity. Enzyme assay was carried out under optimal conditions. Alkaline phosphatase activity was determined by the method of Walter and Schutt.[22] Enzyme activity was expressed as μmol of *p*-nitrophenol liberated per min per mg protein. Protein concentration was determined by the method of Lowry *et al.*[23]

Statistical Analysis —— The significance of differences between values was estimated by Student's *t*-test. *p*-Values of less than 0.05 were considered statistically significant.

RESULTS

Sham-operated rats and OVX rats were given experimental diets containing either soybean protein or natto, with or without supplementation of calcium, calcium plus zinc, calcium plus isoflavone or calcium plus isoflavone and zinc, for 3 months (90 d), and the animals were then sacrificed by bleeding. There was no difference in the dietary intake between sham-operated rats and OVX rats given experimental diets for 3 months. OVX did not cause a significant alteration in serum calcium concentration, while serum inorganic phosphorus concentra-

tion was significantly reduced (Table 3). This reduction was not seen in OVX rats fed experimental natto diets. The serum zinc concentration in OVX rats was significantly increased by feeding the natto diet and this elevation was significantly enhanced by feeding the natto diet supplemented with calcium plus zinc or calcium plus isoflavone and zinc (Table 3).

The change in the dry femoral weight of sham-operated rats and OVX rats given experimental diets is shown in Fig. 1. The dry femoral weight was significantly reduced by OVX and this reduction was largely prevented by feeding a natto diet. This prevention was significantly enhanced by supplementation with isoflavone plus zinc. No such effect was seen in OVX rats fed natto diets supplemented with either calcium, calcium plus zinc or calcium plus isoflavone.

The change in the mineral density of the femur and femoral-metaphyseal tissues of sham-operated rats and OVX rats given experimental diets is shown in Fig. 2. The mineral density of the femur and femoral-metaphyseal tissues was significantly reduced by OVX. This reduction was slightly prevented by feeding a natto diet. This preventive effect was significantly enhanced by feeding natto diets supplemented with isoflavone plus zinc.

The change in calcium content in the femoral-diaphyseal and metaphyseal tissues of sham-operated rats and OVX rats given experimental diets is shown in Fig. 3. The calcium content in the femoral-diaphyseal and metaphyseal tissues was significantly reduced by OVX. This reduction was not seen in OVX rats fed natto diets. The preventive effect of

Table 3. Alteration in Calcium, Inorganic Phosphorus and Zinc Concentrations in the Serum of Ovariectomized Rats Fed the Fermented Soybean (Natto) Diets with Isoflavone and Zinc Supplementation

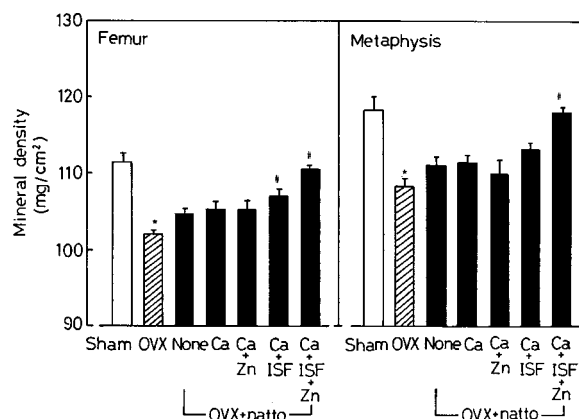| Groups	Calcium (mg/dl)	Inorganic phosphorus (mg/dl)	Zinc (μg/dl)
Sham operation	9.10±0.11	4.47±0.22	125.0±2.9
Ovariectomy (OVX)	8.92±0.14	3.28±0.24*	118.5±4.6
OVX+natto			
None	9.03±0.16	3.92±0.30	147.9±3.5*
Ca	9.07±0.17	4.09±0.25	147.6±2.9*
Ca+Zn	9.06±0.21	3.93±0.16	166.7±3.8*,#
Ca+ISF	9.18±0.15	3.98±0.21	152.2±4.6*
Ca+ISF+Zn	9.14±0.16	4.05±0.14	176.0±8.7*,#

Rats were given dietary natto containing either nothing extra, calcium (Ca), Ca plus Zinc (Zn), Ca plus isoflavone (ISF) or Ca plus ISF and Zn for 3 months, and then sacrificed by bleeding. Each value is the mean±S.E.M. of six rats. * $p < 0.01$ compared with the value for sham-operated rats. # $p < 0.01$, compared with the value for OVX rats fed natto diets.
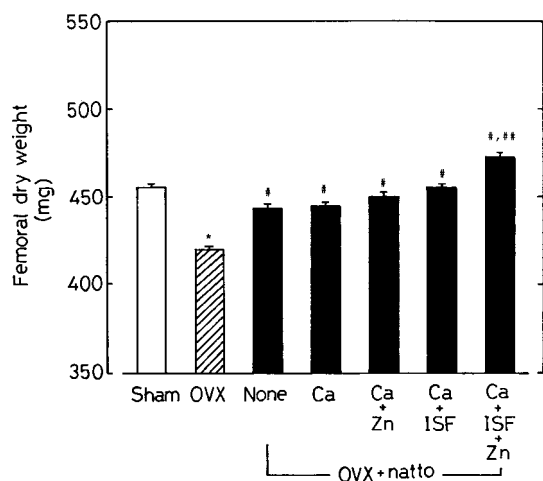
Fig. 1. Alteration in Femoral Dry Weight of Rats Fed the Fermented Soybean (Natto) Diets with Isoflavone and Zinc Supplementation

Sham-operated rats were given experimental diets containing soybean protein. OVX rats were given diets containing either soybean protein or natto, with or without the supplementation of calcium (Ca), Ca plus zinc (Zn), Ca plus isoflavone (ISF) or Ca plus ISF plus Zn, for 3 months. Each value is the mean ± S.E.M. of six rats. * $p < 0.01$, compared with the value for sham-operated rats. # $p < 0.01$, compared with the value for OVX rats without natto supplementation. ## $p < 0.01$, compared with the value for OVX rats fed natto diets supplemented with ISF or Zn.

Fig. 2. Change in the Mineral Density of the Femur and Femoral-Metaphyseal Tissues in Rats Fed Natto Diets Supplemented with Isoflavone and Zinc

Rats were given experimental diets as described in Fig. 1. Each value is the mean ± S.E.M. of six rats. * $p < 0.01$, compared with the value for sham-operated rats. # $p < 0.01$, compared with the value for OVX rats without natto supplementation.

natto diets on the OVX-induced reduction in the femoral-diaphyseal and metaphyseal calcium-content was significantly enhanced by supplementation with isoflavone plus zinc.

The change in zinc content in the femoral-diaphyseal and metaphyseal tissues of sham-operated rats and OVX rats given experimental diets is shown in Fig. 4. OVX caused a significant reduction in zinc content in the diaphyseal and metaphyseal tissues. This reduction was prevented by feeding a natto

diet. The supplementation of zinc, with or without isoflavone, caused a significant increase in the zinc content of the diaphyseal and metaphyseal tissues of OVX rats fed natto diets.

The change in alkaline phosphatase activity in the femoral-diaphyseal and metaphyseal tissues of sham-operated rats and OVX rats given experimental diets in shown in Fig. 5. The alkaline phosphatase activity in the diaphyseal and metaphyseal tissues was reduced by OVX. This reduction was significantly prevented by the feeding of natto diets. The enzyme activity in the diaphyseal and metaphyseal tissues of OVX rats fed natto diets was significantly enhanced by supplementation with isoflavone plus zinc.
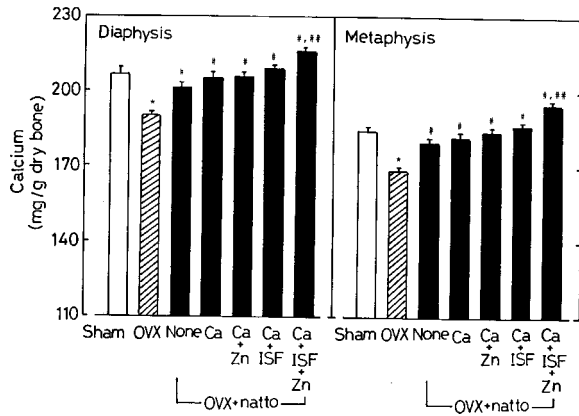
Fig. 3. Change in Calcium Content in the Femoral-Diaphyseal and Metaphyseal Tissues of Rats Fed Natto Diets Supplemented with Isoflavone and Zinc

Rats were given experimental diets as described in Fig. 1. Each value is the mean ± S.E.M. of six rats. * $p < 0.01$, compared with the value for sham-operated rats. # $p < 0.01$, compared with the value for OVX rats without natto supplementation. ## $p < 0.01$, compared with the value for OVX rats fed natto diets supplemented with ISF or Zn.
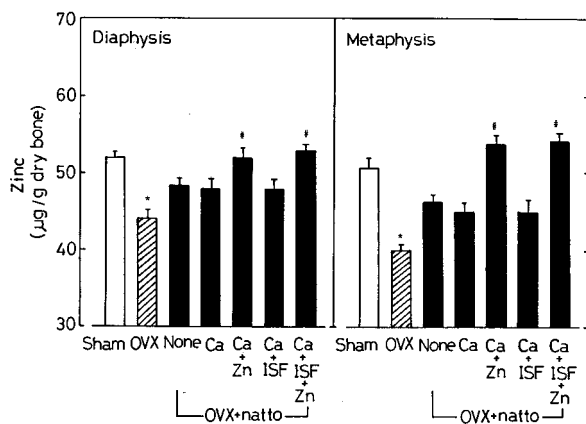

Fig. 5. Change in Alkaline Phosphatase Activity in the Femoral-Diaphyseal and Metaphyseal Tissues of Rats Fed Natto Diets Supplemented with Isoflavone and Zinc

Rats were given experimental diets as described in Fig. 1. Each value is the mean ± S.E.M. of six rats. * $p < 0.01$, compared with the value for sham-operated rats. # $p < 0.01$, compared with the value for OVX rats fed without natto diets. ## $p < 0.01$, compared with the value for OVX rats fed natto diets supplemented with ISF or Zn.

Fig. 4. Change in Zinc Content in the Femoral-Diaphyseal and Metaphyseal Tissues of Rats Fed Natto Diets Supplemented with Isoflavone and Zinc

Rats were given experimental diets as described in Fig. 1. Each value is the mean ± S.E.M. of six rats. * $p < 0.01$, compared with the value for sham-operated rats. # $p < 0.01$, compared with the value for OVX rats without natto supplementation.

DISCUSSION

Bone mass decreases with increasing age and ovarian hormone deficiency at menopause stimulates bone loss. OVX causes a lack of estrogen and this deficiency induces osteoporosis in humans and rats.[24–27] Whether nutritional factors can prevent bone loss due to estrogen deficiency has not been fully clarified.

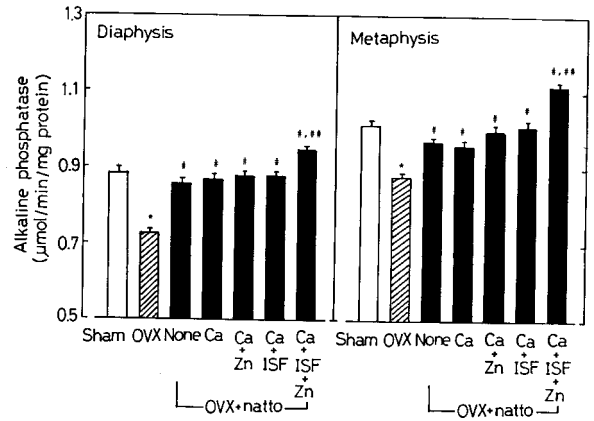The present study demonstrates that bone weight, mineral density and calcium content are reduced in OVX rats, indicating that OVX induces bone loss. These reductions were prevented by the feeding of diets containing fermented soybean (natto) for 3 months. This effect was further enhanced by supplementation with isoflavone and zinc. This finding indicates that the prolonged intake of natto diets with isoflavone and zinc supplementation can prevent OVX-induced bone loss.

Natto contains calcium, zinc and isoflavones, including genistin, genistein, daidzin and daidzein (Table 2). These nutrients may contribute to the prevention of OVX-induced bone loss, since each nutrient have been shown to have an anabolic effect on bone metabolism.[4–12,17,18,28] The preventive effect of natto diets on OVX-induced bone loss was not enhanced by supplementation with calcium, zinc or isoflavone. However, supplementation with isoflavone and zinc significantly enhanced the preventive effect of natto diets on OVX-induced bone loss. The combination of isoflavone and zinc may be important in the prevention of OVX-induced bone loss.

Zinc has been demonstrated to enhance the anabolic effect of genistein or daidzein on bone components in tissue culture *in vitro*; this metal can stimulate the genistein- or daidzein-induced increase in calcium content, alkaline phosphatase activity, and deoxyribonucleic acid (DNA) content in the femoral tissues of rats.[18–20] Zinc enhancement of the anabolic effect of genistein or daidzein on bone components is completely prevented by the presence of

cycloheximide, an inhibitor of protein synthesis, in bone culture systems.[5,17] The synergistic or additive effect of genistein and zinc, or daidzein and zinc, on bone components may be partly due to a newly synthesized protein component in bone tissues.[5,17,19]

The intake of natto diets supplemented with zinc caused a significant elevation in serum zinc concentration and a corresponding increase in the femoral zinc content. These increases were not significantly enhanced by the combination of isoflavone and zinc. Meanwhile, the alkaline phosphatase activity in femoral-diaphyseal and metaphyseal tissues was significantly enhanced by supplementation with isoflavone and zinc. These results suggest that the additive effect of isoflavone and zinc on bone alkaline phosphatase activity results from the action of both isoflavone and zinc in bone tissues. Presumably, the intake of a natto diet supplemented with isoflavone and zinc stimulates bone protein synthesis.

In conclusion, it has been demonstrated that the prolonged intake of natto diets can prevent OVX-induced bone loss, and that this preventive effect is enhanced by supplementation with isoflavone and zinc. The intake of dietary natto supplemented with isoflavone and zinc may be a useful means of preventing osteoporosis.

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