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ORIGINAL CONTRIBUTION

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Comparative activities of daidzein metabolites, equol and *O*-desmethylangolensin, on bone mineral density and lipid metabolism in ovariectomized mice and in osteoclast cell cultures

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Abstract Daidzein, a major isoflavone predominantly found in soybean, is mainly metabolized to equol and *O*-desmethylangolensin (*O*-DMA) by the human gut microflora. Equol exhibits a stronger estrogenic activity than daidzein, however, only approximately 30% of the population has been identified as equol-producers and there are too few direct evidences of the effects of the other major metabolite, *O*-DMA on estrogen-deficient status. The purpose of this study is therefore, to compare the effect of both *O*-DMA and equol on bone and lipid metabolism in vivo and in vitro. For the in vivo study, 8-week-old female mice were assigned to five groups as follows: sham-operated (sham), ovariectomized (OVX), OVX + 0.5 mg/day *O*-DMA (OVX + *O*-DMA), OVX + 0.5 mg/day equol (OVX + Eq), and OVX + 0.03 μg/day 17β-estradiol (OVX + E2) administration. Three weeks after the intervention, *O*-DMA and equol did not affect uterine atrophy in OVX mice. The bone mineral density (BMD) of the femur was lower in the OVX group than in the sham group. The administration of equol but not *O*-DMA, maintained BMD through the intervention. Values of whole body fat mass and plasma lipids were lower in the equol and *O*-DMA treated OVX mice than those in OVX mice. In the in vitro study, equol significantly inhibited the osteoclast formation induced by $1\alpha,25(OH)_2D_3$ in a dose-dependent manner in a co-culture system of mouse bone-marrow cells with primary osteoblastic cells. However, *O*-DMA slightly inhibited osteoclast formation, and the effect was not dose dependent. These results suggest that the effects of *O*-DMA on bone and lipid metabolism in OVX mice and osteoclast cell cultures are weaker than those of equol.

Key words equol – *O*-desmethylangolensin – bone mineral density – osteoclast – microflora

Abbreviations BMD: Bone mineral density; BMC: Bone mineral content; E_2: 17β-Estradiol; Eq: Equol; *O*-DMA: *O*-desmethylangolensin; ER: Estrogen receptor; OVX: Ovariectomized; TC: Total cholesterol; TG: Triacylglycerol; TRAP: Tartrate-resistant acid phosphatase; $1\alpha,25(OH)_2D_3$: 1α,25-Dihydroxyvitamin D_3

Introduction

Osteoporosis is a skeletal disorder in which bone strength is compromised by the loss of bone density and bone quality. It is the leading cause of increased morbidity and functional loss in the elderly. Particularly, postmenopausal women suffer from osteoporosis, being part of the postmenopausal syndrome [12]. Although, the one of the treatment for postmenopausal osteoporosis is hormone replacement therapy, the reported side effects, such as development of hormone dependent breast and uterine cancers [4], have prompted the use of alternative therapies. Epidemiological studies suggested that phytoestrogens [19] have preventive effects for breast cancer and menopausal symptoms [1, 28], much attention being directed to soybean, the main dietary source of isoflavones. Compared with hormone replacement therapy, the risk for side effects of isoflavone treatment seems to be low [17]. Therefore, they have been focused as alternative treatment for prevention of postmenopausal-related diseases [2, 17].

Daidzein, a major soybean isoflavone, is metabolized to *O*-DMA and equol in the gastrointestinal tract by gut microflora [2, 11]. Being structurally similar to E_2, both daidzein and their metabolites are capable to bind to the estrogen receptors, specifically to ERβ, (although the affinity of equol seems to be greater [14, 16].

In this line, recent studies suggest that the clinical effectiveness of isoflavones might depend on the individual's ability to produce equol [7]. However, a correlation between equol/*O*-DMA status and efficacy of isoflavones is still uncertain. Hall et al. [10] reported that soy-isoflavone-enriched foods improved biomarkers of cardiovascular disease risk independently of equol production in postmenopausal women. On the other hand, Setchell et al. [22] hypothesized that maximum clinical responses to soy protein diet were seen in equol producers. We also demonstrated that the positive effect of isoflavones on bone loss depended on the extent of equol production in postmenopausal Japanese women [26], although this was not consistent with the previous report [8]. While, Persky et al. [18] reported that changes in plasma *O*-DMA can be significantly associated with bone mineral density (BMD) in postmenopausal women. However, there was no evidence of a direct effect of *O*-DMA on bone loss in estrogen-deficient status in vivo or in vitro studies. Thus, in the present study we examined the effects of *O*-DMA and equol on bone and lipid metabolism in OVX mice and osteoclast formation in vitro in order to compare the physiological activity of *O*-DMA with that of equol.

Materials and methods

Animals and chemicals

Female ddY strain mice (8 weeks old) were purchased from the Shizuoka Laboratory Animal Center (SLC) (Shizuoka, Japan). The mice were housed in individual cages in a temperature- and humidity-controlled room, and were given free access to food and distilled water. Mice were sham-operated or ovariectomized (OVX) ($n = 5$). Some OVX mice received a daily s.c. administration of *O*-DMA (0.5 mg/day) (synthesized at the Laboratory of Organic Chemistry, University of Helsinki [3] or equol (racemic mixture, 0.5 mg/day) (Funakoshi, Tokyo, Japan) or 17β-estradiol (E_2; 0.03 μg/day) (Sigma, St Louis, MO, USA) using a mini-osmotic pump (Alza Corp., Palo Alto, CA, USA) immediately after surgery (each group, $n = 5$). The same dose of *O*-DMA and equol was used as previously reported for equol, which was effective on prevention of bone loss in OVX mice [9]. Since both *O*-DMA and equol are intestinal metabolites of daidzein, s.c. injection was adopted to ensure targeted plasma concentrations. The mice were fed on AIN-93G diet with corn oil instead of soybean oil (Funabashi Farm, Chiba, Japan) [20] for 3 weeks. 1α,25-Dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) was obtained from Phillips-Duphar (Amsterdam, The Netherlands). 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 femur was removed to measure BMD.

Radiographic analysis of body composition, whole body BMD

The BMD of the entire body and body composition were measured using a PIXImus densitometer (software version 1.4x Lunar, Madison, WI). The coefficient of variation (CV) of BMD of the entire body was 6.2%. The CV for body composition measurement was 5.9% for lean body mass and 3.5% for fat mass.

Radiographic analysis of the femur

Bone mineral density 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 three parts, i.e., the proximal femur, midshaft, and the distal femur.

Biochemical analysis of plasma concentration

Commercially available ELISA kits for E_2 (IBL, Hamburg, Germany), and analytical kits for total cholesterol (TC) and triacylglycerol (TG) (Wako, Osaka, Japan) were used.

Time-resolved fluoroimmunoassay (TR-FIA) for plasma *O*-DMA and equol

Plasma *O*-DMA and equol were analyzed by the TR-FIA method of Brouwers and co-workers [5] and L'homme and co-workers [15], respectively. After enzymatic hydrolysis and extraction by diethyl ether, plasma equol and *O*-DMA concentrations were determined by fluorescence using a DELFIA Victor 1420 multilabel counter (PerkinElmer, Wellesley, MA, USA). The final results were calculated using the following formula: final results = concentration (read) × 1/recovery × dilution factor (nmol/l). Average CV values for the analysis of the equol and *O*-DMA are 5.5 and 5.6%, respectively [5, 15].

Osteoclast formation

Osteoclast formation was carried out by a co-culture of bone-marrow cells with primary osteoblasts according to the method of Takahashi and co-workers [24]. Briefly, bone-marrow cells obtained from the tibiae of 8 weeks ddY male mice were co-cultured with primary osteoblastic cells isolated from the calvariae of the newborn mice in αMEM (phenol red-free, GIBCO, Grand Island, NY, USA) containing 10% fetal bovine serum (JRH, Lenexa, KS) in 24-well plate. An inducer of osteoclasts; $1\alpha,25(OH)_2D_3$ (100 nmol/l); with or without equol (10–1,000 nmol/l), *O*-DMA (10–1000 nmol/l) or E_2 (10 nmol/l) was added to the cultures. After cultured for 6 days, the cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) activity, which is used as a marker for the osteoclasts [24]. The fixed cells were incubated with naphthol AS-MX (Sigma) as a substrate and fast violet LB salt (Sigma) as a stain for the reaction product in the presence of 50 mM sodium tartrate (Wako, Osaka, Japan). TRAP-positive cells containing three or more nuclei were counted as osteoclast-like cells (MNC) with microscope.

Statistical analysis

Stat view 5.0, Abacus Concepts (Calabasas, CA, USA) software was used for statistical analysis. Data are expressed as means ± SEM. The differences between groups were determined by ANOVA and Fisher's protected least-significant difference test. Means without common letter differ. A P value less than 0.05 was considered as statistically significant.

Results

Body and tissue weight

Initial and final body weights of the five groups of the mice did not differ significantly from each other (Fig. 1A). 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. 1B). In contrast, treatment with *O*-DMA and equol did not affect the uterine weight in OVX mice.

Body composition and bone mass of lumbar vertebrae

Bone mineral density of the whole body in OVX mice was significantly lower than that in sham mice ($P < 0.05$). BMD of the whole body in the E_2-treated

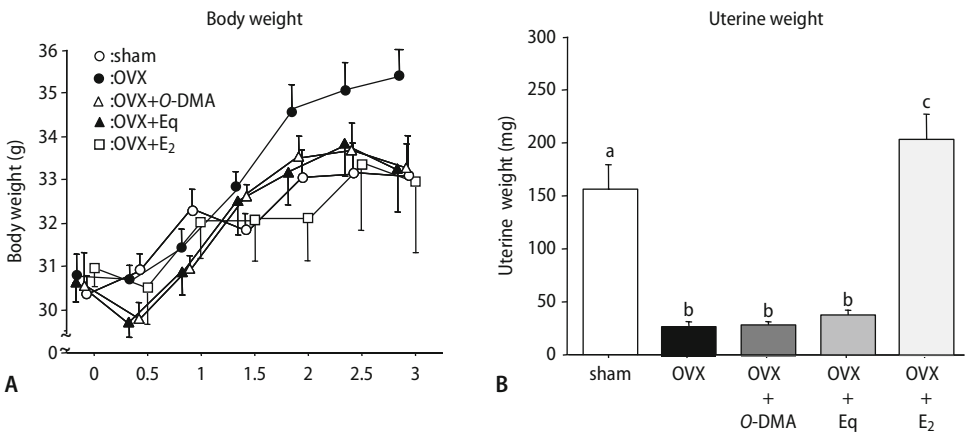
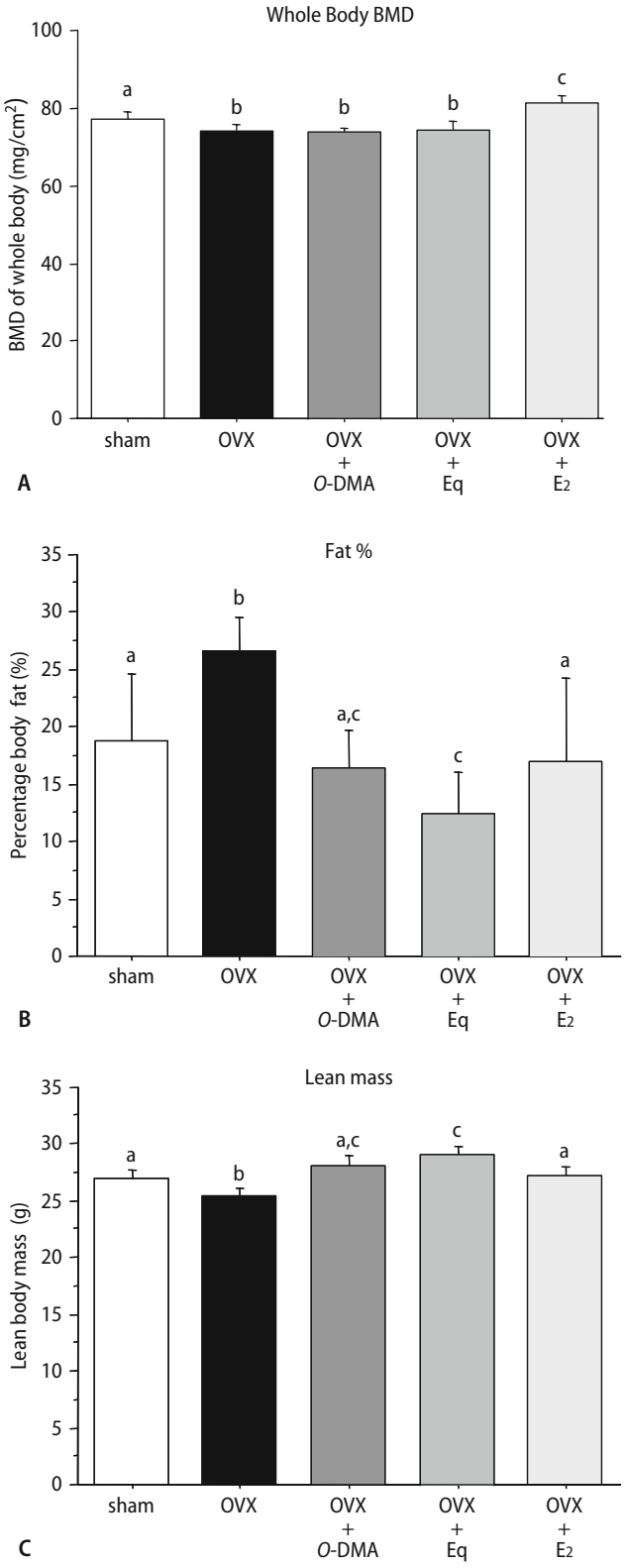


Fig. 1 Body weight and uterine weight of sham-operated (sham) mice, ovariectomized (OVX) mice, OVX mice treated with 0.5 mg/day *O*-DMA (OVX + *O*-DMA), or 0.5 mg/day equol (OVX + Eq), or 0.03 μg/day E_2 (OVX + E_2). **A** Body weight, **B** uterine weight. Values are means ± SEM, $n = 5$ per group. Means with different letters differ significantly, $P < 0.05$



◀ **Fig. 2** Body composition and bone mineral density (*BMD*) of the whole body of sham-operated (sham) mice, OVX mice, and OVX mice treated with 0.5 mg/day *O*-DMA (OVX + *O*-DMA), or 0.5 mg/day equol (OVX + Eq), or 0.03 μg/day E_2 (OVX + E_2) for 3 weeks. **A** BMD of the whole body, **B** body fat (%), **C** lean body mass. Values are means ± SEM, $n = 5$ per group. Means with different letters differ significantly, $P < 0.05$

mice was greater than that in OVX mice ($P < 0.05$), but the BMD of the *O*-DMA and equol-treated mice was equal to that in OVX mice (Fig. 2A). The percentage of body fat in OVX mice was significantly higher than that in sham mice ($P < 0.05$), and the percentage of body fat in the E_2, *O*-DMA, and equol-treated mice were significantly lower than that in OVX mice ($P < 0.05$) (Fig. 2B). The lean body mass in the E_2, *O*-DMA and equol-treated mice was significantly higher than that in the OVX mice ($P < 0.05$) (Fig. 2C).

Bone mineral density of the femur

The BMD of the whole, proximal and distal femur in OVX mice were significantly lower than those in sham mice ($P < 0.05$), and equol administration inhibited the bone loss in the whole, proximal, and distal femur ($P < 0.05$) (Fig. 3A, B, D). The BMDs of the whole, proximal, middle, and distal femur in *O*-DMA treated mice were the same as those in OVX mice. E_2 treatment maintained the BMD over the four regions of femur in OVX mice ($P < 0.05$) (Fig. 3A–D).

Plasma concentrations of E_2, *O*-DMA, equol and lipids

Plasma E_2 concentration was significantly lower in OVX mice than that in sham-operated mice ($P < 0.05$), and *O*-DMA and equol administration did not affect the E_2 concentration in OVX mice (data not shown). Administration of *O*-DMA or equol increased plasma *O*-DMA or equol level in OVX mice (*O*-DMA group; 11,864 ± 2,071 nmol/l of *O*-DMA, equol group; 1,545 ± 453 nmol/l of equol). Plasma TC concentration tended to be higher in OVX mice than that in sham-operated mice, whereas the TC levels in equol and E_2-treated OVX mice were significantly lower than that in OVX mice (Table 1). Plasma TG concentration tended to be higher in OVX mice than that in sham-operated mice, whereas the TG levels in *O*-DMA, equol and E_2-treated OVX mice were significantly lower than that in OVX mice ($P < 0.05$) (Table 1).

Effects of *O*-DMA and equol on osteoclast-like cell formation

In the co-culture system employed, TRAP-positive MNC were induced by 100 nmol/l $1\alpha,25(OH)_2D_3$. The addition of 10 nmol/l E_2 significantly decreased the number of MNC induced by $1\alpha,25(OH)_2D_3$. Equol inhibited the $1\alpha,25(OH)_2D_3$ induced MNC formation in a dose-

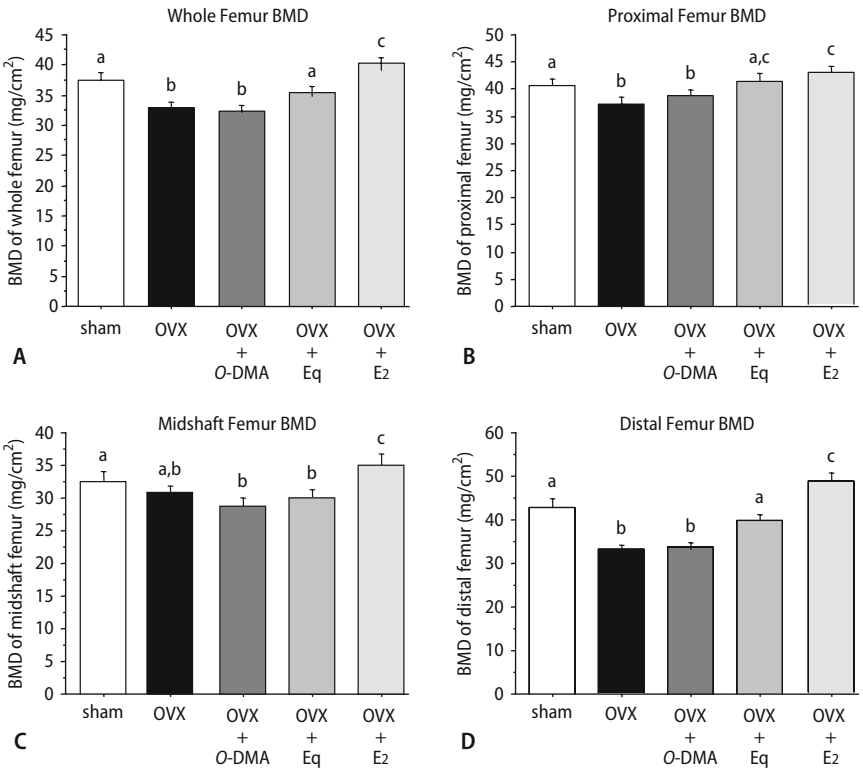


Fig. 3 Bone mineral density of the femur collected from sham-operated (sham) mice, OVX mice, and OVX mice treated with 0.5 mg/day *O*-DMA (OVX + *O*-DMA), or 0.5 mg/day equol (OVX + Eq), or 0.03 μg/day E_2 (OVX + E_2) for 3 weeks. **A** BMD of the whole femur, **B** BMD of the proximal region in the femur, **C** BMD of the midshaft region in the femur, **D** BMD of the distal region in the femur. Values are means ± SEM, $n = 5$ per group. Means with different letters differ significantly, $P < 0.05$

Table 1 Effects of *O*-DMA, Equol, and E_2 on plasma concentration of lipid in sham mice, OVX mice, OVX mice treated with 0.5 mg/day *O*-DMA or equol, or 0.03 μg/day E_2

	sham	OVX	OVX + *O*-DMA	OVX + Eq	OVX + E_2
Total cholesterol (mmol/l)	$3.38 \pm 0.25^{a,b,c}$	4.11 ± 0.37^{a}	$3.55 \pm 0.26^{a,b}$	2.62 ± 0.26^{c}	$2.99 \pm 0.23^{b,c}$
Triacylglycerol (mmol/l)	$1.05 \pm 0.10^{a,b}$	1.30 ± 0.28^{a}	0.70 ± 0.07^{b}	0.70 ± 0.08^{b}	0.78 ± 0.04^{b}

Values are means ± SEM, $n = 5$. Means with different superscript letters differ significantly, $P < 0.05$

dependent manner ranging from 10 to 1,000 nmol/l. *O*-DMA slightly inhibited MNC formation ($P < 0.05$), but the effects were not dose dependent (Fig. 4).

Discussion

Daidzein is metabolized to a variable extent in the human gastrointestinal tract to mainly *O*-DMA and equol. About 70–90% of the population produces *O*-DMA when consuming soy products on a daily basis [6, 7, 22]. The inverse relationship found between *O*-DMA and equol production in humans, has been attributed to variability in intestinal microflora [13, 21], and both human and animal studies have linked this variability with the preventive effect of daidzein on hormone-dependent conditions such as bone health [8, 27]. In Japanese postmenopausal women treated for 1 year with 75 mg/day of isoflavone conjugates (47 mg/day as aglycone form), the decrease in BMD of the inter-trochanter and total hip was significantly ($P = 0.04$) inhibited in equol producers [25]. Similarly, after a 2-year dietary intervention with isoflavones, Setchell and co-workers [22] reported that the BMD of the lumbar spine increased by 2.4% ($P < 0.001$) only in equol-producers. Regarding *O*-DMA, only one study has been published reporting a 6% increase in total BMD among the *O*-DMA producers from a postmenopausal Caucasian population [8]. In animal studies, we have reported that administration of equol inhibited bone loss induced by estrogen deficiency [9]. However, no reports have been published on the effect of *O*-DMA on bone loss induced by estrogen deficiency.

In the present study, BMD of the femur in the OVX mice was significantly lower than that in the sham mice. Administration of equol but not *O*-DMA, maintained the BMD of proximal, distal, and whole femur. Only in the in vitro study, *O*-DMA inhibited the osteoclast formation, although the effect was weaker compared to equol. These results suggest that contrary to equol, administration of *O*-DMA does not prevent bone loss in estrogen-deficient status. *O*-DMA

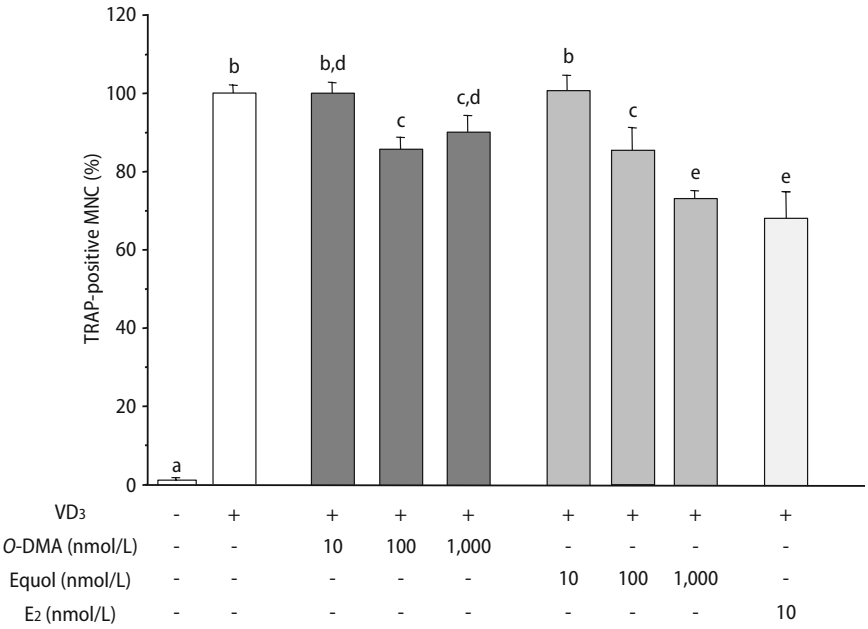


Fig. 4 Dose-response effects of *O*-DMA and equol on VD_3-induced TRAP-positive multinuclear osteoclast-like cells (MNC). Values are means ± SEM, $n = 6$ per each culture. Means with different letters differ significantly, $P < 0.05$

is produced from daidzein by the human microflora through a series of chemical transformation including the cleavage of the C-ring [11]. This cleavage may lead to lower affinity to the estrogen receptor, explaining the lower activity of *O*-DMA on bone metabolism compared with equol bearing an intact C-ring. In this study, the difference in bone effects of *O*-DMA and equol was in agreement with the difference in agonistic activity for the estrogen receptor shown in previous reports [14].

Interestingly, the plasma concentration of *O*-DMA was much higher than that of equol in OVX mice. This result suggests that *O*-DMA might be easier to be dissolved in PEG-DMSO and be absorbed via skin, metabolism of *O*-DMA is slower than that of equol, or binding affinity of these metabolites to SHBG or other plasma proteins might be different. In any case, the plasma concentrations of *O*-DMA in the treated mice were five times higher than that in humans consuming soy foods [18] and similarly, the levels of equol in the respective mice were about 2.5 times higher than that in Japanese women who were equol producer consuming daily soy foods or five times higher than those found in women supplemented with 47 mg/day of isoflavones [25]. These results suggest that the dose used in this study may be relatively higher than dietary levels of isoflavones in humans.

Plasma concentration of TC and TG tended to be higher in OVX mice compared to that in sham mice, and administration of both *O*-DMA and equol decreased or tended to decrease the TG and TC concentrations. Furthermore, administration of *O*-DMA and equol significantly decreased the higher percentage of body fat induced by OVX. There are several reports on isoflavones decreasing plasma levels of LDL-cholesterol in postmenopausal women as well as OVX mice [27, 29]. These reports are mostly in agreement with the findings in the present study.

In OVX mice, equol and E_2 showed good influence on markers of bone and lipid metabolism. On the other hand, administration of *O*-DMA only affected lipid metabolism in this study. These results suggest that the effect of *O*-DMA on lipid metabolism might not occur exclusively via binding to the estrogen receptor. It has been reported that isoflavones competitively inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase in vitro, which may lead to inhibition of cholesterol synthesis [23]. However, the mechanism by which isoflavone including the metabolite *O*-DMA and equol on lipid metabolism especially in estrogen-deficient environment should be examined in future studies.

The present study demonstrated that *O*-DMA did not affect bone loss in OVX mice and showed weak inhibitory effects on osteoclast formation in co-culture system, indicating that the inhibitory effects of daidzein on bone loss may depend on the extent of equol production. However, *O*-DMA decreased the high concentration of plasma lipids induced by estrogen deficiency. Anyhow it is very important to keep daidzein metabolism in mind when we consider the effects of daidzein on human health.

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