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Increase in Bone Components of Rats Orally Administered Isoflovone-containing Soybean Extract (Nijiru)

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The effect of isoflavone-containing soybean extract (nijiru) administration on bone components in rats was investigated. Rats were orally administered nijiru solution (100 mg/ml/100 g body weight) twice a day for 7 and 14 d. Nijiru suspension contained saponin (66 μg/ml), daidzin (77 μg/ml), daidzein (0.6 μg/ml), genistin (58 μg/ml), and genistein (0.6 μg/ml). The solution administered for both periods caused a significant elevation of calcium content, alkaline phosphatase activity, and deoxyribonucleic acid (DNA) content in the diaphyseal and metaphyseal tissues of the femur in rats, while administration for 14 d did not cause a significant alteration in body weight or serum calcium and inorganic phosphorus concentrations, indicating that the solution may not have a toxic effect. This study demonstrates that intake of isoflavone-containing nijiru has an anabolic effect on bone components in rats, suggesting its role in the prevention of osteoporosis.

Key words —— isoflavone, saponin, daidzein, genistein, 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 for which the number 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 micronutrients and macronutrients appears to be strongly implicated in the pathogenesis and the consequences of hip fracture in osteoporotic elderly.[5] Nutritional and pharmacological factors are needed to prevent bone loss with increasing age.

More recently, it has been demonstrated that daidzein and genistein, natural isoflavonoid phytoestrogens found in *Leguminosae*, have an anabolic effect on bone metabolism in rats.[6–10] Isoflavones including daidzin, daidzein, genistin, and genistein are present in soybean at a comparatively higher concentration. Daidzin and genistin are hydrolyzed to daidzein and genistein by β-glucuronidase in gastric juice, respectively. Nijiru is largely produced in the processing of a fermented soybean preparation (natto), and contains great quantities of isoflavones. It is unknown, however, whether nijiru has a functional role in the prevention of osteoporosis.

This study was undertaken to determine the effect of soybean nijiru intake on bone metabolism in rats. We found that the oral administration of nijiru suspension to rats induces an 3 augmentation of bone components in the rat femur, indicating an anabolic effect *in vivo*.

MATERIALS AND METHODS

Animals —— Male Wistar rats, obtained commercially from Japan SLC Inc. (Hamamatsu, Japan) and weighing 95—100 g, were used. The animals were fed commercial laboratory chow (solid) containing 57.5% carbohyrate, 1.1% Ca, and 1.1% P at a room temperature of 25℃, and were allowed distilled water freely.

Administration Procedures —— Nijiru was produced in processing to make a fermented soybean (natto). Soybean was boiled under pressure (1.5 atmospheric pressure) for 40 min at 160℃, and nijiru was produced and was then freeze-dried. Isoflavone content was measured by completely removing it from the nijiru powder by extraction with 80% hot ethanol solution. This ethanol solution was filtered, and the filtrate was injected to HPLC. Isoflavone concentration was expressed as μg per g of nijiru powder.

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Calcium and zinc content in the nijiru powder was measured by atomic absorption spectrophotometry after digestion with HNO_3. Nijiru powder was suspended in distilled water to give a concentration of 50 or 100 mg per ml. Nijiru suspension (50 and 100 mg/ml/100g body weight) was orally administered to rats (weighing 95—100 g) with a stomach tube twice a day (10 a.m. and 4 p.m.) for 7 and 14 d, and 18 h after the last administration the animals were sacrificed by bleeding.

Analytical Procedures —— Rats were killed by cardiac puncture under light anethesia with ether, and the blood and femur were removed immediately. Blood samples were centrifuged for 30 min after collection, and the serum was separated and analyzed immediately. Serum calcium was determined by the method of Willis.[11] Serum inorganic phosphorus was measured by the method of Taussky and Shon.[12]

The femur was removed after bleeding and soaked in ice-cold 0.25 M sucrose solution. It was cleaned of soft tissue and marrow, and diaphysis and metaphysis were separated and dried for 16 h at 110℃ and weighed. The femoral tissues were digested for 24 h at 110℃. Femoral calcium was determined by atomic absorption spectrophotometry.[13] Calcium content was expressed as mg per g dry bone.

To assay alkaline phosphatase activity, the diaphyseal and metaphyseal tissues were immersed in 3.0 ml ice-cold 6.5 mM barbital butter (pH 7.4), cut into small pieces, homogenized with a Potter-Elvehjem homogenizer, and disrupted for 60 s with an ultrasonic device. The supernatant centrifuged at 600 × *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.[14] 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.*[15]

To measure bone DNA content, the diaphyseal and metaphyseal tissues were shaken with 4.0 ml ice-cold 0.1 N NaOH solution for 24 h after homogenization of the bone tissues.[16] After alkali extraction, the samples were centrifuged at 10000 × *g* for 5 min, and the supernatant was collected. DNA content in the supernatant was determined by the method of Ceriotti[17] and expressed as the amount of DNA (mg)/g wet weight of bone tissues.

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

Examination of isoflavone-containing soybean nijiru which has a comparatively higher concentration in foods on bone components in rats showed it to contain great quantities of saponin, daidzin and genistin, and only a slight amount of daidzein and genistein (Table 1). Nijiru suspension (50 and 100 mg/ml 100 g body weight) was orally administered to rats twice a day for 7 d, and 18 h after the last administration the animals were sacrificed by bleeding. The administration of nijiru suspension (100 mg/ml/100 g body weight) caused a significant increase in calcium content, alkaline phosphatase activity, and DNA content in the femoral-diaphyseal and -metaphyseal tissues of rats (Figs. 1, 2, and 3). As shown, these bone components in the diaphyseal

Table 1. Composition of Isoflavones in Nijiru Prepared from Soybeans

Isoflavone	Content (μg/g dry powder)
Saponin	660
Daidzin	770
Daidzein	6
Genistin	580
Genistein	6
Phospholipid	N.D.
Vitamin K_1	N.D.
Calcium	820
Zinc	2

Each value is the mean of three experiments. N.D., not detected.

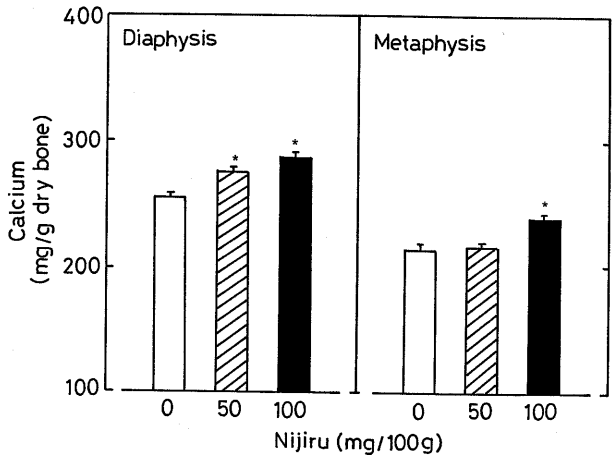

Fig. 1. Alteration in Calcium Content in the Femoral-Diaphyseal and Metaphyseal Tissues of Rats Orally Administered Soybean Nijiru Suspension

Rats were orally administered nijiru suspension (50 and 100 mg/ml/100 g body weight) twice a day for 7 d, and 18 h after the last administration the animals were sacrificed by bleeding. Each value is the mean ± S.E.M. of six rats. *$p<0.01$, compared with the control value.

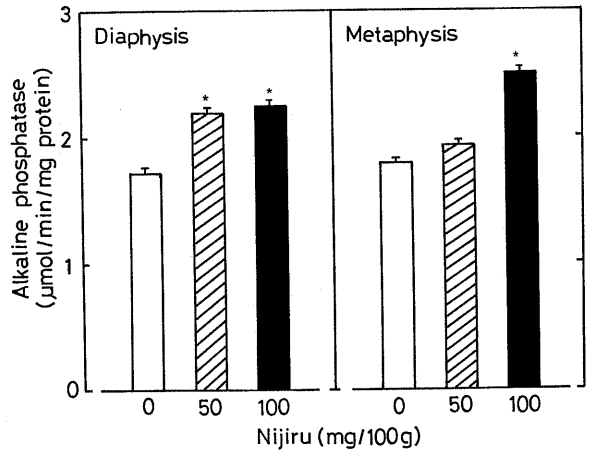

Fig. 2. Alteration in Alkaline Phosphatase Activity in the Femoral-Diaphyseal and -Metaphyseal Tissues of Rats Orally Administered Soybean Nijiru Suspension

Rats were orally administered nijiru suspension (50 and 100 mg/ml/100g body weight) twice a day for 7 d, and 18 h after the last administration the animals were sacrificed by bleeding. Each value is the mean ± S.E.M. of six rats. $*p<0.01$, compared with the control value.

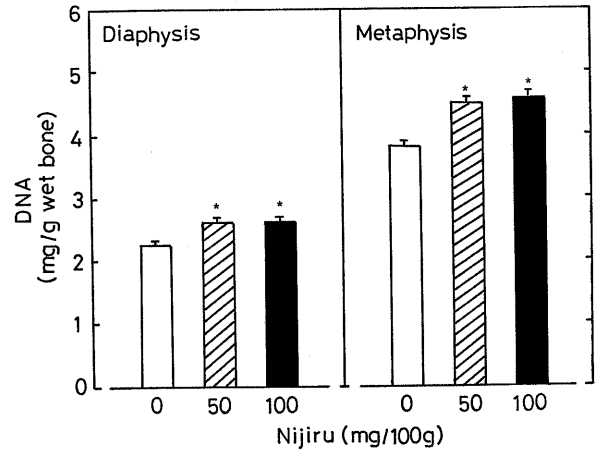

Fig. 3. Alteration in DNA Content in the Femoral-Diaphyseal and -Metaphyseal Tissues of Rats Orally Administered Soybean Nijiru Suspension

Rats were orally administered nijiru suspension (50 and 100 mg/ml/100 g body weight) twice a day for 7 d, and 18 h after the last administration the animals were sacrificed by bleeding. Each value is the mean ± S.E.M. of six rats. $*p<0.01$, compared with the control value.

tissues were significantly elevated by the dose of 50 mg/ml/100 g body weight.

Nijiru suspension was orally administered to rats twice a day for 14 d, and 18 h after the last administration the animals were sacrificed by bleeding. The administration caused a significant elevation of calcium content, alkaline phosphatase activity, and DNA content in the femoral-diaphyseal and metaphyseal tissues (Table 2).

Table 2. Alteration in Bone Components in the Femoral-Diaphyseal and -Metaphyseal Tissues of Rats Orally Administered Soybean Nijiru Suspension

Treatment	Calcium (mg/g dry bone)	Alkaline phosphatase (μmol/min mg/ protein)	DNA (mg/g wet bone)
Diaphysis			
Control	251.1±11.5	1.538±0.081	1.886±0.048
Nijiru	294.9± 8.6*	1.934±0.087*	2.259±0.039*
Metaphysis			
Control	223.0± 6.6	1.411±0.097	3.219±0.042
Nijiru	253.1± 7.3*	2.078±0.061*	3.493±0.014*

Rats were orally administered nijiru suspension (100 mg/ml/100 g body weight) twice a day for 14 d, and 18 h after the last administration the animals were sacrificed by bleeding. Each value is mean ± S.E.M. of six rats. $*p<0.01$, compared with the control value.

Table 3. Body Weights and Serum Calcium and Inorganic Phosphorus Concentrations in Rats Orally Administered Soybean Nijiru Suspension

Treatment	Body weight (g)	Serum calcium (mg/dl)	Serum inorganic phosphorus (mg/dl)
Control	172.7±10.1	10.3±0.10	8.47±0.07
Nijiru	162.5± 5.0	10.4±0.06	7.98±0.17

Rats were orally administered nijiru suspension (100 mg/ml/100 g body weight) twice a day for 14 d, and 18 h after the last administration the animals were sacrificed by bleeding. Each value is the mean ± S.E.M. of six rats. Data were not significant.

Nijiru administration at 100 mg/ml/100 g body weight, however, had no appreciable effect on the body weight, or the serum calcium and inorganic phosphorus concentrations as compared with those of control rats (Table 3).

DISCUSSION

Bone mass decreases with age.[1-3] Osteoporosis with decrease of bone mass is widely recognized as a major puplic health problem. Malnutrition or undernutrition is often observed in the elderly, and deficiency in both micronutrients and macronutrients appears to be strongly implicated in the pathogenesis and the consequences of hip fracture in the osteoporotic elderly.[4-6] Nutritional factors are important to prevent bone loss with increasing age. The chemical compounds that act on bone metabolism as nutrients in food, however, are poorly understood. Recent-

ly it has been shown that daidzein and genistein, natural isoflavonoid phytoestrogens found in *Leguminosae*, have an anabolic effect on bone components, suggesting a role of isoflavonone in the prevention of osteoporosis.[6-10]

Nijiru, which is produced in the process of making fermented soybean (natto), contains great quantities of isoflavone including saponin, daidzin, and genistin. Daisdin and genistin are hydrolyzed by β-glucosidase in gastric juice, and are converted to daidzein and genistein which can induce a potent anabolic effect on bone metabolism.[7,10] The oral administration of nijiru suspension to rats caused a significant increase in calcium content, alkaline phosphatase activity, and DNA content in the diaphyseal and metaphyseal tissues of the femur. Alkaline phosphatase is a marker enzyme of osteoblasts, and the enzyme participates in bone mineralization.[18] DNA content is an index of bone growth and a number of bone cells including osteocytes, osteoblasts, and osteoclasts in bone tissues.[19] Nijiru administration may stimulate bone formation and mineralization in the femoral tissues of rats.

Saponin is converted to sapogenin by β-glucosidase in gastric juice. At present, the effect of saponin and sapogenin on bone metabolism is unknown, although we have preliminary results that saponin (100 μg/100 g body weight) administration has a significant effect on bone components in rats (unpublished data). Moreover, daidzin and genistin can increase bone calcium content.[7,10] Thus, the anabolic effect of nijiru administration on bone components may be the result of saponin, daidzin, and genistin. It is possible that the combination of these chemicals have an additive and/or synergistic effect on bone components.

In conclusion, it has been shown that the intake of isoflavone-containing soybean nijiru has an anabolic effect on bone metabolism, suggesting its nutritional role in the prevention of osteoporosis.

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