Equol, a Metabolite of Daidzein, Inhibits Bone Loss in Ovariectomized Mice[1]

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ABSTRACT Soybean isoflavones have structures similar to that of estrogen and have received attention as alternatives to hormone replacement therapy for the prevention of postmenopausal osteoporosis. Daidzein, a major isoflavone found in soybean, is metabolized to equol by gut microflora, and the metabolite exhibits a stronger estrogenic activity than daidzein. However, there is no direct evidence that equol affects bone metabolism. In this study, we examined the effect of equol on the inhibition of bone loss in ovariectomized (OVX) mice. Female mice (8 wk old) were assigned to 5 groups as follows: sham-operated (sham), OVX, OVX + 0.1 mg/d equol administration (0.1 Eq), OVX + 0.5 mg/d equol administration (0.5 Eq), and OVX + 0.03 μg/d 17β-estradiol administration (E_2). Equol and E_2 were administered s.c., using a mini-osmotic pump. At 4 wk after the intervention, uterine weight was less in the OVX mice than in sham-operated mice ($P < 0.05$). The weight was maintained in the E_2 group. In contrast, administration of equol at doses used in this study did not affect uterine atrophy in OVX mice. Bone mineral density (BMD) for the whole body in the OVX group measured by dual-energy X-ray absorptiometry was lower than that in the sham group, whereas administration of 0.5 mg/d Eq as well as E_2 maintained the BMD. The BMD of the femur and lumbar spine in the OVX group was also lower than those in the sham group, and treatment with 0.5 mg/d Eq maintained it. Notably, the BMD of the proximal femur in the 0.5 Eq group was the same as that of the sham group. E_2 inhibited bone loss from all regions induced by OVX. These results suggest that equol, a major metabolite of daidzein, inhibits bone loss apparently without estrogenic activity in the reproductive organs of OVX mice. J. Nutr. 134: 2623–2627, 2004.

KEY WORDS: • *equol* • *daidzein* • *postmenopause* • *osteoporosis*

Osteoporosis is characterized by bone loss and increases the risk of fracture. The most effective treatment for the prevention of osteoporosis is hormone replacement therapy. However, because of an increased risk of breast cancer and an unacceptable rate of undesirable outcomes, a large scale clinical intervention study was terminated early on the advice of the Data and Safety Monitoring Board in the United States (1).

Soybean isoflavones have structures similar to that of estrogen and have a weak affinity for the estrogen receptor; for this reason, they are called phytoestrogens. They have received much attention for prevention of postmenopausal symptoms such as osteoporosis and cardiovascular disease. Soybean isoflavones exhibit estrogenic and antiestrogenic activities depending on the hormonal environment in vitro (2–4). Daidzein, a soybean isoflavone, is metabolized to equol in the gastrointestinal tract by gut microflora. Equol possesses a stronger affinity for estrogen receptors than daidzein (2) and has the strongest transcriptional activity among soybean isoflavonoids in yeast 2-hybrid systems (5). Sathyamoorthy et al. (4) observed that equol possesses estrogenic activity ~100-fold stronger than that of daidzein using a pS2 mRNA expression assay. Thus, equol is a biological active metabolite of daidzein.

Recent studies suggest that the clinical effectiveness of isoflavones might be due to their ability to produce equol in the intestine (6). Maximal responses to isoflavone intake are observed in people who are good producers of equol (6). However, only 30–50% of the population can produce equol (6–8). Epidemiologic studies suggest that high equol producers are at lower risk of breast cancer than low equol producers (9–14). Furthermore, a higher intake of isoflavones is positively correlated with bone mineral density (BMD)[3] (15,16)

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[3] Abbreviations used: BMC, bone mineral content; BMD, bone mineral density; BV/TV, bone volume/tissue volume; E_2, 17β-estradiol; Eq, equol; N.OC/B.Pm, osteoclast number/bone length; OVX, ovariectomized; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; TR-FIA, time-resolved fluoroimmunoassay.

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and attenuates bone loss (17). It is notable that postmenopausal high equol producers had smaller bone loss changes than low producers (18).

A number of animal studies demonstrated that isoflavones play a role in bone metabolism. The intake of soy protein with isoflavone prevents bone loss in ovariectomized (OVX) rats (19). We also reported that genistein prevents bone loss in OVX and castrated male mice (20–22). Furthermore, we recently reported that fructooligosaccharides, indigestible sugars, increase the bioavailability of genistein, daidzein, and equol by stimulating gut microflora (23,24). In particular, equol may have an indirect effect on bone loss in OVX mice (24). However, direct effects of equol on bone metabolism have not been established. In this study, we examined the effect of equol on bone loss in OVX mice to determine whether equol has estrogenic properties related to bone metabolism.

MATERIALS AND METHODS

Animals and chemicals. Female ddY strain mice (8 wk old) were purchased from the Shizuoka Laboratory Animal Center. The mice were housed in individual cages in a temperature- and humidity-controlled room (23 ± 1°C and 60 ± 5% relative humidity) with a 12-h light:dark cycle, and were given free access to food and distilled water. Mice were sham-operated or OVX. Some OVX mice received a daily s.c. administration of equol (Eq; 0.1 mg/d or 0.5 mg/d) (Funakoshi) or 17β-estradiol (E_2; 0.03 μg/d) (Sigma) dissolved in 20% dimethyl sulfoxide in polyethylene glycol-300 using a mini-osmotic pump (Alza) immediately after surgery. The mice were fed an AIN-93G diet with corn oil instead of soybean oil (Funabashi Farm Chiba) (25) for 4 wk. Equol is a metabolite of daidzein, a typical soybean isoflavone, and is structurally similar to E_2 (**Fig. 1**).

All procedures were undertaken in accordance with the National Institute of Health and Nutrition Guidelines for the Care and Use of Laboratory Animals. In each experiment, body and uterine weight were measured, and the right and left femora were removed to measure BMD or to carry out histomorphological analysis, respectively.

Radiographic analysis of body composition, whole-body and lumbar BMD, and bone mineral content (BMC). The BMD and BMC of the entire body and lumbar spine and body composition were measured using a PIXImus densitometer (software version 1.4x Lunar). Through whole-body scanning, the BMD and BMC of the entire body and L4–L6 vertebrae as well as body composition, including fat and lean body mass, were analyzed. The CV of BMD of the entire body and lumbar vertebrae were 0.8% and 0.7%, respectively. The CV for body composition measurement was 0.2 for lean body mass and 2.8% for fat mass.

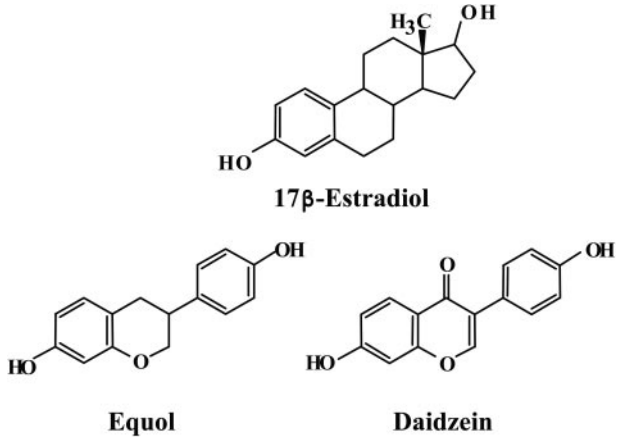

FIGURE 1 The molecular structure of equol, estradiol and daidzein.

Radiographic analysis of the femur. Radiographic analysis of the right femur was performed using a soft X-ray system. BMD of the femur was measured by dual-energy X-ray absorptiometry (model DCS-600EX-R, Aloka). BMD was calculated using the BMC of the measured area. The BMC of the mouse femur was closely correlated with its ash weight ($r = 0.978$). The scanned area of the mouse femur was equally divided into 3 parts (5.3 mm each), i.e., the proximal femur, midshaft, and the distal femur.

Histomorphometry. The femur was fixed in 70% ethanol and embedded in methyl methacrylate. Undecalcified 5-μm sections were cut longitudinally using a microtome (Model 2050, Reichert Jung) and stained for tartrate-resistant acid phosphatase. Histomorphometry was performed using a semiautomatic image-analysis system (System Supply) linked to a light microscope. Using sections of the distal femur, histomorphometric parameters were quantified for cancellous bone tissue, and 1 cortical width from the endosteal surface was excluded from the measurements. Trabecular bone volume (BV)/tissue volume (TV), trabecular number (Tb.N), trabecular separation (Tb.Sp), and osteoclast number/bone length (N.Oc/B.Pm) were calculated. Nomenclature and units that were used are those recommended by the Nomenclature Committee of the American Society for Bone and Mineral Research (26).

Time-resolved fluoroimmunoassay (TR-FIA). Plasma equol was analyzed by the TR-FIA method of Brouwers et al. (27). For the recovery calculation and hydrolysis, 20 μL of ^{3}H-estradiol glucuronide and 100 μL of 0.1 mol/L (pH 5.0) acetate buffer containing 200 U/L glucuronidase and 2000 U/L sulfatase were added to 100 μL of plasma, respectively. The mixture was incubated overnight at 37°C, and then extracted by diethyl ether. The ether phases were completely evaporated using a 45°C water bath. Then, 100 μL of 50 mmol/L Tris-HCl buffer containing 5 g/L bovine serum albumin (assay buffer; pH 7.8) was added to the tubes containing the dry residues and 20 μL of the solution was taken as the TR-FIA compound. Another 20 μL of solution was taken for the determination of recovery. A 20-μL volume of standard or hydrolyzed and extracted plasma samples was pipetted into the microstrips; 100 μL of antiserum in the assay buffer for equol and 100 μL of europium-labeled equol were added to each well. The strips were slowly shaken at room temperature for 90 min and then washed. A 200-μL volume of DELFIA enhancement solution was added to each well. After an additional 5 min of shaking, fluorescence was determined using a DELFIA Victor 1420 multilabel counter (Wallac). The final results were calculated using the following formula: final results = concentration (read) × 1/recovery × dilution factor (nmol/L)

Statistical analysis. Data are expressed as the means ± SEM. The significance of the differences was determined by ANOVA and Fisher's protected least-significant difference test (StatView 5.0, Abacus Concepts). Differences were considered significant when $P < 0.05$.

RESULTS

Body and tissue weight. The initial body weights of mice in the sham, OVX, 0.1 Eq, 0.5 Eq, and E_2 groups were 29.4 ± 0.3, 29.3 ± 0.5, 29.7 ± 0.2, 29.6 ± 0.2, and 29.8 ± 0.3 g, respectively, and the final body weights were 32.7 ± 1.3, 33.9 ± 1.9, 33.9 ± 1.2, 33.7 ± 0.8, and 31.8 ± 0.2 g, respectively. Initial and final body weights of the 5 groups of the mice did not differ.

Administration of equol and E_2 did not affect the body weight of OVX mice. Uterine weight was lower in OVX mice than that in sham-operated mice ($P < 0.05$), whereas E_2 administration inhibited uterine atrophy induced by OVX ($P < 0.05$) (**Fig. 2**). In contrast, treatment with 0.1 and 0.5 mg/d equol did not affect the uterine weight in OVX mice.

Body composition and bone mass of lumbar vertebrae. BMD of the whole body in OVX mice was significantly lower than that in sham mice ($P < 0.05$). BMD of the 0.5 mg/d but not of the 0.1 mg/d equol-treated group was greater than that in the OVX group ($P < 0.05$), and the BMD in E_2 mice was

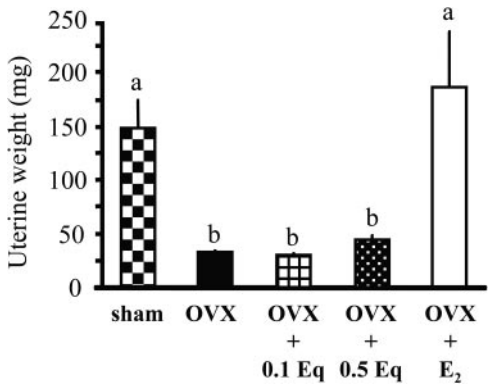

FIGURE 2 Wet weight of the uterus in sham-operated (sham) mice, ovariectomized (OVX) mice, OVX mice treated with 0.1 mg/d equol (OVX + 0.1 Eq), OVX mice treated with 0.5 mg/d equol (OVX + 0.5 Eq), and OVX mice treated with 0.03 μg/d E_2 (OVX + E_2) for 4 wk. Values are means ± SEM, $n = 6$. Means with different letters differ, $P < 0.05$.

equal to that in sham mice (**Fig. 3***A*). BMC of the whole body in the 0.5 Eq-treated group was also higher than that in the OVX group ($P < 0.05$) (Fig. 3*B*). The BMD of the lumbar spine in OVX mice was significantly lower than that in sham mice ($P < 0.05$), but the mice in the 0.5 Eq-treated group had higher BMD than those in the OVX group ($P < 0.05$) (Fig. 3C). The BMC of the lumbar spine showed similar results to BMD (Fig. 3*D*). Similarly, E_2 treatment inhibited OVX-induced bone loss over the entire body and lumbar spine (Fig. 3*A–D*). Body fat (%) and lean body mass at the end of the 4-wk experimental period are shown in Figure 3*E* and *F*, respectively. The percentage of body fat and lean body mass in the sham and OVX group did not differ. The percentage of body fat in the E_2 group was lower than those in the sham, OVX, and 0.1 Eq groups ($P < 0.05$) (Fig. 3*E*). The lean body mass in the 0.5 Eq group was higher than that in the sham group ($P < 0.05$) (Fig. 3*F*).

Bone mass and histomorphometry of the femur. Administration of 0.5 mg/d of equol also inhibited bone loss in the femur in OVX mice (**Fig. 4**). The BMDs of the whole, proximal, and distal femur in OVX mice were lower than those in sham mice ($P < 0.05$), and 0.5 mg/d but not 0.1 mg/d equol administration inhibited the bone loss ($P < 0.05$) (**Fig. 5***A*, C, *E*). The BMC of the whole femur showed similar results to that of BMD (Fig. 5). E_2 treatment maintained the BMD and BMC over the 3 regions of femur in OVX mice ($P < 0.05$) (Fig. 5*A–E*).

Histomorphometric analysis of the femoral metaphysis is shown in **Table 1**. Bone volume/tissue volume (BV/TV), trabecular thickness (Tb.Th), and trabecular number (Tb.N) in the OVX group were lower than those in sham mice ($P < 0.05$), and these differences were not observed in the E_2 group ($P < 0.05$). These indices in the 0.5 Eq group tended to be higher ($P = 0.2$ for BV/TV and Tb.N) than those in the OVX group. Trabecular separation (Tb.Sp) and osteoclast number (N.Oc) in the OVX group were greater than those in the sham group ($P < 0.05$). N.Oc in the 0.5 Eq group was significantly lower than that in the OVX group ($P < 0.05$). Tb.Sp and N.Oc in the E_2 group did not differ from those in the sham group.

Plasma equol concentrations. Subcutaneous administration of equol using a mini-osmotic pump for 4 wk increased the plasma equol level in a dose-dependent manner in OVX mice ($P < 0.05$) (0.1 Eq group, 431.5 ± 61.7 nmol/L and 0.5 Eq group, 1550.6 ± 202.5 nmol/L).

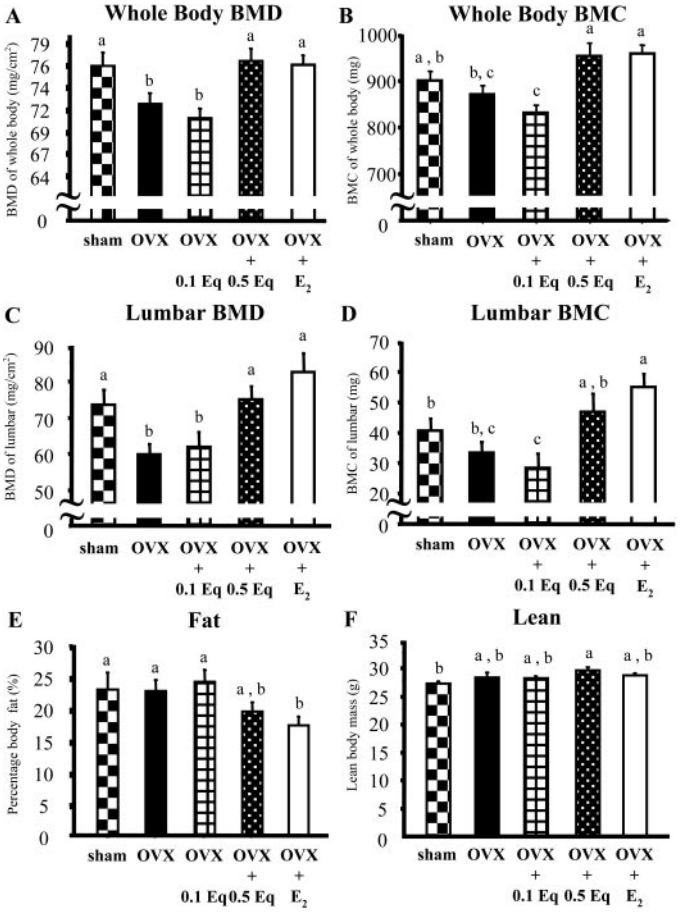

FIGURE 3 Body composition and BMD and BMC of the whole body and lumbar spine of sham-operated (sham) mice, OVX mice, OVX mice treated with 0.1 mg/d equol (OVX + 0.1 Eq), OVX mice treated with 0.5 mg/d equol (OVX + 0.5 Eq), and OVX mice treated with 0.03 μg/d E_2 (OVX + E_2) for 4 wk. (*A*) Whole body BMD; (*B*) whole body BMC; (*C*) lumbar spine BMD; (*D*) lumbar spine BMC; (*E*) body fat (%); (*F*) lean body mass. Values are means ± SEM, $n = 6$. Means with different letters differ, $P < 0.05$.

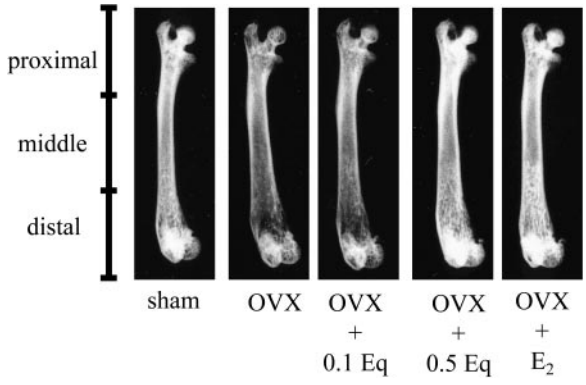

FIGURE 4 Radiogram of the femora collected from sham-operated (sham) mice, OVX mice, and OVX mice treated with 0.1 mg/d equol (OVX + 0.1 Eq), OVX mice treated with 0.5 mg/d equol (OVX + 0.5 Eq), and OVX mice treated with 0.03 μg/d E_2 (OVX + E_2) for 4 wk.

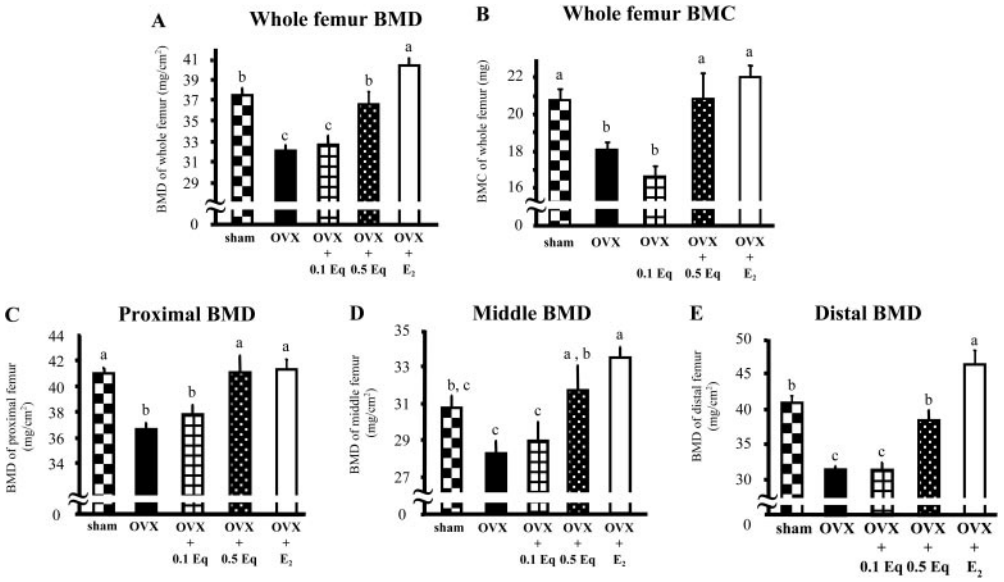

FIGURE 5 BMD and BMC of the femur collected from sham-operated (sham) mice, OVX mice, and OVX mice treated with 0.1 mg/d equol (OVX + 0.1 Eq), OVX mice treated with 0.5 mg/d equol (OVX + 0.5 Eq), and OVX mice treated with 17β-estradiol (OVX + E_2) for 4 wk. (*A*) BMD of the whole femur; (*B*) BMC of the whole femur; (*C*) BMD of the proximal region in the femur; (*D*) BMD of the midshaft region in the femur; (*E*) BMD of the distal region in the femur. Values are means ± SEM, $n = 6$. Means with different letters differ, $P < 0.05$.

DISCUSSION

The present study demonstrates that equol inhibits bone loss induced by OVX in mice without substantial effects on the uterus, indicating that the effect of daidzein on bone metabolism is due at least in part to equol.

Setchell et al. (18) reported that the lumbar spine BMD of equol producers increased by 2.4% compared with the control group, whereas there were no changes in BMD in the nonproducers after a 2-y intervention with isoflavones. In animal studies, there are many reports showing that soy isoflavones such as daidzein and genistein inhibit bone loss induced by estrogen deficiency. However, there are no reports that have examined the direct effects of equol on bone loss induced by estrogen deficiency. In this study, we demonstrated that equol had the ability to inhibit bone loss in vivo when estrogen status was deficient.

Both BMD and BMC of the entire body, lumbar spine, and femora of the OVX mice were significantly lower than those in the sham mice, but administration of 0.5 mg/d Eq maintained the bone mass (Fig. 3*A–D*, Fig. 5). In particular, 0.5 Eq maintained bone mass of the proximal femur as E_2 did (Fig. 5C). Eq administration did not affect uterine weight in OVX mice, although plasma equol concentration increased significantly. Therefore, Eq administration inhibited bone loss without estrogenic effects on the reproductive organs in OVX mice. These results suggest that equol may contribute to the effectiveness of daidzein in inhibiting bone loss when estrogen status is deficient.

In radiographic and histomorphometric analysis of the femoral metaphysis, trabecular bone volume in OVX mice was significantly lower than that in sham mice, and osteoclast numbers in OVX mice were greater than those in sham mice. However, the number of osteoclasts in mice treated with 0.5 mg/d equol was lower than that in OVX mice (Table 1). These results support the data of the BMD in the distal femur (Fig. 5*E*).

Postmenopausal estrogen deficiency increases plasma lipid levels, which in turn increases the risk for cardiovascular diseases. Potter et al. (28) reported that soy protein–containing isoflavones decreased plasma LDL cholesterol and inhibited lipid oxidation in postmenopausal women. In this study, there were no differences in the concentration of plasma LDL cholesterol and triglyceride among the sham, OVX, 0.1 Eq, and 0.5 Eq groups (data not shown). Similarly, the percentage of body fat did not differ in the 4 groups, although the lean body mass in the 0.5 Eq group was significantly greater than that in sham group (Fig. 3*E*, *F*). These results suggest that the effects of isoflavones on lipid metabolism are likely exerted through the combination of the original soy isoflavones and their metabolites. Further studies are warranted to clarify the

TABLE 1

Histological analysis of the trabecular bone collected from sham-operated (sham) mice, ovariectomized (OVX) mice, and OVX mice treated with 0.1 mg/d equol (OVX + 0.1 Eq), OVX mice treated with 0.5 mg/d equol (OVX + 0.5 Eq), and OVX mice treated with 17β-estradiol (E_2)[1]

	BV/TV	Tb.Th	Tb.N	Tb.Sp	N.Oc/B.Pm
	%	μm	per mm	mm	per mm
Sham	15.4 ± 1.8^{a}	59.0 ± 3.5^{a}	2.6 ± 0.2^{a}	0.33 ± 0.03^{c}	0.9 ± 0.1^{d}
OVX	4.3 ± 1.1^{c}	37.0 ± 4.0^{b}	1.1 ± 0.3^{b}	1.10 ± 0.38^{ab}	4.2 ± 0.8^{a}
OVX + 0.1 Eq	3.4 ± 0.7^{c}	39.2 ± 4.8^{b}	0.9 ± 0.2^{a}	1.29 ± 0.28^{a}	3.3 ± 0.5^{ab}
OVX + 0.5 Eq	9.3 ± 3.1^{bc}	45.4 ± 2.3^{b}	1.8 ± 0.5^{ab}	0.62 ± 0.13^{bc}	2.2 ± 0.1^{bc}
OVX + E_2	13.2 ± 3.5^{ab}	52.4 ± 5.2^{ab}	2.4 ± 0.4^{a}	0.41 ± 0.09^{c}	1.4 ± 0.2^{cd}

[1] Values are means ± SEM, $n = 6$. Means in a column with different superscript letters differ, $P < 0.05$.

mechanism responsible for the effects of isoflavones on lipid metabolism when estrogen status is deficient.

Equol is produced from daidzein in the gastrointestinal tract, but interindividual variation exists in its metabolism in humans (7). About 30–50% of the adult population excretes equol in the urine when consuming soy products on a daily basis (6–8). This interindividual variation is due to gut microflora. Ueno et al. (29) identified 3 strains of bacteria that convert pure daidzein into equol in Japanese equol producers. We also found that fructooligosaccharides, indigestible sugars, increase plasma equol and BMD of the femora when normal and OVX mice are treated simultaneously with isoflavones (24). This suggests that indigestible sugars or dietary components promote the growth or activity of bacteria responsible for equol production in the colon (24). Because the production of equol is a key factor in clinical studies investigating the effects of isoflavone on bone turnover, enhancing the bioavailability of isoflavones using dietary components might be an effective and less expensive regimen for obtaining maximal effects of isoflavones on bone metabolism. In the future, it may be possible to establish a tailor-made regimen for the prevention of osteoporosis based on an individual's constitution.

In conclusion, we demonstrated that equol inhibited bone loss without affecting the reproductive organs in ovariectomized mice. It is important to promote or activate gut microflora that produce equol to obtain the maximal effects of isoflavones on prevention of bone loss when estrogen status is deficient.

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