# Prolonged Intake of Fermented Soybean Diets with Supplementation of Isoflavone and Saponin Prevents Bone Loss in Ovariectomized Rats

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**The effect of experimental diets with fermented soybean (natto) containing soybean extract (nijiru) on ovariectomy (OVX)-induced bone loss was investigated. Experimental diets containing either natto (46%), natto with 3.8% nijiru supplementation or natto with 9.8% nijiru were freely given to sham-operated rats or OVX rats for 3 months. Nijiru contained saponin (660 $\mu$g/g of dry nijiru) and isoflavone including daidzin (770 $\mu$g/g) and genistin (580 $\mu$g/g) of comparatively higher concentration. OVX caused a significant reduction in the dry weight, mineral density and mineral content of the femur. The calcium content in the femoral-metaphyseal tissues was significantly reduced by OVX and these reductions were largely prevented by the feeding of natto diets with nijiru (9.8%) supplementation. This study demonstrates that the intake of natto diets supplemented with nijiru containing isoflavone and saponin has a preventive effect on OVX-induced bone loss, suggesting that it may have a role in the prevention of osteoporosis.**

**Key words** —— isoflavone, genistein, daidzein, saponin, soybean, bone metabolism, osteoporosis

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## INTRODUCTION

In recent years, it has been shown that bone loss with increasing age induces osteoporosis. Osteoporosis is widely recognized as a major public health problem and the most dramatic expression of this disease is represented by fractures of the proximal femur with the incidence of which increases as the population ages.[1,2] Malnutrition or undernutrition is often observed in the elderly, and it appears to be more severe in patients with hip fracture than in the general ageing population.[3,4] Deficiency in both micro- and macronutrients appears to be strongly implicated in the pathogenesis and the consequences of hip fracture in osteoporotic elderly patients.[5]

Nutritional factors are needed to prevent bone loss with increasing age, although this has still not been fully clarified More recently, it has been demonstrated that isoflavone found in Leguminosae has an anabolic effect on bone metabolism in rats.[6-10] Isoflavones including daidzin, daidzein, genistin and genistein are present in soybean at comparatively high concentrations. Daidzin and genistin are hydrolyzed to daidzein and genistein by $\beta$-glucosidase in gastric juice, and daidzein and genistein have been shown to increase osteoblastic bone formation[11] and to inhibit osteoclastic bone resorption.[8,12,13] Therefore, isoflavone may prevent bone loss with increasing age. Moreover, saponin, which is present in soybean, has been shown to have an anabolic effect on bone metabolism *in vitro* and *in vivo*.[14] Isoflavone and saponin in soybean may be important as nutritional factors in preventing osteoporosis.

Soybean extract (nijiru) contains large quantities of isoflavone and saponin[15] and administration of nijiru can have an anabolic effect on bone metabolism in rats *in vivo*.[15] Intake of soybean foods, including isoflavone and saponin, may have an important role in the prevention of osteoporosis with increasing age.

This study was undertaken to determine the preventive effect of soybean food intake on bone loss in ovariectomized (OVX) rats. Experimental diets containing fermented soybean (natto) with supplementation of nijiru were given to OVX rats for 3 months. We found that the intake of these experimental diets can prevent OVX-induced bone loss.

## MATERIALS AND METHODS

**Animals** —— Female Wistar rats (conventional) weighing 120—130 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℃, with free access to distilled water. Rats were divided into four groups of seven rats each. Animals were given a sham-ovariectomy, and other animals had bilateral ovariectomy (OVX) under ether anesthesia. In the sham-operated animals, both ovaries were handled, but not removed.

**Experimental Diets** —— Nijiru is produced in the process used to make fermented soybean (natto).[15] Soybean was boiled under pressure (1.5 atmospheric pressure) for 40 min at 160℃, and the nijiru produced was then freeze-dried. The isoflavone and saponin content was measured by completely removing these compounds from the nijiru powder by extraction with hot 80% ethanol solution.[15] This ethanol solution was filtered, and the flitrate was subjected to HPLC. The isoflavone and saponin concentration was expressed as $\mu$g per g nijiru powder.

Freeze-dried natto powder usually contains 40.2 % protein, 22.3% lipids, 24.3% carbohydrate, 0.23% calcium, 0.06% phosphorus, 0.243% isoflavone, and 0.580% saponin. The experimental diets contained freeze-dried natto powder (the natto content was 46%) or natto powder (46%) with nijiru supplementation (3.8 or 9.8%). The isoflavone and saponin content of the experimental diets containing natto powder, with or without nijiru supplementation, is shown in Table 1.

**Animal Experiments** —— Animals in group 1 (sham OVX) and group 2 (OVX) were freely given experimental diets containing natto. OVX animals in groups 3 and 4 were freely given natto diets supplemented with nijiru, 3.8 and 9.8%, respectively. All animals were fed matched amounts of the chow previously described for 3 months (90 days), but no pair feeding was camed out.

**Analytical Procedures** —— Rats were killed by cardiac puncture under light ether anesthesia, and blood and femur were removed immediately. Blood samples were centrifuged for 30 min after collection, and the serum was determined by the method of Willis.[16] Serum inorganic phosphorus was measured by the method of Taussky and Shon.[17]

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℃ to measure the mineral density and bone dry weight. The mineral denisty was measured in total sections of the femur using a dual X-ray bone densitometer (XR-26 ; Norland Co., Ltd.).[18] After this measurement, the femurs were separated into diaphysis and metaphysis (not containing epiphyseal tissues) and weighed to determine the bone calcium content. The femoral-metaphyseal tissues were digested with nitric acid

**Table 1.** Composition and Analysis of Experimental Diets

| | Sham-operated rats | Ovariectomized rats | | |
|---|---|---|---|---|
| | | None | Nijiru 3.8% | Nijiru 9.8% |
| Ingredients (%) | | | | |
| Natto powder | 46.0 | 46.0 | 46.0 | 46.0 |
| Nijiru powder | — | — | 3.8 | 9.8 |
| USP-vitamin mixture[a] | 1.0 | 1.0 | 1.0 | 1.0 |
| USP-mineral mixture[b] | 4.0 | 4.0 | 4.0 | 4.0 |
| Cellulose | 3.0 | 3.0 | 3.0 | 3.0 |
| Glucose | 46.0 | 46.0 | 46.0 | 46.0 |
| Analytical values (mg/100 g diets) | | | | |
| Daidzin | 39.0 | 39.0 | 46.0 | 57.0 |
| Daidzein | 1.5 | 1.5 | 1.5 | 1.6 |
| Genistin | 70.0 | 70.0 | 76.0 | 86.0 |
| Genistein | 3.7 | 3.7 | 4.0 | 4.1 |
| Saponin | 263 | 263 | 280 | 290 |

*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*) USP-mineral mixture (100 g diet) : NaCl, 557.2 mg ; KI, 3.16 mg ; KCl, 852.9 mg ; $MgSO_4$, 229.2 mg ; $FeSO_4 \cdot 7H_2O$, 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.

**Table 2.** Body Weight and Serum Calcium and Inorganic Phosphorus Concentrations in Ovariectomized Rats following Intake of Fermented Soybean (Natto) Diets.

| | Sham-operated rats | Ovariectomized rats | | |
|---|---|---|---|---|
| | | None | Nijiru 3.8% | Nijiru 9.8% |
| Body weight (g) | 181.1±3.6 | 219.3±3.2* | 212.4±5.4* | 209.7±4.9* |
| Serum | | | | |
| Calcium (mg/dl) | 10.2±0.24 | 9.5±0.27 | 10.0±0.22 | 9.4±0.21 |
| Inorganic phosphorus (mg/dl) | 5.4±0.27 | 4.6±0.12* | 4.1±0.17* | 4.5±0.14* |

Rats were given natto diets containing nijiru for 3 months, and then sacrificed by bleeding. Each value is the mean±S.E.M. of seven rats. *$P<0.01$ compared with the value for sham-operated rats.

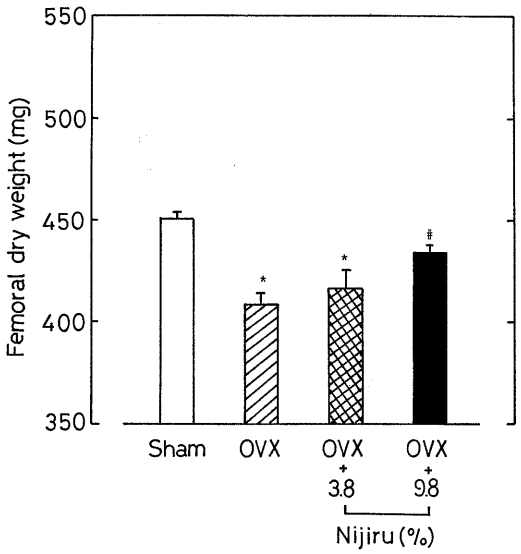


**Fig. 1.** Alteration in Femoral Dry Weight of Rats Fed Fermented Soybean (Natto) Diets with Soybean Extract (Nijiru) Supplementation

Sham-operated rats were freely given experimental diets containing natto. OVX rats were given natto diets, with or without supplementation of 3.8% nijiru or 9.8% nijiru, for 3 months. Each value is the mean ± S.E.M. of seven rats. *$p<0.01$, compared with the value for sham-operated rats. #$p<0.025$, compared with the value for OVX rats without nijiru supplementation.

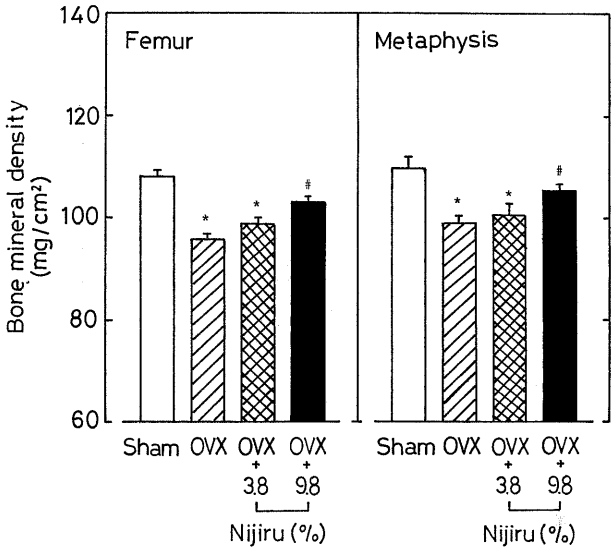


**Fig. 2.** Change in the Mineral Density of the Femur and Femoral-Metaphyseal Tissues in Rats Fed Natto Diets Supplemented with Soybean Extract (Nijiru)

Rats were freely given experimental diets as described in Fig. 1. Each value is the mean ± S.E.M. of seven rats. *$p<0.01$, compared with the value for sham-operated rats. #$p<0.025$, compared with the value for OVX rats without nijiru supplementation.

and calcium was determined by atomic absorption spectrophotometry (Perkin-Elmer Model 303).

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

## RESULTS

Sham-operated rats and OVX rats were freely given experimental natto diets containing soybean extract (nijiru) for 3 months (90 days), and the animals were then sacrificed by bleeding. The amount of dietary intake did not result in any difference between sham-operated rats and OVX rats given experimental natto diets, with or without nijiru supplementation, for 3 months. OVX caused a significant increase in body weight compared with that of sham-operated rats (Table 2). The body weight of OVX rats given experimental diets for 3 months was not significantly different from that of a nijiru groups. Serum inorganic phosphorus concentration was significantly reduced by OVX and this reduction was also seen in OVX rats fed experimental diets containing nijiru (3.8 and 9.8%) (Table 2).

The change in the femoral dry weight of sham-operated and OVX rats given experimental diets is shown in Fig. 1. The femoral dry weight was significantly reduced by OVX and this reduc-

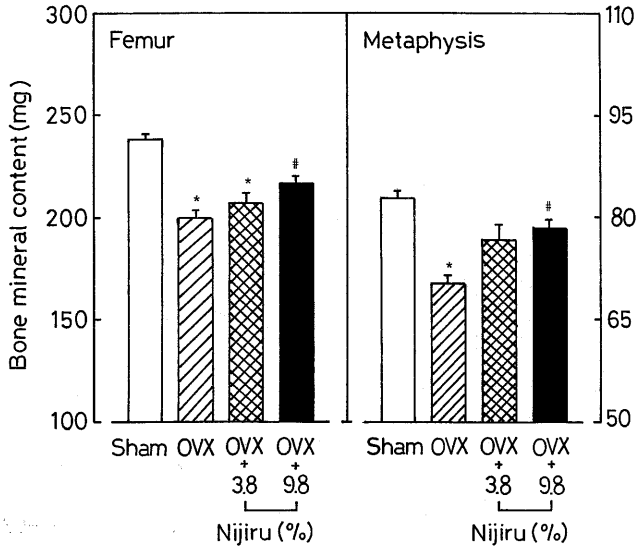


**Fig. 3.** Change in the Mineral Content of the Femur and Femoral-Metaphyseal Tissues in Rats Fed Natto Diets Supplemented with Soybean Extract (Nijiru)

Rats were freely given experimental diets as described in Fig. 1. Each value is the mean ± S.E.M. of seven rats. $^{*}p<0.01$, compared with the value for sham-operated rats. $^{\#}p<0.025$, compared with the value for OVX rats without nijiru supplementation.

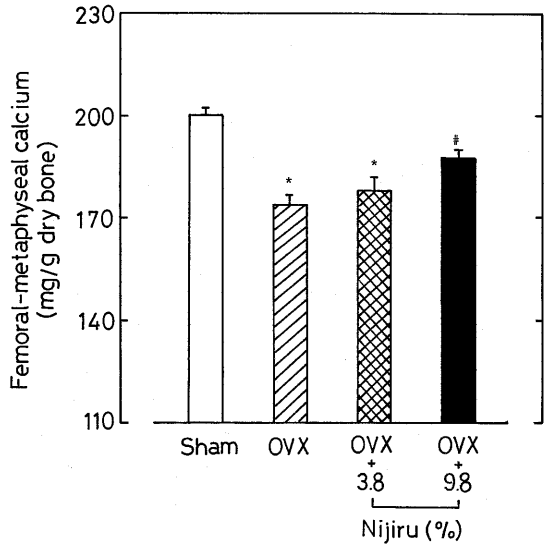


**Fig. 4.** Change in Calcium Content in the Femoral-Metaphyseal Tissues of Rats Fed Natto Diets Supplemented with Soybean Extract (Nijiru)

Rats were freely given experimental diets as described in Fig. 1. Each value is the mean ± S.E.M. of seven rats. $^{*}p<0.01$, compared with the value for sham-operated rats. $^{\#}p<0.025$, compared with the value for OVX rats without nijiru supplementation.

tion was largely prevented by supplementation.

The mineral density and mineral content of the femur and femoral-metaphyseal tissues in sham-operated rats was significantly reduced by OVX (Figs. 2 and 3). This reduction was significantly prevented by the feeding of natto diets with nijiru supplementation (9.8%).

The calcium content in the metaphyseal tissues of the femur of sham-operated rats was significantly reduced by OVX (Fig. 4). Feeding of natto diets with nijiru supplementation (9.8 %) significantly prevented the OVX-induced reduction in metaphyseal calcium content (Fig. 4).

## DISSCUSSION

Bone mass decreases with increasing age and ovarian hormone deficiency at menopause stimulates bone loss.[19-21] Ovariectomy (OVX) causes a lack of estrogen and it has been established that estrogen deficiency induces osteoporosis in humans and in rats.[22] Current hypotheses have linked ovarian hormone deficiency to defects in calcium and bone regulation by the calcitropic hormones.[19-21]

The present study demonstrates that bone weight, bone mineral density and bone mineral content are reduced in OVX rats, indicating that OVX induces bone loss. These reductions were significantly prevented by the feeding of dietary fermented soybean (natto) with supplementation of nijiru containing isoflavon and saponin for 3 months. This finding indicates that OVX-induced bone loss can be prevented by the prolonged intake of dietary natto with isoflavone and saponin supplementation.

The femoral mineral content and femoral-metaphyseal calcium content in OVX rats were significantly increased by the feeding of dietary natto with nijiru supplementation. Nijiru contains large quantites of saponin and isoflavone (including daidzin, daidzein, genistin, and genistein)[15] and isoflavone and saponin can stimulate bone formation and bone mineralization in the femoral tissues of rats.[14,15] Presumably, the preventive effect of natto diets with nijiru supplementation on OVX-induced bone loss results from the action of isoflavone and saponin. More recently, it has been demonstrated that genistein and daidzein can stimulate osteoblastic bone formation and these can inhibit osteoclastic bone resorption,[11-13], thereby inhibiting bone loss.

Natto contains isoflavone, saponin and vitamin $K_2$ (menaquinone-7) which have an anabolic effect on bone metabolism in rats.[14,15,23] Intake of

dietary natto may have a partial preventive effect on OVX-induced bone loss in rats.[23] Isoflavone and saponin supplementation may further enhance the preventive effect of dietary natto on OVX-induced bone loss. Such supplementation is significant in preventing osteoporosis by nutrient factors. The present finding, that the intake of dietary natto with nijiru supplementation has a preventive effect on OVX-induced bone loss in rats, further supports the view that soybean foods are a useful for the prevention of osteoporosis with increasing age.

In conclusion, it has been shown that the intake of dietary natto supplemented with isoflavone- and saponin-containing soybean extract (nijiru) can prevent OVX-induced bone loss in rats, suggesting that it has a nutritional role in the prevention of osteoporosis.

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