

Effects of Dietary Intake of Isoflavone Aglycone-rich Fermented Soybeans on Bone Metabolism in Ovariectomized Rats

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We compared the effect of dietary intake of isoflavone aglycone-rich fermented soybeans (FS) with that of glucoside-rich non-fermented soybeans (NFS) on bone metabolism, using ovariectomized (OVX) rats. Female Sprague-Dawley rats were given OVX or a sham operation at 8 weeks of age. After a 2-week recovery, sham-operated rats were given a NFS diet for 57 days, while OVX rats were given the NFS or FS diet (Sham-NFS, OVX-NFS, and OVX-FS group, $n = 10$). The two diets had the same level of total isoflavone (228 $\mu\text{mol}/100\text{ g}$). The percentage of isoflavone aglycone to total isoflavone was 100% in the FS diet, and approximately 5% in the NFS diet. Tibial bone calcium and phosphorus contents were significantly lower in the OVX-NFS group than in the Sham-NFS group. This reduction was significantly prevented in the OVX-FS group. Tibial trabecular bone density was also significantly reduced by OVX, but the reduction tended to be prevented in the OVX-FS group ($p = 0.1$). The level of urinary deoxypyridinoline (DPD), a bone resorption marker, was significantly increased by OVX; this increase was significantly prevented in the OVX-FS group. Moreover, urinary total isoflavone levels tended to be higher in the OVX-FS group than in the OVX-NFS group ($p = 0.1$). In conclusion, this study suggested that intake of FS prevented excessive bone resorption and bone loss following OVX in rats. It was also suggested that these beneficial effects on bone resulted from higher absorption of isoflavone aglycone.

Key words — isoflavone aglycone, fermented soybean, bone metabolism, ovariectomized, rat

INTRODUCTION

Osteoporosis has become a major health problem worldwide. Especially in women, estrogen deficiency after menopause is a major risk for osteoporosis.¹⁾ Hormone replacement therapy has been used for many years and can be quite effective. However, there is currently a controversy over using such estrogen preparations because of their possible side effects.^{2,3)} Thus, it is necessary to develop alternatives, such as natural food products, which are more moderate and have no side effects.

Isoflavones are flavonoids that are contained mainly in soybeans. They are structurally similar to estrogen, and have weak binding abilities to estrogen receptors.⁴⁾ Thus, they exhibit moderate estrogen-like effects. Many studies have been

reported the beneficial effects of isoflavones on bone in animals^{5,6)} and humans.^{7,8)} Isoflavones are present in soybeans and most soy foods primarily in the glucose-conjugated form (glucoside).⁹⁾ Isoflavone is not absorbed in this form. Once ingested, glucoside must be hydrolyzed to absorbable aglycone by β -glucosidase in the intestinal microflora.^{10,11)} Therefore, it is commonly considered that aglycone is absorbed faster than glucoside.¹²⁾ On the other hand, the bioavailability of aglycone and glucoside remains controversial. Izumi *et al.*¹³⁾ reported that the amount of isoflavones absorbed is higher for the aglycone than the glucoside form. Kano *et al.*¹⁴⁾ reported that the amount of isoflavones absorbed was higher in aglycone-rich fermented soymilk than in glucoside-rich non-fermented soymilk; however, other studies have reported no significant difference in the bioavailability of aglycone and glucoside forms.^{15,16)} These apparently different results may be partly because of the amount of isoflavone intake or the food matrix containing the isoflavone.¹²⁾ For example, Setchell

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*et al.*¹⁷⁾ suggested that intake of large doses of isoflavones at one time did not necessarily enhance the bioavailability of isoflavones from the pharmacokinetic view. Cassidy *et al.*¹⁸⁾ reported the greater bioavailability of isoflavones in soymilk than in textured vegetable protein. There has not been enough systematic research considering the various factors potentially affecting the pharmacokinetics of isoflavones.

Nonetheless, it makes sense to speculate that aglycone is superior to glucoside in that its first step of absorption is independent of intestinal microflora, which can be influenced by many factors, such as aging, food, mental condition, and so on.^{19,20)}

We developed a new type of fermented soybeans produced by fermenting soybeans with koji (*Aspergillus oryzae*) under salt-free conditions. The product is unique in that more than 90% of isoflavones are present in the aglycone form. This is because the activity of koji considerably increases under salt-free conditions, which enables hydrolyzation of isoflavone to aglycone in a very short time.²¹⁾ At present, we call this new material “isoflavone aglycone-rich fermented soybeans” (FS). Traditional fermented soy foods, like miso, natto, and tempeh, have isoflavone aglycone, too, but at lower rates. Miso has about 65% aglycone-form isoflavone,²²⁾ natto has about 9%,²²⁾ and tempeh has about 37%.²³⁾ Therefore, it is speculated that FS will exhibit superior beneficial effects on bone through the high absorption of isoflavone aglycone. To our knowledge, there have been few studies clearly showing the superior bone-protective effects of aglycone or aglycone-rich soy foods as compared to glucoside or glucoside-rich soy foods.

In this research, we compared the effects of isoflavone aglycone-rich FS with isoflavone glucoside-rich non-fermented soybeans (NFS) on bone metabolism in ovariectomized (OVX) rats. We also investigated the isoflavone absorption by prolonged intake of FS and NFS.

MATERIALS AND METHODS

Animals—Female Sprague-Dawley rats (7 weeks old) were obtained from Charles River Laboratories Japan (Yokohama, Japan). All rats were kept in a room with controlled lighting (lights on 08:00–20:00), temperature ($22 \pm 3^\circ\text{C}$), and humidity ($50 \pm 20\%$). They were individ-

ually housed in standard stainless steel cages ($225 \times 185 \times 200$ mm). Rats were given free access to water and commercial diets (CRF1; Oriental Yeast, Tokyo, Japan). After a 7-day adaptation period, rats were given a bilateral OVX or sham operation. In the sham-operated rats, both ovaries were handled, but not removed. After a 14-day recovery period, experimental diets were initiated. The rats were maintained in accordance with the guidelines of the ethical committee for the Animal Experiments of Ajinomoto Co. Inc. (Kawasaki, Japan) Institute.

Diets—FS was produced in a similar way as miso, but without using salt. Soybeans were steamed and fermented with koji. The obtained paste was vacuum drum dried, then milled into powder. All of the isoflavones in the FS powder were aglycones (Table 1). At the same time, NFS was produced by steaming the same soybeans, and then drying in the same way. As a result, 5% of its isoflavones were aglycones, and 95% were glucosides (Table 1). Isoflavones were analyzed by HPLC-UV, using the method of Kudou *et al.*²⁴⁾

FS powder (39.5%) or NFS powder (40.7%) was added to the diets so that both diets had the same amount of isoflavones ($228 \mu\text{mol}/100$ g diet). Both diets were adjusted to contain 0.5% calcium (Ca), 0.3% phosphorus (P), 10.7% fat, and 15.5% protein. The composition of the diets is shown in Table 2.

Experimental Protocol—After a 14-day post-operative recovery, sham-operated rats were given

Table 1. Isoflavone Concentration in NFS and FS powder

$\mu\text{mol}/100$ g	NFS powder	FS powder
daidzin	173.1	— ^{a)}
genistin	295.1	—
glycitin	6.4	—
acetyldaidzin	17.4	—
acetylgenistin	34.8	—
acetylglycitin	—	—
malonyldaidzin	—	—
malonylgenistin	6.1	—
malonylglycitin	—	—
daidzein	11.2	230.3
genistein	15.6	338.9
glycitein	—	5.8
total isoflavone	559.6	575.0
total glucoside	532.9	—
total aglycone	26.8	575.0

a) Detection limit was $0.5 \text{ mg}/100$ g.

Table 2. Composition of NFS and FS Diet

Ingredients (g/100 g)	Diet	
	NFS	FS
dextrin	38.2	39.5
sucrose	10.0	10.0
soy oil ^{a)}	0	0.2
AIN93G mineral mixture ^{b)}	3.5	3.5
AIN93 vitamin mixture ^{c)}	1.0	1.0
BHT	0.0014	0.0014
cellulose	5.0	5.0
CaCo ₃ ^{a)}	1.049	1.057
KH ₂ PO ₄ ^{a)}	0.158	0.178
NFS powder	40.7	0
FS powder	0	39.5
defatted soy protein ^{a, d)}	0.487	0
Total	100	100
Calculated Values		
Fat (%)	10.7	10.7
Protein (%)	15.5	15.5
Carbohydrate (%)	65.0	64.4
Isoflavones (μmol/100 g)	228	228
aglycone	11	228
glycoside	217	0
Ca (%)	0.5	0.5
P (%)	0.3	0.3

a) Contents were adjusted and added so that the total amounts of fat, protein, Ca, and P in the two diets were equal (FS and NFS themselves were unequal). b) Sucrose was substituted for CaCO₃ and KH₂PO₄. c) Contained 25% choline bitartrate. d) Isoflavone-free defatted soy protein (<1 μmol/100 g).

the NFS diet (Sham-NFS) for 57 days. Half of the OVX rats were given the NFS diet (OVX-NFS), and the other half was given the FS diet (OVX-FS). Each group had 10 rats. The Sham-NFS group was given free access to the diet. The OVX-NFS and OVX-FS groups were pair-fed.

After 3 weeks of the experimental diet, blood and urine samples were collected to analyze the markers of bone turnover. On day 19, blood samples were collected from the subclavian vein. Serum samples were obtained by centrifugation at 1500 × g at 4°C for 15 min and stored at -70°C until analysis for osteocalcin. On day 24, urine samples were collected for 24 hr, using metabolic cages, and stored at -20°C until analysis for deoxyypyridinoline (DPD).

On day 53, urine samples were collected for 24 hr and stored at -20°C until analysis for isoflavones. On the final day of the experiment, after overnight fasting, rats were anaesthetized with diethyl ether. Blood samples were collected from the inferior vena cava, and then the rats were sacrificed. The organs were removed immediately. The

right tibial bone was carefully cleaned of surrounding tissue, and kept in 70% ethanol until the bone density analysis. The left tibial bone was stored with surrounding tissue at -70°C until the Ca and P analysis.

Analytical Methods—

Ca and P Contents in Tibia: The left tibial bone was cleaned of surrounding tissue, and dried at 110°C for 12 hr. Dried samples were ashed at 680°C for 24 hr. Ashed samples were dissolved by 1.1 ml of 6N HCl, and diluted appropriately with distilled water for measurement. Ca and P contents in these samples were measured with commercial kits [Ca-C-test Wako; Wako Pure Chemical Industries (Osaka, Japan), Phospha-C-test Wako; Wako Pure Chemical Industries].

Bone mineral density in tibia by peripheral quantitative computed tomography (pQCT): The bone mineral density at the metaphysis of the right tibia was estimated by using pQCT (model XCT research SA+; Stratec Medizintechnik, Pforzheim, Germany). The metaphysis was defined as 3 mm below the articular surface. The voxel size was 0.12 × 0.12 × 0.77 mm. The trabecular bone density was calculated as the area not over 395 mg/cm³.

Urinary DPD and Creatinine: Urinary DPD from the 24-hr sample, collected during on day 24, was measured with the commercial kit (Osoteolinks-DPD; DS Pharma Biomedical, Osaka, Japan). Urinary creatinine of the same sample was measured with 7150 Hitachi Clinical Analyzer (Hitachi High-technologies, Tokyo, Japan).

Urinary Isoflavones and Creatinine: Urinary isoflavones in the 24-hr sample collected on day 53 were analyzed by using HPLC, as previously reported.²⁵⁾ All samples were analyzed at SRL, Tokyo, Japan.

Two hundred μl of appropriately diluted urine sample was added to acetate buffer (pH 4.5) containing β-glucuronidase and sulfatase (Nippon Bio-Test Laboratories, Tokyo, Japan) and incubated overnight at 37°C. Released aglycone was extracted with ethyl acetate. The organic layer was isolated and dried under nitrogen stream at 40°C. The residue was dissolved in a solvent containing methanol, acetonitrile, dimethyl sulfoxide, and water, and used for HPLC analysis. The isoflavones daidzein, glycitein, genistein, and equol were separated with a column-switching HPLC system (LC-10Avp series; Shimadzu, Kyoto, Japan), and detected by UV absorption. The pre-column was Wakosil-2 RS 4.6 × 50 mm (Wako Pure Chemical

Industries). The main column was CAPCELL PAK AQ 4.6 × 250 mm (Shiseido, Tokyo, Japan). The separate condition was isocratic, using phosphate buffer containing acetonitrile and sodium perchlorate as the mobile phase. Creatinine of the same sample was measured with 7170 Hitachi Clinical Analyzer (Hitachi High-technologies).

Serum Osteocalcin: Serum osteocalcin from blood sample, collected on day 19, was measured with the commercial kit (Rat-MID Osteocalcin ELISA, IDS Nordic a/s, Herlev, Denmark).

Statistical Analysis—Data were expressed as means and standard error of mean (SEM). For urinary isoflavone data, significant differences between OVX two groups were determined by unpaired *t*-test (Statview-J 5.0, SAS institute, Cary, NC, U.S.A.). For the other data, significant differences among three groups were determined by one-way analysis of variance (ANOVA) and Fisher’s PLSD test as a post-hoc test (Statview-J 5.0, SAS institute). Differences were considered significant at *p* < 0.05.

RESULTS

Body Weight, Food Intake, and Uterine Weight

The OVX-NFS and OVX-FS groups had significantly higher mean food intake and body weight than the Sham-NFS group, but were not significantly different from each other for those values (Table 3). The OVX-NFS and OVX-FS group had significantly lower uterine weight than the Sham-NFS group, but were not significantly different from each other.

Bone Density

The trabecular bone density at the metaphysis of the tibia is shown in Fig. 1. Compared to the Sham-NFS group, the trabecular bone density was significantly reduced in the OVX-NFS group. This reduction tended to be prevented in the OVX-FS group; the OVX-FS group tended to have higher trabecular bone density than the OVX-NFS group.

Bone Ca and P Contents in the Tibia

Ca and P contents in the tibia are shown in Fig. 2. Compared to the Sham-NFS group, Ca content in the tibia was significantly lower in the OVX-NFS group. This reduction was significantly prevented in the OVX-FS group; the OVX-FS group had significantly higher Ca content in the tibia than the OVX-NFS group. Likewise, P content in the tibia was significantly reduced by OVX and significantly prevented in the OVX-FS group. The OVX-FS group had a significantly higher P content in the tibia than the OVX-NFS group.

Urinary DPD and Serum Osteocalcin

The day-24 urinary excretion of DPD, a bone resorption marker, is shown in Fig. 3 in terms of its ratio to endogenous creatinine excretion. Compared to the Sham-NFS group, urinary excretion of DPD was significantly increased in the OVX-NFS group. This increase was significantly prevented in the OVX-FS group; the OVX-FS group had a significantly lower DPD level than the OVX-NFS group.

The day-19 serum osteocalcin, a bone formation marker, is shown in Fig. 4. Compared to the Sham-NFS group, serum osteocalcin was signifi-

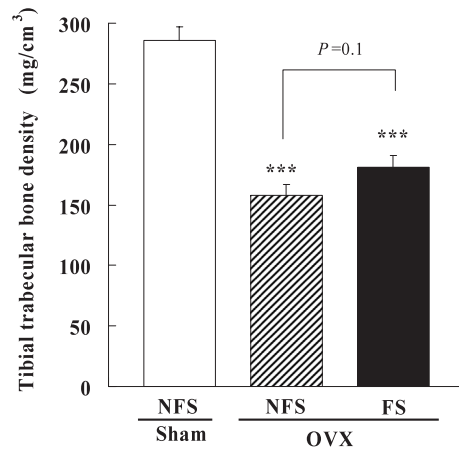


Fig. 1. Tibial Trabecular Bone Density in Rats Fed NFS or FS Diet for 57 Days

Values are means ± SEM for ten rats. *** *p* < 0.001, Significantly different from Sham-NFS group.

Table 3. Final Body Weight, Food Intake, and Uterine Weight in Rats Fed NFS or FS Diet for 57 Days

	Sham-NFS	OVX-NFS	OVX-FS
Final body weight (g)	261.4 ± 4.4 a	368.0 ± 8.5 b	366.5 ± 7.9 b
Average food Intake (g)	13.6 ± 0.2 a	17.4 ± 0.5 b	18.1 ± 0.3 b
Uterine (g/100 g BW)	0.210 ± 0.016 a	0.031 ± 0.002 b	0.033 ± 0.003 b

Values are means ± SEM for ten rats. Values in a row not sharing a common letter differ significantly (*p* < 0.001).

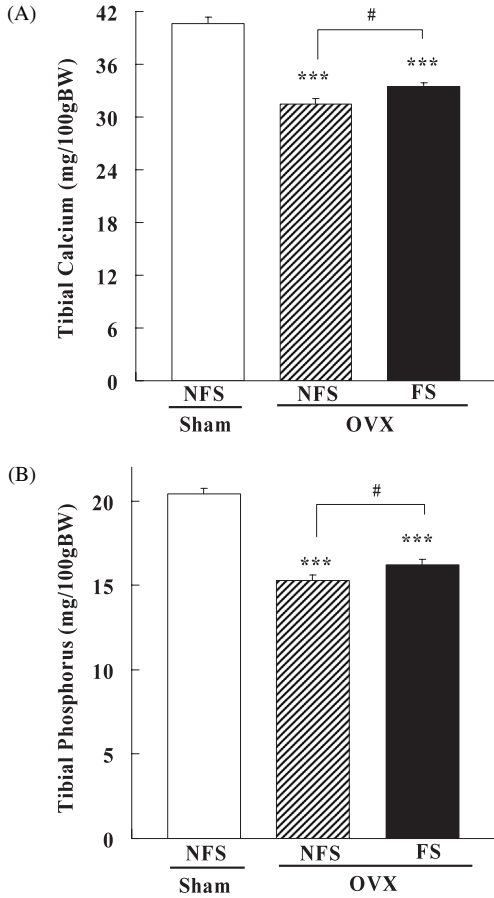


Fig. 2. Tibial Mineral Contents in Rats Fed NFS or FS Diet for 57 Days

A, Calcium; B, Phosphorus. Values are means \pm SEM for ten rats. *** $p < 0.001$, Significantly different from Sham-NFS group. # $p < 0.05$, Significantly different from OVX-NFS group.

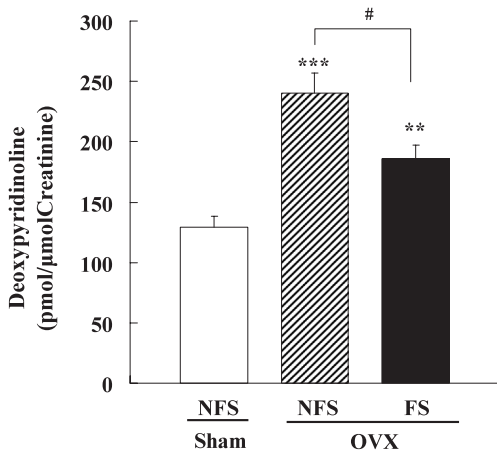


Fig. 3. Urinary deoxyypyridinoline at day 24 in Rats Fed NFS or FS Diet

Values are means \pm SEM for ten rats. *** $p < 0.001$, ** $p < 0.01$, Significantly different from Sham-NFS group. # $p < 0.05$, Significantly different from OVX-NFS group.

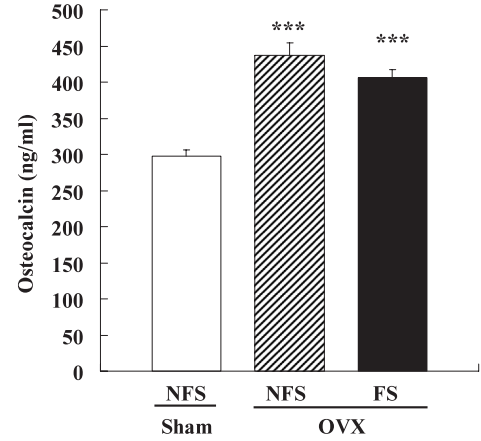


Fig. 4. Serum osteocalcin at day 19 in Rats Fed NFS or FS Diet

Values are means \pm SEM for ten rats. *** $p < 0.001$, Significantly different from Sham-NFS group.

cantly increased in the OVX-NFS group. There was no difference between the OVX-NFS and OVX-FS groups.

Urinary Isoflavones

The day-53 urinary excretion of isoflavones in the OVX groups is shown in Fig. 5 in terms of their ratios to endogenous creatinine excretion. The urinary daidzein level in OVX-FS group was significantly higher than that in OVX-NFS group. Genistein and total isoflavone level in OVX-FS group also tended to be higher than those in OVX-NFS group ($p = 0.1$ for both). The amount of total isoflavone was estimated as the sum of genistein, daidzein, glycitein, and equol. Glycitein and equol levels didn't differ significantly between the two groups.

DISCUSSION

The purpose of this study was to compare the effects of prolonged dietary intake of FS and NFS on bone metabolism, using OVX rats. Compared to the Sham-NFS group, the trabecular bone density and mineral contents in the tibia of rats in the OVX-NFS group were significantly reduced. However, these reductions were prevented in the rats fed FS for 8 weeks. This result suggested that FS exhibited more effective isoflavone action than did NFS.

The bone loss caused by OVX has been reported to be prevented by isoflavone glucoside administration.²⁶⁾ Arjmandji *et al.*²⁷⁾ reported the bone-protective effect of dietary ingestion of isoflavone

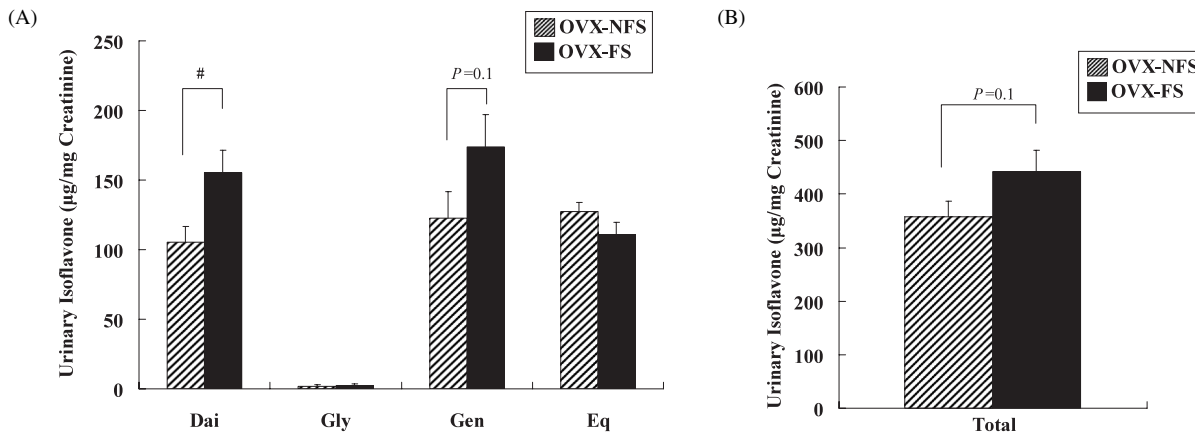


Fig. 5. Urinary isoflavones at day 53 in Rats Fed NFS or FS Diet

A, respective isoflavones; B, Total isoflavone. Values are means \pm SEM for ten rats. # $p < 0.05$, Significantly different from OVX-NFS group. Dai, Daidzein; Gly, Glycitein; Gen, Genistein; Eq, Equol. Total isoflavone: Dai, Gly, Gen, Eq.

glucoside-rich soy protein. The bone-protective effects of isoflavone aglycone have also been reported. Mathey *et al.*²⁸⁾ reported that isoflavone aglycone administration improved bone mineral density in OVX rats. Lien *et al.*²⁹⁾ reported that dietary ingestion of aglycone had a favorable effect on bone mineral contents in OVX rats. In addition, Kawakami *et al.*³⁰⁾ compared the effects of aglycone and glycoside on lipid metabolism in rats and suggested the superiority of aglycone. These previous reports agree with the results of the present study, in which prolonged dietary intake of FS showed protective effects on bone loss in OVX rats. The effects of FS appeared to be greater than those of NFS.

In this study, an 8-week intake of FS showed protective effects against bone loss. Mathey *et al.*³¹⁾ reported that a 12-week intake of isoflavone at the same level as that in the present study (228 $\mu\text{mol}/100\text{ g} = 0.06\% \text{w/w}$ diet) improved femoral bone strength. A longer intake of FS may result in even more improvement of bone strength.

In our preliminary study, a 4-week intake of FS tended to prevent the loss of mineral contents, but not at a significant level (data not shown). In general, the bone-sparing effects were reported after more than a 4-week intake. Deyhim *et al.*³²⁾ reported that at a low dose (about 0.014%), isoflavones had no effect on bone mineral contents in such a short time. Using the same dose of isoflavones as we used in the present study, Hidaka *et al.*³³⁾ reported that a 7-week intake improved the tibial bone density. Therefore, we extended the intake period to 8 weeks in this study and clearly confirmed the bone-sparing effects of FS.

Food intake and body weight were significantly higher in the OVX group than in the Sham group. In general, OVX has been reported to cause excessive eating.³⁴⁾ However, the bone density and mineral contents were significantly lower in the OVX group than in the Sham group. This established that the bone loss was definitely caused by OVX. Moreover, the primary goal of this study was to compare the effect on bone of FS and NFS, and the food intake of the OVX-FS and OVX-NFS groups was not significantly different.

OVX is commonly known to induce excessive bone resorption and formation in rats.³⁵⁾ Bone resorption exceeds formation, thereby causing bone loss. That is, a drastic change in bone turnover markers is observed in advance of change in bone mineral contents, especially shortly after OVX. Picherit *et al.*³⁶⁾ reported that the urine DPD level increased by 1.5-fold at 5.7 weeks after OVX and returned to the baseline at 11 weeks. Serum osteocalcin level also increased by 1.6-fold at 5.7 weeks, and returned to the baseline at 17 weeks. Therefore, in this study, the bone turnover markers were measured at 5 weeks after OVX (= 3 weeks after the start of dietary intake) to properly evaluate the changes. Urinary DPD results in this study agree with this previous knowledge. Compared to the Sham-NFS group, the urinary DPD level was significantly increased in the OVX-NFS group. This increase was significantly prevented in the rats fed FS, suggesting that dietary intake of FS inhibits the excessive bone resorption induced by OVX. We also measured serum osteocalcin, as a bone formation marker and found that it was significantly increased by OVX, but there was no difference between the OVX-

FS and OVX-NFS groups. Therefore, we speculated that FS exerted bone-protective effects mostly through preventing the excessive bone resorption caused by OVX. On the other hand, isoflavones have been reported to affect bone resorption and formation *in vitro*.^{37–40} Some authors have suggested that the actions of daidzein and genistein are slightly different,⁴¹ and the detailed mechanism of their action is still unclear. Further studies on the mechanism of the bone protective effects by FS are necessary.

Urinary isoflavone levels showed a trend of being higher in rats fed FS for 8 weeks than in rats fed NFS. It was suggested that the bone-protective effects of FS may result from higher absorption of isoflavone aglycone. Kawakami *et al.*³⁰ also reported that the serum isoflavone level was higher in rats fed an aglycone-rich diet than in rats fed a glucoside-rich diet, speculating that this increase might be attributed to the beneficial effects on lipid metabolism. In this study, FS and NFS had equal amounts of fat, protein, Ca, and P but different isoflavone types. Therefore, it was speculated FS enabled the higher absorption of isoflavone because its isoflavones were in the aglycone form. Of course, the two diets were also different in the decomposition level of their protein contents. Proteins in FS were mostly degraded, with largely free amino acids. On the other hand, proteins in NFS were kept as proteins with few free amino acids. To our knowledge, there have been few studies reporting that free amino acids affect the absorption of isoflavones. Further studies should be done, with careful consideration of what other factors might affect the absorption of isoflavones.

In conclusion, this study revealed that prolonged intake of FS prevented excessive bone resorption and subsequent bone loss in OVX rats. It was also suggested that these beneficial effects on bone resulted from the higher absorption of aglycone-form isoflavones.

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