A Combination of Dietary Fructooligosaccharides and Isoflavone Conjugates Increases Femoral Bone Mineral Density and Equol Production in Ovariectomized Mice

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ABSTRACT Fructooligosaccharides (FOS) stimulate the growth of bifidobacteria, which cleave isoflavone conjugates to yield the corresponding aglycones and metabolites. In a previous study, FOS modified the absorption and enterohepatic recirculation of isoflavones in rats. In the present study, we determined the effect of the combination of dietary FOS and isoflavone conjugates on bone mass in ovariectomized (OVX) and surgical control mice. After undergoing OVX or sham operation, female ddY mice (8 wk old, n = 64) were randomly assigned to four groups: a purified control diet (AIN-93G) group, a FOS diet (AIN-93G + 5% FOS) group, an isoflavone diet (AIN-93G + 0.2% isoflavone conjugates) group, or a FOS and isoflavone diet (AIN-93G + 5% FOS + 0.2% isoflavone conjugates) group. After 6 wk, the mice were killed and the blood and femora were sampled immediately. In OVX mice, both isoflavone conjugates and FOS prevented femoral bone loss. An additive effect of dietary isoflavone conjugates and FOS was observed by dual-energy X-ray absorptiometry in the distal part of the femur and in trabecular bone, by peripheral quantitative computed tomography. Moreover, FOS increased cecal β-glucosidase activity and equal production from daidzein in both OVX and surgical control mice fed isoflavone conjugates. These results suggest that FOS increase the bioavailability of isoflavones, leading to cooperative effects in the prevention of osteopenia in OVX mice. J. Nutr. 132: 2048–2054, 2002.

KEY WORDS: • bone mineral density • fructooligosaccharides • genistein • daidzein • equol • mice

All people experience bone loss with aging, but it is especially pronounced in early-stage postmenopausal women, who show a rapid decrease in bone mineral density (BMD).2 Osteoporosis, which can be due to estrogen deficiency after menopause, is a serious public health problem worldwide. Osteoporosis is a major contributor to the high frequency of bone fracture in elderly people. Therefore, many drugs have been developed to prevent osteoporosis. In exercise therapy, the mechanical stress of exercise stimulates bone formation (1–3); however, this method has some risk for fracture in the elderly. Another approach is the supplementation of nutrients such as calcium (Ca) and vitamin D. These nutrients are required for normal bone formation, and insufficient intake may be a cause of osteopenia (4,5). It has been reported that osteopenia arises not only due to a decrease in Ca intake but also due to a decrease in intestinal Ca absorption (6). Vitamin D, which is involved in Ca absorption, can have hypercalce- mia as a side effect (7,8). The most effective approach for preventing postmenopausal osteoporosis is hormone (estrogen) replacement therapy (9,10). However, this therapy increases the risk of breast and uterine cancers (11,12).

The medical community must establish a safer and more efficient therapy for preventing or improving osteoporosis. One solution to this problem is the use of selective estrogen receptor modulators, which selectively act on bone and cardiovascular systems without exhibiting substantial estrogenic action in the reproductive organs (13). Isoflavonoids are structurally similar to estrogens and bind to estrogen receptors, suggesting that they exhibit estrogenic action in various tissues. The isoflavones in soybeans are called phytoestrogens, and it has been suggested that they prevent sex hormone-related cancers, such as breast and prostate cancer (14–18).

Previously, Ishimi et al. (19) reported that genistein, a major soybean isoflavone, acted on bone as a selective estrogen receptor modulator in ovariectomized (OVX) mice. Several lines of study also have demonstrated that soybean protein and isoflavones (ISO) prevent bone loss in OVX rats.

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and mice (20–26). Furthermore, some reports suggested that soybean protein or ISO supplementation prevents postmenopausal bone loss in humans (27–30). It has been also reported that a synthetic isoflavone derivative is rather useful and safe compared with estrogen therapy in treating low bone mass or osteoporosis in postmenopausal women (31).

Fructooligosaccharides (FOS), the indigestible sugar that increases intestinal absorption of Ca, magnesium (Mg) and iron by stimulating growth of beneficial intestinal bacteria such as bifidobacteria (32,33), also have been shown to prevent osteopenia in several animal models. We reported that FOS stimulates bone formation by increasing Ca absorption from the large intestine in normal (34), OVX (35) and gastrectomized rats (36,37). The stimulatory effect of FOS on Ca absorption also was confirmed in human clinical studies (38,39). Thus, we propose that FOS supplementation would improve osteoporosis.

Recently, we reported that FOS improves the bioavailability of genistein and daidzein in rats given ISO conjugates (40). This evidence suggests that the combination of dietary FOS and ISO may be more efficient than either alone in the prevention of bone loss in osteoporosis. The purpose of this study was to determine whether FOS and ISO show a cooperative effect on the prevention of osteoporosis by measuring femur BMD in OVX mice fed both FOS and crude soybean ISO conjugates or each supplement alone.

MATERIALS AND METHODS

This study was approved by the Animal Studies Committee of Meiji Seika Bioscience Laboratories, and the mice were maintained in accordance with their guidelines for the care and use of laboratory animals. Female ddY strain mice (6 wk old; n = 64) were fed an AIN-93G diet (41) for a 2-wk adaptation period before surgery. The mice were randomly assigned to two groups of 32 each. All mice were anesthetized by flutamide inhalation and underwent either OVX or surgery in which the ovaries were not removed (sham-operated surgical control: SH). Dietary treatments began on d 7 post-surgery, at which time the 32 mice in each surgical treatment group were randomly divided into four diet groups [a control diet, a 5.0% FOS diet (F diet), a 0.5% crude ISO conjugate diet (I diet), and a FOS and ISO-containing diet (FI diet)]. Table 1 shows the composition of the experimental diets. Corn oil was used to eliminate any possible contamination with isoavones in soybean oil. FOS (Meioligo-P; Meiji Seika Kaisha, Tokyo, Japan) was a mixture of 42% I-kestose, 46% nystose and 9% 1F-betafructofuranosylnystose. Supplemented Fuji-avone P40 (ISO content: 40%, Fuji-Jeco, Japan), the crude ISO conjugate, contained daidzin (20.4%), malonyldaizin (0.1%), acetyldaizin (1.1%), daidzein (0.3%), genistin (4.6%), acetylgensitin (0.3%), genistein (0.1%) and glycinin plus glycitin (13%). As their aglycones, 33 mg of daidzein, 8.5 g of genistein and 15 mg of glycinin were included in 100 mg of conjugates. The mice were fed their respective diets for 6 wk.

The mice were allowed free access to deionized water throughout the experiment. On the final day of the experiment, all mice were anesthetized by exposure to diethyl ether. After laparotomy, whole blood was collected by abdominal vein puncture, and the mice were killed. The femora, uterus and blood were sampled immediately. The femora, uterus and blood were sampled immediately.

Ca, Mg, and P contents of femora. Each left femur removed was first dried at 80°C for 18 h in a ceramic pot to evaluate the dry bone weight, and then ashed at 550°C for 24 h to determine the ash weight. The ashed samples were dissolved in 4 mL of 2 mol/L HCl, then diluted appropriately with distilled water for atomization. After these pretreatments, the amounts of Ca, Mg and P in the femur sample solution were quantitated with an inductively coupled plasma emission spectrophotometer (ICPS-7000, Shimadzu, Kyoto, Japan).

Bone mineral content, bone area, and bone mineral density in femora by dual energy X-ray absorptiometry (DXA). Bone mineral content (BMC; mg), BMD (mg/mm²), and bone area (BA; mm²) of the right femur of each mouse were measured by dual-energy X-ray absorptiometry (DXA; model DCS-600A; Aloca, Tokyo, Japan). The BMD was calculated by BMC of the measured BA. The scanned area of each mouse femur was divided into three equal parts (5.3 mm each): proximal, midshaft and distal femur.

Bone mineral density in femora by peripheral quantitative computed tomography (pQCT). Various parameters were assessed in cross section using pQCT (model XCT-960A Norland Stratec, Birkenfeld, Germany). The measurement in the right femur was started at the metaphysis 3 mm below the articular surface, visualized with the help of the scout-view. The cross section was made at a distance of 1 mm. The section for analysis was defined with a clearly complete cortical ring. The voxel size was 0.08 mm and the threshold was 0.464 (at contour mode 2, peak mode 2). The measured parameters were total bone mineral content (mg), total mineral density (mg/cm³), cortical and subcortical mineral density (mg/cm³), trabecular mineral density (mg/cm³), total bone area (mm²), cortical area (mm²) and area of the medullary cavity (trabecular bone; mm²).

Time-resolved fluoroimmunoassay (TR-FIA) for measuring plasma genistein, daidzein and equol. TR-FIA (42) is a solid phase fluoroimmunoassay, based on competition between europium-labeled phytoestrogens and sample phytoestrogens for polyclonal antiphytoestrogen antibodies (derived from rabbits). Using the methods of Wang et al. (43), plasma genistein and daidzein were analyzed. Equol in plasma was analyzed by the TR-FIA method of Brouwers et al. (unpublished data). Comparing the equal assay results obtained by the TR-FIA method and those obtained by gas chromatography-mass spectrometry, a strong correlation was evident (r = 0.977, P < 0.001, n = 61). Immunoassay, immunization and labeling of isoflavanoid derivatives with europium chelate (in the case of genistein and daidzein), and standards of genistein, daidzein and equol were performed as previously described (43,44). For the recovery calculation, 20 μL of "H-estradiol glucuronide was added to tubes containing 100 μL of plasma. After mixing and equilibrating for 30 min at room temperature, 100 μL of 0.1 mol/L (pH 5.0) acetate buffer containing 200 μL glutaronidase and 2000 μL sulfatase was added to the tubes. After mixing with a Vortex mixer and incubating overnight at 37°C, 2.0 mL of diethyl ether was added, and the phytoestrogens were extracted after equilibrating the phases with a Vortex mixer. The phase was transferred into disposable glass tubes. After thawing, the water phase was reextracted with the same amount of ether, and the
RESULTS

Body and uterine weights. There were no significant differences in initial body weight among the groups (Table 2). Final body weight was lower in the OVX-I mice than in the OVX-F mice (P < 0.05). Uterine weight was decreased by OVX (P < 0.05), but there were no differences among the four OVX groups.

Femur Ca, Mg, and P contents. Ash weight was decreased by OVX (P < 0.05) (Table 3). There were no differences in ash weight between SH and OVX mice fed F and FI diets. Ash weight was greater in the OVX-I and OVX-FI groups than in the OVX-control group (P < 0.05). Ca and P contents in femora were decreased by OVX (P < 0.05). Ca content was greater in the OVX-I and OVX-FI groups than in the OVX-control group (P < 0.05). There was no difference in femoral Ca content between the SH-F and OVX-F groups. Femoral Mg content was higher in the OVX-I and OVX-FI groups than in the OVX-control group (P < 0.05).

Femur bone mineral content and bone mineral density by DXA and by pQCT. The three main effects (O, F, I) and the interaction O × I were significant (P < 0.05) for total and midshaft BMC. Among SH groups, total BMC was greater in mice fed the I (1.48 ± 0.11 mg) and the FI diets (1.56 ± 0.06 mg) than in mice fed the control diet (1.30 ± 0.06 mg) (P < 0.05). In OVX mice, total BMC was greater in mice fed the F, I or FI diet (F, 1.38 ± 0.10; I, 1.36 ± 0.07; FI, 1.44 ± 0.11 mg) than in mice fed the control diet (1.26 ± 0.04) (P < 0.05). BMC at the proximal femur was affected by FOS (F) and the O × F × I interaction (P < 0.05). In OVX mice, proximal BMC was greater in mice fed the F diet (0.53 ± 0.05 mg) than in mice fed the control diet (0.47 ± 0.02 mg) (P < 0.05). In midshaft femur, BMC was greater in SH mice fed the F (0.38 ± 0.03 mg) and FI diets (0.40 ± 0.02) compared with mice fed the control diet (0.33 ± 0.03) (P < 0.05). OVX mice did not differ in midshaft femur BMC. Distal femur BMC was greater in SH mice fed the I (0.62 ± 0.07 mg) or FI diet (0.62 ± 0.04 mg) compared with SH mice fed the control diet (0.47 ± 0.02 mg) or F diet (0.51 ± 0.06 mg). Among OVX groups, distal femur BMC was greater in mice fed the I (0.51 ± 0.04 mg) or the FI (0.57 ± 0.06 mg) diet than in mice fed the control diet (0.44 ± 0.03 mg).

The BMD in both the total femur and the distal regions was decreased by OVX (P < 0.05).

TABLE 2

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<th>OFI</th>
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<td>Control</td>
<td>F</td>
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<td>36.0 ± 2.2</td>
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<td>NS</td>
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<td>NS</td>
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<tr>
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1 Values are means ± SD, n = 8; means with different superscript letters differ, P < 0.05. NS, not significant, P > 0.05.
decreased by OVX (Fig. 1; P < 0.05) and increased by ingestion of the FI diet as assessed by DXA. Furthermore, mice fed the F diet and the FI diet differed in the BMD of the total femur and of the distal region. In SH groups, the BMD of the total and distal regions was greater in mice fed the I and the FI diets compared with mice fed the control diet.

The metaphysis total, cortical and trabecular BMD were decreased by OVX and increased by the I and FI diets as assessed by pQCT (Fig. 2). In SH groups, feeding mice the I or FI diet increased the total BMD. In cortical bone, the BMD was also decreased by OVX (P < 0.05). Only feeding the I diet to OVX mice improved the decreased cortical BMD. Trabecular bone results were similar to those of total BMD (Fig. 2).

Plasma genistein, daidzein and equol concentrations. Both genistein and daidzein concentrations were significantly increased by ISO feeding (Figure 3). In OVX and SH mice, there were no differences in genistein and daidzein concentration between mice fed the I and FI diets. Equol, a metabolite of daidzein, was increased by feeding the mice the FI diet compared with the I diet (P < 0.05).

β-Glucosidase activity in cecal contents. Activity was elevated in OVX and SH mice fed FOS (Fig. 4; P < 0.05).

DISCUSSION

In this experiment, diets supplemented with FOS, crude soybean ISO conjugates or a combination of the two efficiently prevented the decrease in BMD in OVX mice. FOS also increased cecal β-glucosidase activity and equol production from daidzein in both OVX and SH mice, indicating that FOS increased the bioavailability of isoflavones.

Previously, we reported that diets containing 2.5 and 5% FOS prevented post-OVX bone loss in mature rats in a dose-dependent manner (35). These effects were observed at both

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### TABLE 3

<p>| Ash weights, and calcium (Ca), magnesium (Mg) and phosphorus (P) contents in the femora of sham-operated and overiectomized (OVX) mice fed a control diet, a fructooligosaccharide (FOS)-containing diet (F), an isoflavone conjugate (ISO)-containing diet (I), or a combination diet (FI) for 6 wk |
|---|---|---|---|---|---|---|</p>
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<th>Control</th>
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<td>34.1 ± 3.3ab</td>
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<td>262.0 ± 20.3abc</td>
<td>290.0 ± 30.2a</td>
<td>269.0 ± 22.3ab</td>
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<td>Mg</td>
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<td>P</td>
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</table>

1 Values are means ± sd, n = 8; those with different superscript letters differ, P < 0.05. NS, not significant, P > 0.05.

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**FIGURE 1** Bone mineral density (BMD) of the total (panel A), proximal region (panel B), midshaft (panel C) and distal region (panel D) of the femora from sham-operated and overiectomized (OVX) mice fed a control diet, a fructooligosaccharide (FOS)-containing diet (F), an isoflavone conjugate (ISO)-containing diet (I) or a combination diet (FI) for 6 wk, measured by DXA. Values are means ± sd, n = 8. Main factors O: OVX, F: dietary FOS, I: dietary isoflavone conjugates. Bars not sharing a letter differ, P < 0.05.
ends (one-third proximal and distal ends) of the femur. In this study, we observed similar effects on BMD in OVX mice fed a 5% FOS diet. Because the preventive effect of ISO conjugates on the OVX-induced bone loss was observed in both rats and mice, we can compare the effects of FOS and ISO conjugate diets across species. Picherit et al. (46) reported that neither BMD nor cancellous bone area was greater in rats fed soybean ISO conjugates than in OVX rats. They concluded that daily consumption of soybean ISO conjugates did not reverse established osteopenia. Thus, whether osteopenia is established is an important variable in this kind of study.

Feeding growing mice soybean ISO conjugates markedly prevented the post-OVX bone loss, both with and without FOS (i.e., FI and I diets). Ishimi et al. (47) reported a dose-dependent preventative effect of genistein on bone loss in OVX mice by using a mini-osmotic pump infusion method. The report showed that the minimum effective dose of genistein in mice was 0.4 mg/d and the effective dose for recovering normal BMD level was 0.7 mg/d. Picherit et al. (26) reported that the preventative effect of daidzein on post-OVX bone loss was greater than that of genistein in rats. In our study, pure ISO conjugates accounted for ~40% of the content of crude soybean ISO conjugate extract, and the content of crude soybean ISO conjugate extract in the experimental diet was 0.5 g/100 g. In this experiment, a mouse ate ~2 g diet/d and ingested ~4 mg soybean ISO conjugates/d (~80 mg/kg body). In our previous study (40), 48-h urinary recovery of genistein and daidzein were ~12 and 22%, respectively, after the administration of crude soybean ISO conjugates extract to rats. We calculated that at least 15% of soybean ISO conjugates was absorbed as aglycones. In the present experiment, ~0.6 mg/mouse of soybean ISO conjugates appears to have been absorbed. Therefore, in the present study, a rather high, nonphysiologic amount of ISO may have been absorbed. Indeed, an increase in BMD was observed in the SH mice fed the I and FI diets, and plasma concentrations of genistein and daidzein were much higher than reported elsewhere. In this experiment, BMD in OVX mice fed the I diet did not differ from that in SH mice fed the control diet, but BMD in OVX mice fed the FI diet was significantly higher than that of SH mice fed the control diet. The mean BMD in OVX mice fed the FI diet was 5% greater (P = 0.120) than that in OVX mice fed the I diet. This suggests an additive effect of FOS and soybean ISO conjugates in the prevention of

**FIGURE 2** Total (panel A), cortical (panel B) and trabecular (panel C) bone mineral density (BMD) of the femora from sham-operated and ovariectomized (OVX) mice fed a control diet, a fructooligosaccharide (FOS)-containing diet (F), an isoflavone conjugate (ISO)-containing diet (I) or a combination diet (FI) for 6 wk, measured by pQCT. Values are means ± SD, n = 8. Main factors: O: OVX, F: dietary FOS, I: dietary isoflavone conjugates. Bars not sharing a letter differ, P < 0.05.

**FIGURE 3** Plasma genistein (panel A), daidzein (panel B) and equol (panel C) concentration from sham-operated and ovariectomized (OVX) mice fed a control diet, a fructooligosaccharide (FOS)-containing diet (F), an isoflavone conjugate (ISO)-containing diet (I) or a combination diet (FI) for 6 wk. Values are means ± SD, n = 8. Main factors: O: OVX, F: dietary FOS, I: dietary isoflavone conjugates. Bars not sharing a letter differ, P < 0.05.
and pQCT, respectively. Especially in the pQCT result for trabecular bone, three-way ANOVA showed a significant interaction between FOS and ISO conjugate feeding. Future studies may reveal a stimulatory effect of FOS on the production of equol by the intestinal microflora in agreement with the observation that a carbohydrate-rich diet increases equol production (51,52). Several investigators have analyzed equol concentration in urine, but few have measured it in plasma or serum. For this kind of experiment, ISO availability is estimated more reliably using plasma rather than urine analysis. However, more detailed investigations are required.

In conclusion, preventing the post-OVX bone loss in mice was most effectively achieved with a diet containing both FOS and soybean ISO conjugates. These results suggest that intake of the combination of FOS and soybean foods may be useful in preventing bone loss in postmenopausal women.

**LITERATURE CITED**


