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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 miniosmotic 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

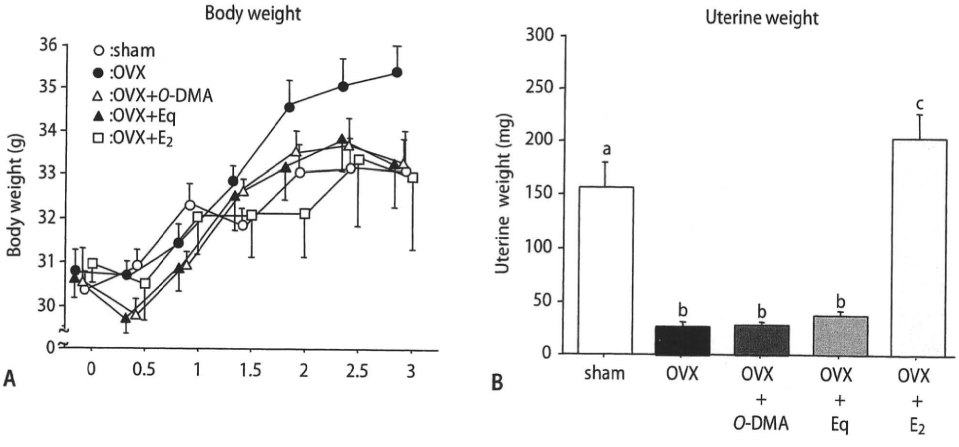

Fig. 1 Body weight and uterine weight of sham-operated (sham) mice, ovariectomized (OVX) mice, OVX mice treated with 0.5 mg/day *O*-DMA (OVX + *O*-DMA), or 0.5 mg/day equol (OVX + Eq), or 0.03 μg/day E_2 (OVX + E_2). **A** Body weight, **B** uterine weight. Values are means ± SEM, $n = 5$ per group.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

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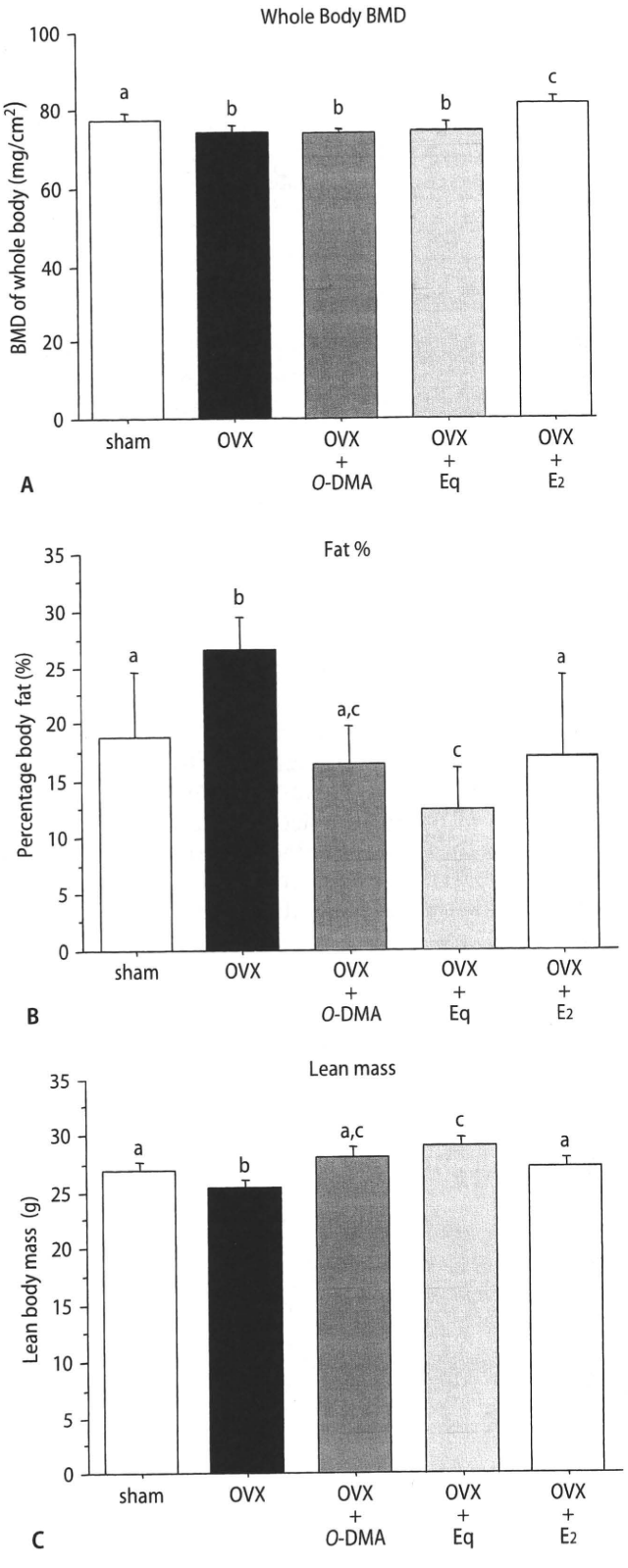

◀ **Fig. 2** Body composition and bone mineral density (*BMD*) of the whole body of sham-operated (sham) mice, OVX mice, and OVX mice treated with 0.5 mg/day *O*-DMA (OVX + *O*-DMA), or 0.5 mg/day equol (OVX + Eq), or 0.03 μg/day E_2 (OVX + E_2) for 3 weeks. **A** BMD of the whole body, **B** body fat (%), **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-

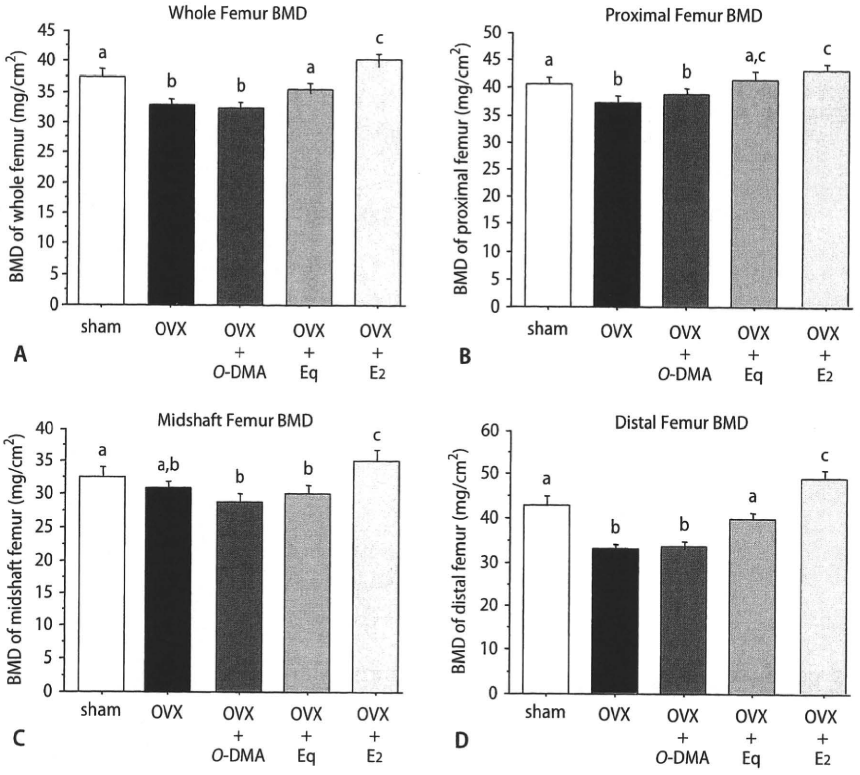

Fig. 3 Bone mineral density of the femur collected from sham-operated (sham) mice, OVX mice, and OVX mice treated with 0.5 mg/day *O*-DMA (OVX + *O*-DMA), or 0.5 mg/day equol (OVX + Eq), or 0.03 μg/day E_2 (OVX + E_2) for 3 weeks. **A** BMD of the whole femur, **B** BMD of the proximal region in the 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 intertrochanter 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

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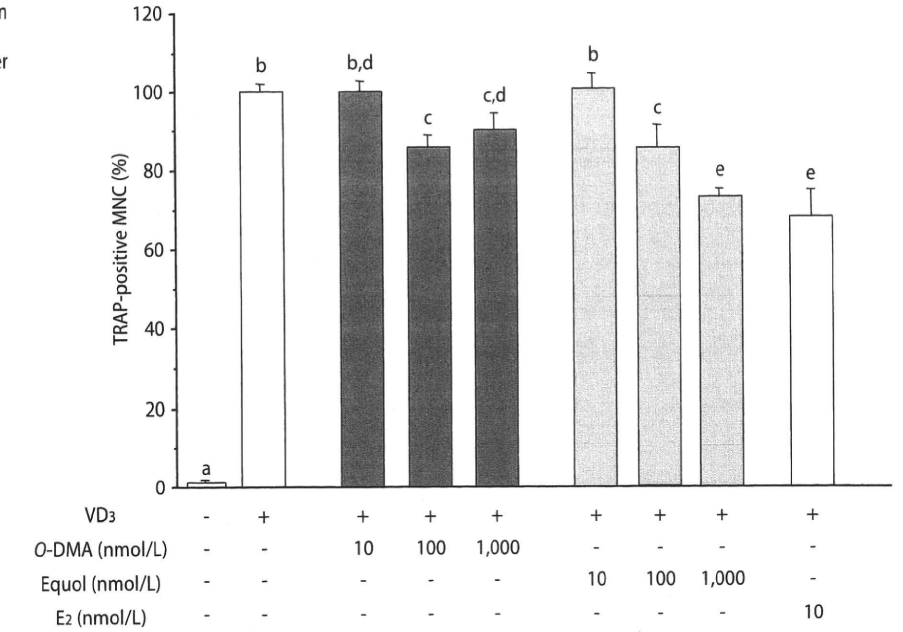

Fig. 4 Dose-response effects of *O*-DMA and equol on VD_3-induced TRAP-positive multinuclear osteoclast-like cells (MNC). Values are means ± SEM, $n = 6$ per each culture. Means with different letters differ significantly, $P < 0.05$

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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Effects of extracted soy isoflavones alone on blood total and LDL cholesterol: Meta-analysis of randomized controlled trials

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Abstract: When provided concurrently with soy protein for 1–3 months, soy isoflavones exert synergistic or additive cholesterol-lowering effects. This meta-analysis was performed to evaluate the effects of extracted soy isoflavones alone (not ingested concurrently with soy protein) on total and low density lipoprotein (LDL) cholesterol. MEDLINE (1966–2007), EMBASE (1966–2007), CENTRAL (1966–2007), ICHUSHI (1983–2008), and CNKI (1979–2007) were searched for randomized placebo-controlled trials published in English, Japanese, and Chinese, describing the changes in lipid profiles in adult humans resulting from ingestion of extracted soy isoflavones for 1–3 months. Reference lists of relevant systematic reviews and meta-analyses were hand-searched. Meta-analysis of 10 and 9 trials with usable information using REVMAN found that an average of 70 mg soy isoflavones/day (27–132 mg, as the aglycone form) alone had a nonsignificant effect on total (0.01 mmol/L [95% CI: −0.12, 0.14]; $P = 0.86$) and LDL (0.03 mmol/L [95% CI: −0.11, 0.16]; $P = 0.71$) cholesterol in menopausal women, respectively. It is concluded that ingestion of about 70 mg extracted soy isoflavones/day alone for 1–3 months does not improve total and LDL cholesterol levels in normocholesterolemic menopausal women; further studies are needed to verify the effects of extracted soy isoflavones.
Keywords: extracted soy isoflavones, lipid, total cholesterol, LDL cholesterol

Introduction

Meta-analysis of 23 randomized controlled trials (RCTs) with durations of 3–26 weeks has found that soy protein ingested with isoflavones intact significantly decreased serum levels of total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C); subgroup analyses demonstrated that studies employing intakes of >80 mg isoflavones/day showed better effects, and that the lowering effects were most marked within 3–12 weeks (Zhan and Ho 2005). Our previous meta-analysis of 11 RCTs found that ingestion of soy protein with a high isoflavone content reduced serum LDL-C to a greater degree than ingestion of the same amount of soy protein with a low isoflavone content for 1–3 months (Taku et al 2007). These two meta-analyses suggested that soy isoflavones would have synergistic or additive effects on cholesterol lowering when provided concurrently with soy protein for 1–3 months. However, the effects of extracted soy isoflavones alone (not ingested concurrently with soy protein) are unclear.

Meta-analysis of RCTs employing isoflavones in tablet form demonstrated nonsignificant effects on TC and LDL-C; however, only 5 of the 10 studies evaluated soy-derived isoflavones (Yeung and Yu 2003). So far, only one meta-analysis of RCTs has evaluated the effects on lipid of soy isoflavones without soy protein. This included 9 trials lasting 4–26 weeks, and revealed a nonsignificant effect of extracted soy isoflavones on LDL-C, although there was a high degree of heterogeneity in mean net changes across the studies (Balk et al 2005).

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We have identified several other recently published RCTs of soy-derived isoflavones, and the present meta-analysis was performed to clarify the effects of extracted soy isoflavones ingested alone for 1–3 months on TC and LDL-C, independent of any effect of soy protein on the same parameters.

Subjects and methods

Study identification and selection

MEDLINE (1966–2007), EMBASE (1966–2007), CENTRAL (1966–2007), ICHUSHI (1983–2008), and CNKI (1979–2007) were searched for randomized placebo-controlled trials published in English, Japanese, or Chinese describing the effects of ingesting extracted soy isoflavones for 1–3 months in adult humans. Reference lists of relevant systematic reviews (Balk et al 2005; Cassidy et al 2006; Sacks et al 2006), and meta-analyses (Yeung and Yu 2003; Zhan and Ho 2006) were hand-searched.

After excluding two RCTs (Tormala et al 2006; Badeau et al 2007) that were subset analyses of an original trial (Nikander et al 2004) and two (Lissin et al 2004; Petri Nahas et al 2004) in which actual endpoint TC and LDL-C values were not clear, 12 RCTs (Nestel et al 1997; Simons et al 2000; Dewell et al 2002; Uesugi et al 2002, 2003, 2004; Nikander et al 2004; Yildiz et al 2005; Garrido et al 2006; Hall et al 2006; Cheng et al 2007; Gonzalez et al 2007) and 10 RCTs (Nestel et al 1997; Simons et al 2000; Uesugi et al 2002, 2004; Nikander et al 2004; Yildiz et al 2005; Garrido et al 2006; Hall et al 2006; Cheng et al 2007; Gonzalez et al 2007) that had respectively evaluated the effects of ingesting extracted soy isoflavones alone on TC and LDL-C in adult humans for 1–3 months, and had reported actual endpoint values for each of the comparison groups, were included in the meta-analysis. Two trials (Yildiz et al 2005; Garrido et al 2006) and one trial (Garrido et al 2006) were subsequently withdrawn, leaving 10 and 9 RCTs with usable information that were finally selected for meta-analysis of TC and LDL-C, respectively (see Figure 1). Two reviewers independently reviewed and evaluated the studies, and consensus was reached by discussion when there were disagreements.

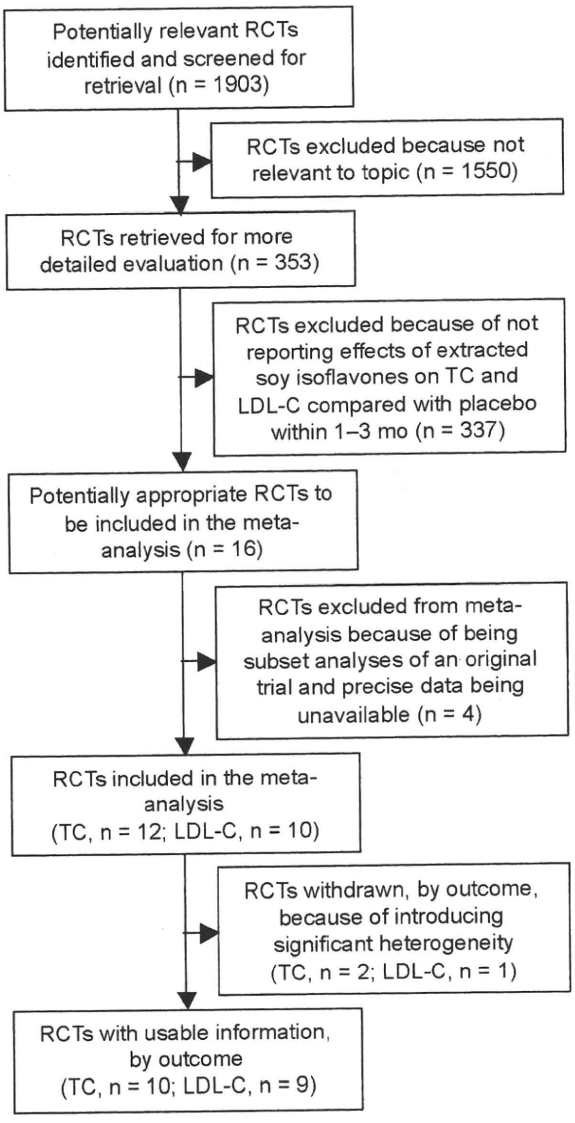

Figure 1 The QUOROM statement flow diagram.
Notes: Potentially relevant randomized controlled trials (RCTs) were identified from database PubMed (n = 837), CENTRAL (n = 386), EMBASE (382), ICHUSHI (n = 62) and CNKI (n = 236).
Abbreviations: QUOROM, quality of reports of meta-analyses of randomized controlled trials; RCTs, randomized controlled trials; TC, total cholesterol; LDL-C, low density lipoprotein cholesterol.

Data extraction

Data on study design and duration, subjects, total soy isoflavones, baseline TC and LDL-C for the isoflavone group, the numbers of participants (N) in each of the comparison groups, and the means and SDs of the endpoint TC and LDL-C values were independently extracted for meta-analysis by two reviewers with double-checking. Normally, serum cholesterol and TG concentrations are about 3% higher than corresponding plasma concentrations (LMC 1977), but because we were interested in mean differences in each study, we analyzed plasma and serum concentrations without correction for this difference; all results are reported as blood concentrations here.

Assessment of study quality

Sensitivity analyses on the basis of methodological quality demonstrated that poor-quality studies indicated a positive effect

of treatment, whereas no benefit was observed in high-quality studies (Khan et al 1996). We used the Jadad scale (Jadad et al 1996) to assess the quality of RCTs included in the meta-analysis. The Jadad scale assesses randomization, blinding, and reporting of withdrawal and dropouts, and assigns studies quality scores ranging from 0 to 5, with a score of >2 indicating high quality (Lind et al 2001; Sjogren and Halling 2002). Concealment of treatment allocation in RCTs was assessed as adequate, inadequate, or unclear (Schulz et al 1995).

Meta-analysis

We performed the meta-analysis to determine the combined effect of extracted soy isoflavones alone on TC and LDL-C using the weighted mean difference method in REVMAN (version 4.2.10; Cochrane Collaboration, Oxford, UK), by inputting the N and the means and SDs of endpoint lipid concentrations for two groups in each comparison. The treatment effect was estimated as the mean difference in endpoint values between comparison groups (ie, the value for subjects ingesting extracted soy isoflavones alone minus that for subjects ingesting a placebo). We did not use mean change from baseline outcomes instead of mean endpoint value outcomes, because mean changes were not available in most of the included studies, and imputed SDs of the changes should not be used for a majority of studies in a meta-analysis (Cochrane 2006). Although 2 of the 12 RCTs reported both endpoint values and changes (Nikander et al 2004) or percentage changes (Gonzalez et al 2007) from the baseline, we used endpoint value outcomes to maintain consistency across trials.

We used either a fixed effect model or a random effects model to calculate weighted mean differences (WMD), 95% CIs for each comparison, a combined overall effect with *P*-value, and the *P*-value for testing heterogeneity ($P < 0.1$ was considered significant). We presented the results based on the random effects model when there was heterogeneity that could not be readily explained (Zhan and Ho 2005; Cochrane 2006); otherwise, the results obtained using the fixed effect model were presented.

To explore the possible reasons for differences in results across trials, especially when the test for heterogeneity was significant ($P < 0.1$ or $I^2 > 25\%$) (Cochrane 2006; Nelson et al 2006), we performed subgroup meta-analyses based on variability in the participants' baseline TC and LDL-C, intervention duration, dose of soy isoflavones, study design and study quality (Yeung and Yu 2003; Zhan and Ho 2005). Sensitivity analyses were performed on the basis of participants' baseline TC and LDL-C, dose of soy isoflavones, and study quality. We examined potential publication bias by using funnel plots of the SEs of the studies against their corresponding effect sizes.

Results

Study and treatment group characteristics

The characteristics of the 12 RCTs included in the meta-analysis are summarized in Table 1. Six trials used a parallel design, and the remaining trials used a crossover design. Concealment of treatment allocation for all trials was assessed as "unclear" due to insufficient information. All trials focused on menopausal women. Doses of total soy isoflavones were 42–150 mg/day (27–132 mg/day expressed as the aglycone form). One trial (Yildiz et al 2005) reported extremely small SDs for endpoint TC values, compared with SDs for other lipids in the trial and SDs reported in remaining trials. Significantly different endpoint TC and LDL-C values between the two comparison groups were reported in two trials (Yildiz et al 2005; Garrido et al 2006) and one trial (Garrido et al 2006), respectively, possibly due to their significantly different baseline values; the remaining trials did not reported significantly different TC and LDL-C values at the baseline and endpoint between comparison groups.

In addition, subjects in most of the comparisons had similar diets, with similar amounts of fat (total and saturated), cholesterol, and fiber. Most of the studies were designed to maintain subjects' usual diets and lifestyles. No significant differences in age, body weight, or body mass index were reported between the comparison groups.

Adverse events of extracted soy isoflavones were evaluated according to the evidence report (Balk et al 2005). Of the 12 RCTs included, one trial (Garrido et al 2006) that used 100 mg soy isoflavones/day reported one case of abdominal bloating and one case of nicturia; one trial (Nikander et al 2004) using 114 mg soy isoflavones/day reported two cases of gastralgia and one recurrence of breast cancer; one trial (Simons et al 2000) using 80 mg soy isoflavones/day reported one case of paresthesia and one case of brief menstrual period 5 weeks into the treatment (the subject being >2 years postmenopausal).

Meta-analysis

Meta-analysis of the 12 RCTs that reported the endpoint TC demonstrated significant heterogeneity ($P < 0.00001$). Use of the random effects model did not reveal any significant effect of extracted soy isoflavones alone on TC. Excluding two outlying trials (Yildiz et al 2005; Garrido et al 2006)

Table 1 Characteristics of 10 included randomized placebo-controlled trials with usable information

Study and reference	Design and duration[a]	Subjects[b]	Total soy isoflavones (mg/d)[c]	Jadad scale	Baseline lipids[d]	
					TC	LDL-C
Cheng et al 2007	P; R+; DB+; WD (15%); 3 mo	60 healthy PoW	60 [37 (De, 41%; Ge, 52%; Gle, 7%)]	5	5.70	3.10
Dewell et al 2002	P; R; DB; WD (0%); 2 mo	36 PoW	150 (Ge, 27%; De and Gle, 33%; glycosides, 40%) [128]	3	6.80	NR
Garrido et al 2006	P; R; DB+; 12 wk	29 healthy PoW	[95 (De, 49%; Ge, 51%)]	3	5.50*	3.40*
Gonzalez et al 2007	CO; R+; DB+; WD (19%); 12 wk	32 type 2 diabetes PoW	[132 (De, 37%; Ge, 53%; Gle, 10%)]	5	5.40	3.40
Hall et al 2006	CO; R; DB+; WD (12%); 8 wk	117 healthy PoW	[50 (De, 33%; Ge, 67%)]	4	6.03	3.88
Nestel et al 1997	CO; R; SB; WD (9%); 5 wk	23 MPW	[80 (De, 42%; Ge, 54%; Gle, 4%)]	2	5.54	3.57
Nikander et al 2004	CO; R+; DB+; WD (10%); 3 mo	62 PoW with a history of breast cancer	[114 (De, 36%; Ge, 6%; Gle, 58%)]	5	5.88	3.87
Simons et al 2000	CO; R; DB+; WD (13%); 8 wk	23 healthy PoW	[80 (De and Ge, 100%)]	4	5.86	3.94
Uesugi et al 2002	P; R; DB; WD (0%); 4 wk	23 healthy PW	62 [38 (De, 52%; Ge, 11%; Gle, 37%)]	3	5.85	3.83
Uesugi et al 2003	P; R; WD (4%); 3 mo	22 PoW	62 [38 (De, 52%; Ge, 11%; Gle, 37%)]	2	5.79	NR
Uesugi et al 2004	CO; R; DB+; 4 wk	58 CW	42 [27 (De, 46%; Ge, 13%; Gle, 41%)]	3	5.87	3.62
Yildiz et al 2005	P; R; SB; WD (0%); 3 mo	80 healthy PoW	[40 mg/d (Ge, 100%)]	2	5.81*	3.98

Notes: [a]P, Parallel; CO, crossover; R, randomized; R+, randomized by appropriate method; DB, double-blinded; DB+, double-blinded by appropriate method; SB, single-blinded; WD, withdrawals and dropouts described. [b]Number randomized; PoW, postmenopausal women; MPW, menopausal and perimenopausal women; PW, perimenopausal women; CW, climacteric women. [c]Values in brackets are expressed as the aglycone form; De, daidzein; Ge, genistein; Gle, glycitein. [d]Total cholesterol (TC) and LDL cholesterol (LDL-C) concentration (mmol/L) for soy isoflavones treatment group; to convert mg/dL to mmol/L, multiply by 0.02586; *$P < 0.05$ vs. placebo.

showing the poorest overlap (apparently due to the extremely small SDs and significant difference in baseline values) on the forest plot, meta-analysis of 10 RCTs with usable information (see Figure 1) using the fixed effect model demonstrated nonsignificant heterogeneity and a nonsignificant effect of an average intake of 73 (27–132, as the aglycone form) mg soy isoflavones/day on TC (0.01 mmol/L [95% CI: –0.12, 0.14]; $P = 0.86$). Results obtained using the random effects model were identical.

Meta-analysis of the 10 RCTs that reported the endpoint LDL-C demonstrated significant heterogeneity ($P = 0.003$), and use of the random effects model revealed no significant effect of extracted soy isoflavones alone on LDL-C. Excluding one outlying trial (Garrido et al 2006) with the poorest overlap (apparently due to the significant difference in baseline values) on the forest plot, meta-analysis of 9 RCTs with usable information (see Figure 1) demonstrated nonsignificant heterogeneity and a nonsignificant effect of an average intake of 67 (27–132, as the aglycone form) mg soy isoflavones/day using the fixed effect model (see Figure 2) on LDL-C (0.03 mmol/L [95% CI: –0.11, 0.16]; $P = 0.71$). Results obtained using the random effects model were similar.

Subgroup analyses for the effect on LDL-C found that trials lasting >2 months resulted in significant heterogeneity and that trials with a parallel design resulted in a significant effect (see Table 2). Subgroup analyses for the effect on TC on the basis of the same variables did not reveal any subgroups with significant results. Sensitivity analyses excluding one trial with LDL-C ≤ 3.36 mmol/L (Cheng et al 2007) or one trial with TC > 6.21 mmol/L (Dewell et al 202), trials that evaluated 20–40 (or > 80) mg soy isoflavone aglycones/day, or trials of poor quality did not significantly influence the results. The funnel plots for the effects on TC and LDL-C did not indicate any obvious publication bias (data not shown).

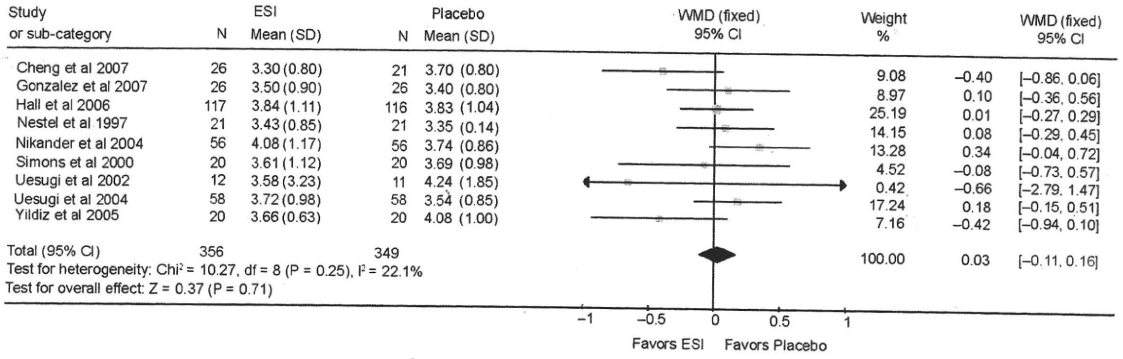

Figure 2 Effect of extracted soy isoflavones (ESI) alone on blood LDL cholesterol.
Notes: Weight assigned by REVMAN software based on N and SD. WMD, Weighted mean difference in endpoint values between the ESI alone (not ingested concurrently with soy protein) and placebo groups; fixed, fixed effect model. Horizontal lines denote the 95% CI. ■, point estimate (size of the square corresponds to its weight); ◆, combined overall effect of ESI treatment.

Discussion

Although two previous meta-analyses have suggested that soy isoflavones have synergistic or additive effects in lowering TC and LDL-C when provided concurrently with soy protein for 1–3 months, especially in hypercholesterolemic (TC > 6.21 mmol/L or LDL-C > 4.14 mmol/L) subjects (Zhan and Ho 2005; Taku et al 2007), the present meta-analysis found that ingestion of an average of 70 mg extracted soy isoflavones/day (as the aglycone form) alone (not ingested concurrently with soy protein) for 1–3 months did not significantly change TC and LDL-C compared with placebo in normocholesterolemic menopausal women. The

Table 2 Combined treatment effect of extracted soy isoflavones alone on LDL cholesterol in subgroup analyses

Variables	No. of trials	Sample size (ESI/PLA)	WMD (95% CI) (mmol/L)	*P* for effect	*P* for heterogeneity
Baseline LDL cholesterol					
≤3.36 mmol/L	1	26/21	−0.04 (−0.86, 0.06)	0.09	Not available
>3.36 mmol/L	8	330/328	0.07 (−0.08, 0.21)	0.35	0.47
Study duration					
≤2 mo	5	228/226	0.06 (−0.11, 0.24)	0.49	0.87
>2 mo	4	128/123	−0.07 (−0.46, 0.31)	0.70	0.04
Total isoflavone aglycones					
20–40 mg/d	4	116/110	−0.11 (−0.35, 0.13)	0.35	0.11
41–80 mg/d	3	158/157	0.02 (−0.19, 0.23)	0.83	0.91
>80 mg/d	2	82/82	0.24 (−0.05, 0.54)	0.10	0.43
Daidzein and genistein					
20–40 mg/d	4	116/110	−0.11 (−0.35 0.13)	0.35	0.11
41–80 mg/d	4	214/213	0.10 (−0.09, 0.28)	0.30	0.52
>80 mg/d	1	26/26	0.10 (−0.36, 0.56)	0.67	Not available
Study design					
Parallel	3	58/52	−0.42 (−0.75, 0.08)	0.02	0.97
Crossover	6	298/297	0.11 (−0.04, 0.27)	0.14	0.79
Study quality					
High	7	315/308	0.06 (−0.10, 0.21)	0.47	0.30
Poor	2	41/41	−0.09 (−0.39, 0.21)	0.57	0.12

Abbreviations: WMD, weighted mean difference; ESI, extracted soy isoflavones; PLA, placebo.

inconsistency of the results might be due mainly to the differences in effects between extracted soy isoflavones alone and soy isoflavones provided concurrently with soy protein. Another explanation might be that the two previous meta-analyses included more trials evaluating the effects of soy protein using larger amounts of soy-associated isoflavones in hypercholesterolemic subjects and used different approaches to determine the combined treatment effects. The lack of any beneficial effects of extracted soy isoflavones alone on TC and LDL-C was consistent with a previous meta-analysis that evaluated RCTs of soy isoflavones without soy protein (Balk et al 2005). However, that study noted a high degree of heterogeneity with mean net changes across the 9 included trials with durations of 4–26 weeks using the inverse variance method and by arbitrarily assuming the correlation coefficient between baseline and endpoint lipid values to be 0.5.

A large cohort study reported that high isoflavone intake from soy foods was associated with a reduced risk of cerebral and myocardial infarction in Japanese women, the risk reduction being pronounced for postmenopausal women (Kokubo et al 2007). It is still unclear why intake of isoflavones as a component of intact soy protein or soy foods had beneficial effects on lipid profile, whereas extracted soy isoflavones alone had no such effects. When producing the soy isoflavone extract, alcohol extraction may have removed the active agent, the isoflavones may have been inactivated during the process of purification, or some enabling factor in soy protein may be required for the beneficial effects of soy isoflavones on lipid profiles (Clarkson and Anthony 1998). The beneficial effects of soy protein might require synergistic interaction between isoflavones and other soy components (Clarkson 2002). Isoflavone glycosides are not absorbed intact across the enterocytes of healthy adults, and their bioavailability requires initial hydrolysis by intestinal β-glucosidases for uptake to the peripheral circulation (Setchell et al 2002). Absorption of isoflavones is affected by food matrix and processing in humans (de Pascual-Teresa et al 2006), and differences in bioavailability between soy isoflavones contained in intact soy protein and in various extract supplements (without soy protein) may account for the differences in effects on lipid profiles.

Subgroup and sensitivity analyses based on participants' baseline lipid values, soy isoflavone dose, and study quality revealed no significant influence of extracted soy isoflavones on TC and LDL-C. However, interpretation of the results for any of the variables considered is limited because of the small number of trials available. We did not conduct subgroup analysis to evaluate the effect of isoflavone metabolites (equol and O-DMA) because only one trial had reported that being an equol producer or nonproducer was not a factor influencing the effects of extracted soy isoflavones on lipid profiles (Nikander et al 2004); another trial reported the urinary excretion of equol and O-DMA for equol producers and nonproducers, but did not address the effect on lipids.

In conclusion, our meta-analysis has shown that ingestion of about 70 mg extracted soy isoflavones/day (as the aglycone form) alone (not ingested concurrently with soy protein) for 1–3 months does not decrease blood TC and LDL-C levels in normocholesterolemic menopausal women. Further studies are needed to verify the long-term effects of extracted soy isoflavones alone in other subjects and possible adverse events.

Acknowledgments

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Effects of Soybean Isoflavones on Bone Health and its Safety in Postmenopausal Japanese Women

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Sumamry **Recently, phytoestrogens have received a great deal of attention for their potential role in preventing osteoporosis and hypercholesterolemia. On the other hand, it is well established that exercise is also effective in preventing bone loss and hypercholesterolemia resulting from estrogen deficiency. In this regard, we examined the combined effects of isoflavones and walking exercise on bone in postmenopausal Japanese women. A total of 136 postmenopausal women at <5 years after the onset of menopause were randomly assigned to 1) placebo, 2) walking (45 min/day, 3 days/week) with placebo, 3) isoflavone intake (75 mg of isoflavone conjugates/day; equivalent to 47 mg of aglycone form), and 4) combination of isoflavone plus walking groups. The results showed that the combination of isoflavone plus walking for 1 year had a greater effect on the bone than either alone. Isoflavones did not affect serum sex and thyroid hormones. These findings suggest that the combined intervention of isoflavone plus walking may offer a potential regimen for the primary prevention of osteoporosis in postmenopausal women without unfavorable effects. Regarding the effect of equol producing status, % change in bone loss in total hip was lower in equol producers than in non-producers. Additionally, we found that a certain bacteria, *Lactococcus* 20-92 strain, which can produce equol from daidzin via daidzein, existed in the feces of the subjects.**

Key Words**: isoflavone, exercise, BMD, equol, safety**

Introduction

Soybean isoflavones are structurally similar to estrogen having the ability to bind to estrogen receptor and exhibit weak estrogen activity. Since, isoflavones is of benefits on bone health in postmenopausal women as well as estrogen-deficient animals, it was approved as a principle ingredient of Foods for Specified Health Uses (FOSHU) which is the functional foods with health claim approved by Japanese Ministry of Health, Labour and Welfare (MHLW) in 2001.

The factors which influence bone metabolism include genetics, hormone, nutrition, physical activity and other lifestyles. Frost hypothesized that estrogen deficiency increased the set point for the skeleton to response to loading, causing the skeleton to be less sensitive to mechanical force and de-

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creasing bone mass [1]. We have recently reported that, in the prevention of bone loss and fat gain in estrogen-deficient animals, a combined intervention of moderate-intensity exercise and isoflavone administration was more advantageous than either treatment alone [2]. To assess this issue in humans, we examined the cooperative effects of soy isoflavone intake and walking on bone metabolism in postmenopausal Japanese women.

Recently, it was suggested that equol, a gut bacterial metabolite of daidzein, may play an important role in the effects of isoflavones [3]. Thus, we also analyzed the effects of isoflavones on BMD by equol status in placebo and isoflavone treated groups. Furthermore, hormone levels in the study participants were evaluated, since the safety evaluation of FOHSU containing isoflavones was conducted by the Japanese Food Safety Commission of the Cabinet in 2006.

Materials and Methods

Effects of isoflavones and exercise on BMD

One hundred thirty-six subjects (average age was 55 years old), who were postmenopausal within 5 years of natural menopause, were randomly assigned to four groups: placebo; walking combined with placebo (3 times/week, 6 km/h); isoflavone intake (75 mg conjugates/day; equivalent to 47 mg of aglycone, Fujicco Co. Ltd, Kobe, Japan) in addition to the normal diet, and isoflavone combined with walking exercise. After 1 year intervention, 108 subjects were completed the study. BMD of the lumber spine, left hip, and sub-whole body were assessed by DXA using Hologic QDR-4500 (Hologic Inc., Waltham, MA) at baseline and after 1 year. Serum isoflavone concentrations in each participant were determined by reverse-phase high-performance liquid chromatography (HPLC).

Possible role of equol status in the effects of isoflavones on bone

Fifty-four women (29 in the placebo, 25 in the isoflavone groups) completed the 1 year intervention, and their data were used for equol analysis. Equol production was assessed by fecal suspension incubated with daidzein under anaerobic condition and the released equol in the medium was analyzed by HPLC. The number of equol producers and nonproducers in each group was 15 and 14 in the placebo group, respectively; and 15 and 10 in the isoflavone group, respectively. Serum sex hormones and thyroid hormones were analyzed by ELISA methods. *Lactococcus garvieae* (*Lc. garvieae*) in the feces of the study participants was detected by real time-PCR method using the particular primer for *Lc. garvieae* [4]. Statistical analyses were performed SPSS for Windows (version 13.0J), and a p value was set at <0.05.

Results and Discussion

Effects of isoflavones and walking intervention on BMD in postmenopausal women

There was no difference in height, weight, BMI and physical activities among the 4 groups, and between baseline and after 1 year intervention in each group. Average daily intake of isoflavone aglycone from soy foods were around 28 mg per day in the subjects in 4 groups at baseline and after 1 year. There were no significant differences in daily intake of isoflavones and other nutrients among the groups at baseline, and between baseline and after 1 year in each group. Of the % change in BMD, walking showed significant main effects on the preservation of BMD in the total hip region after 1year. Interventions with isoflavones or walking showed significant main effects on the preservation of BMD at Ward's triangle in the hip after 1 year. However, no interaction was observed between the two interventions [5].

Analysis of the effects of isoflavones on BMD by equol producing status

In the next study, we stratified the subjects in placebo and isoflavone intervention groups based on their fecal equol production and serum equol concentrations in order to investigate whether any difference exists in the effects of isoflavone on BMD between equol producers and nonproducers in postmenopausal Japanese women. Percentage of equol producers in our subjects were 55% by assessment of equol production in fecal suspension incubated with daidzein under anaerobic condition. Serum daidzein dramatically increased in isoflavone group after 1 year. However, there was no difference in serum daidzein between equol producer and nonproducers. On the other hand, serum equol was increased only in equol producer in isoflavone group after 1 year. Serum equol did not change in equol producer in placebo group after 1 year. The % change in bone loss at total hip and inter-trochanter of the hip of equol producers was significantly lower than that of equol non-producers in isoflavone intervention group by student's t-test. However, none of the difference between producer and non-producers was observed in placebo group. From these results the effects of isoflavone on bone mass might depend on equol producing activity in postmenopausal women [6].

Isolation of equol producing bacteria from human feces

Ueno and Uchiyama have found mainly four equol producing bacteria from human feces, first in the world [7], and concluded *Lactococcus* 20-92 strain (*Lc.* 20-92) as homologous to *Lc. garvieae* is the most appropriate bacteria for food usage, because we have dietary habit of *Lc. garvieae*, which is in Italian cheese without toxicity and bad smell.

We examined equol production in soymilk fermented with *Lc.* 20-92 (Fig. 1A). In soy milk, daidzin, which is a glyco-

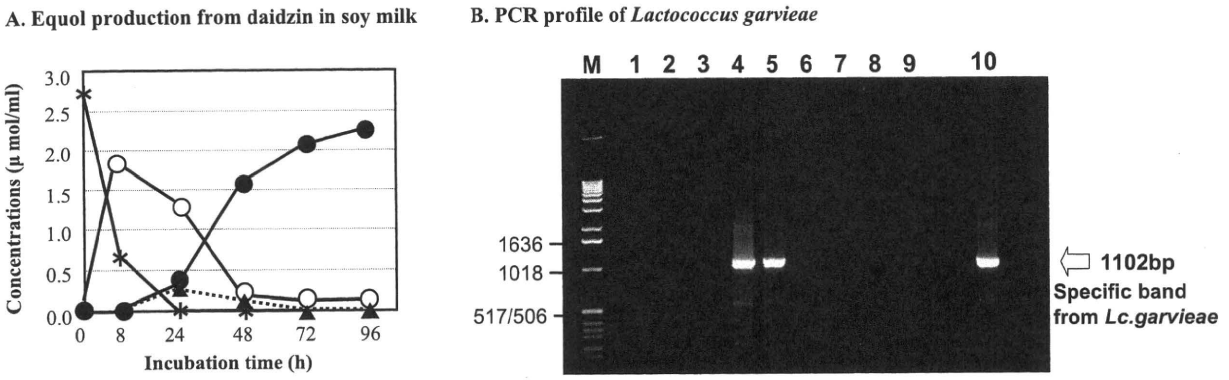

Fig. 1. (A) Equol production from daidzin in soymilk fermented with strain *Lactococcus* 20-92*; daidzin, ○; daidzein, ▲; dihydrodaidzein, ●; equol. Data show the mean of 3 experiments. (B) Detection of *Lactococcus garvieae* using PCR specific products of *Lactococcus garvieae* existed in the feces of postmenopausal Japanese women. *Lactococcus garvieae* PCR assay was analyzed by real time-PCR method using species-specific primers of *Lactococcus garvieae*. 16S rRNA specific primer of *Lactococcus garvieae* used pLG-1 (5'-CAT AAC AAT GAG AAT CGC-3') and pLG-2 (5'-GCA CCC TCG CGC GTT G-3'). The results of RT-PCR in some fecal samples; Lane M: 1 Kb DNA Ladder, Lane 1–9: Fecal samples, Lane 10: Positive Control (*Lactococcus garvieae* type strain ΔJCM10343)

side form of daidzein, was mainly existed. After the fermentation with *Lc.* 20-92, the peak for daidzin disappeared, and daidzein and dihydrodaidzein elevated within 24 h, and after that a peak for equol came out very clearly and the production increased linearly until 96 h. Another metabolite, *O*-DMA was not detected. Interestingly, *Lc.* 20-92 can cleave glycosidic bond. Then we confirmed if *Lc.* 20-92 exists in the feces of our study participant by real time-PCR method using the particular primer for *Lc. garvieae*. Fig. 1B shows an example which *Lc. garvieae* was found in human feces. The bacteria was detected in 47 of 133 samples, this means *Lc. garvieae* existed in 35.3% postmenopausal Japanese women in this study [*8*]. Interestingly, the people with *Lc. garvieae* were not always equol producer, suggesting that some other bacteria and several factors such as hydrogen gas and short chain fatty acids, which can affect the environmental conditions in the colon, might be also important for equol production in humans.

Safety evaluation of isoflavones in postmenopausal Japanese women

Soybean isoflavone was approved in 2001 as the principle ingredient in FOSHU as targeted to individuals concerned about bone health. There are tea, soymilk and soft drink containing 40 mg of isoflavone conjugates, which is equivalent to 25 mg of aglycone form. In 2004, applications of a tablet containing soy isoflavone aglycones as its principal ingredient, and a fermented food containing isoflavone aglycone in contents exceeding the usual amounts in FOSHU were filed for the approval. Foods with fortified or condensed isoflavones had not been consumed before. And there is possibility that tablet and capsules would be excessively consumed. Accordingly, the Japanese Food Safety Commission of the Cabinet conducted a safety evaluation on soy isoflavones, and issued a Notice: "Basic approaches to evaluating the safety of FOSHU containing soy isoflavones" in 2006. The main contents of the report are summarized in 3 points. Firstly, the upper limit of isoflavone aglycone intake from FOSHU was set at 30 mg/day for additional consumption with a normal diet. Secondly, the maximum recommended level for safe isoflavone aglycone intake in a daily diet was set at 70 to 75 mg/day at the present time. Thirdly, it was not recommended that pregnant women, infants and children take soy isoflavone from FOSHU [*9*]. These criteria were also adapted to the so-called Health Foods fortified with isoflavones.

In order to examine the effects of isoflavone intake on hormone levels in postmenopausal women, we evaluated serum concentrations of estrogen, FSH, LH, progesterone and thyroid hormones in our participants. Table 1 shows that there were no significant differences in estrogenic hormone levels between placebo and isoflavone treatment groups. The same results were obtained in thyroid hormone levels. These results suggest that isoflavone intake for additional consumption with normal diet (total intake was about 75 mg of aglycone equivalent a day) for a year did not affect serum hormone levels in postmenopausal Japanese women.

It is well known that there are a lot of benefits, when we take soy foods. For example, they are good source of protein, calcium, and also soy protein decreases the serum cholesterol and isoflavones preserve the bone health in postmenopausal women. Therefore, MHLW set for the goal at 100 g/day of intake of beans in Health Japan 21 Program since 2000. Soybeans have been consumed since ancient times in Asia, and have left no report of any problems even when used excessively. Soy isoflavone intake from soy foods in a normal daily diet, therefore, is considered free of concerns for safety.

Table 1. Effects of 1 year isoflavone intake on serum sex and thyroid hormone concentrations in postmenopausal japanese women

		Placebo (n = 29)	Isoflavone (n = 25)	Placebo vs Iso
Estradiol (pg/mL)				
	Baseline	12.66 (4.03)	12.32 (3.34)	NS
	After 1 year	12.98 (7.31)	11.98 (2.94)	NS
	% change	6.09 (57.71)	1.20 (23.80)	NS
FSH (U/L)				
	Baseline	70.36 (26.02)	68.19 (18.66)	NS
	After 1 year	60.00 (19.83)*	58.12 (17.86)*	NS
	% change	−12.36 (8.40)	−14.05 (9.01)	NS
LH (U/L)				
	Baseline	26.68 (13.87)	27.70 (9.32)	NS
	After 1 year	22.43 (11.12)*	22.33 (7.90)*	NS
	% change	−12.16 (15.98)	−19.10 (14.44)	NS
Progesterone (ng/mL)				
	Baseline	0.27 (0.11)	0.29 (0.16)	NS
	After 1 year	0.21 (0.10)*	0.24 (0.12)*	NS
	% change	−25.54 (29.47)	−14.07 (18.08)	NS
T3 (ng/mL)				
	Baseline	1.13 (0.16)	1.08 (0.13)	NS
	After 1 year	1.10 (0.16)	1.03 (0.16)	NS
	% change	−1.60 (60.11)	−3.92 (7.96)	NS
T4 (μg/dL)				
	Baseline	8.57 (1.12)	8.19 (1.28)	NS
	After 1 year	8.46 (1.13)	7.54 (1.42)*	NS
	% change	−1.85 (7.46)	−4.48 (6.23)	NS
TSH (mU/L)				
	Baseline	2.34 (1.10)	2.30 (0.74)	NS
	After 1 year	2.75 (2.81)	2.25 (1.10)	NS
	% change	12.71 (69.59)	−9.22 (32.74)	NS

* Significantly different from baseline by paired t test, $p<0.05$

Conclusions

First, isoflavone conjugate intake (47 mg aglycone equivalent) in addition to the normal diet (average intake was 28 mg aglycone equivalent a day) combined with walking exercise for a year prevented bone loss in total hip region in postmenopausal Japanese women. Second, preventive effects of daidzein on bone loss in postmenopausal Japanese women might depend on equol – producing activity. Third, isoflavone intake for additional consumption with normal diet for a year did not affect serum hormone levels in postmenopausal Japanese women.

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Isoflavone Regulates Lipid Metabolism *via* Expression of Related Genes in OVX Rats Fed on a High-fat Diet[1]

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Objective To investigate the effects of isoflavone on body weight, fat mass, and gene expression in relation to lipid metabolism. **Methods** Thirty-six female SD rats were ovariectomized or sham-operated and fed on a high-fat diet. Two months later, abdominal incision was made, blood was collected to separate serum, and the liver and adipose tissue were immediately collected and weighed. Some portions of these tissues were frozen in liquid nitrogen and stored at -80℃. **Results** Ovariectomy (OVX) with a high-fat diet could induce obesity in rats, while treatment with isoflavone significantly inhibited the increase in body weight and fat mass in abdomen. Serum total cholesterol and leptin were significantly decreased in isoflavone group, compared with the OVX group. The mRNA expression of liver fatty acid synthase (FAS) in the OVX group was significantly higher than that in sham-operated group, while this difference was not observed in the isoflavone group. The mRNA expression of liver hormone-sensitive lipase (HSL) in the OVX rats tended to be lower than that in the sham-operated rats. Furthermore, a large amount of isoflavone maintained the mRNA expression at a sham level. **Conclusion** Isoflavone may prevent obesity induced by ovariectomy with a high-fat diet, in part by modulating gene expression related to lipid metabolism.

Key words: Isoflavone; Lipid metabolism; Ovariectomy; Obesity; Gene expression

INTRODUCTION

Ovariectomy (OVX) causes the lack of estrogen and increases the body weight and white adipose tissue (WAT) in rodents[1]. Estrogen reverses these changes. Similar changes have been found in postmenopausal women on hormone replacement therapy[2].

Recently, there has been a considerable interest in the effects of phytoestrogen isoflavone on human health. Isoflavone is a type of phytoestrogen that primarily exists in soybeans and their products. It was reported that isoflavone can prevent menopausal symptoms, osteoporosis, cardiovascular diseases, and many types of cancers[3]. It has been shown that a higher daily isoflavone intake is associated with a lower body mass index (BMI) in postmenopausal women, and isoflavone-containing soy protein diets significantly improve plasma lipid concentrations[4]. These biological effects are most likely regulated through the changes in gene expression[5].

Lipid metabolism is a complicated process highly regulated by both peptides and steroid hormones and also influenced by diets[6]. There is evidence that dietary isoflavone may play a beneficial role in lipid metabolism. It was reported that consumption of isoflavone genistein and daidzein is associated with a lower BMI and fasting insulin concentration and a higher high-density lipoprotein (HDL) cholesterol in postmenopausal women with normal body weight[7] and consumption of genistein is associated with decreased BMI, body weight, waist circumference, and total body fat mass in postmenopausal women[8]. Genistein and daidzein also decrease insulin response to an oral glucose load. These findings indicate that isoflavones have beneficial effects on excess body weight, hyperinsulinemia, and hyperlipidemia, which are the major cardiovascular risk factors commonly associated with human obesity.

The present study was designed to investigate the effects of isoflavone on body weight, adipose tissue,

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serum lipids, and regulation of the expression of lipid metabolism-related genes in ovariectomized rats fed on a high-fat diet in order to clarify the mechanism of action of isoflavone on lipid metabolism.

MATERIALS AND METHODS

Animals

Two-month-old female Sprague-Dawley (SD) rats were obtained from Shizuoka Laboratory Animals Center (Shizuoka, Japan). The animals were group-caged in a specific pathogen-free (SPF) animal room and fed with standard laboratory chow (CE-2, Nippon Clea, Tokyo, Japan) for 1 week and their body weight was measured. Anesthetized with pentobarbital sodium (50 mg/kg body weight) through an abdominal incision, the rats were assigned to OVX group, L-ISO group, M-ISO group, H-ISO group, and sham-operated group. Subsequently, the rats were individually caged at 22 ℃ in a light-controlled environment in a 12 h light and 12 h dark cycle and switched to the assigned diet for 8 weeks. In order to evaluate the effects of interventions on lipid metabolism, all the rats were fed on a high-fat diet containing 150 g lard per kg based on AIN-93M[9], and an isoflavone-supplemented diet containing 0.17%, 0.33%, and 0.5% of Fujiflavone P40 (40% soy isoflavone content, Fujicco Co. Ltd., Kobe, Japan). The diet contained 0.68 g/kg, 1.3 g/kg, and 2.0 g/kg isoflavone, respectively, along with a high-fat diet. Fujiflavone P40 contains isoflavones, such as daidzin (20.4%), genistin (4.6%), and glycitin plus glycitein (13%). The rats were pair-fed with free access to water. Their body weight and food intake were assessed every week throughout the study. At week 8, the rats fasted overnight.

All procedures were undertaken in accordance with the National Institute of Health and Nutrition Guidelines for the Care and Use of Laboratory Animals.

Sample Collection and Analytical Methods

An abdominal incision was made and blood was collected into a plastic tube. Serum was prepared and stored at -20℃ for analysis. After the rats were sacrificed by exsanguination, the liver and adipose tissue were immediately collected and weighed. Some portions of these tissues (~1 g) were frozen in liquid nitrogen and stored at -80℃ for total RNA extraction.

Measurement of Serum Lipids and Adipokines

Total cholesterol (TC) and triglycerides (TG) in the serum were determined with commercially available kits (cholesterol C-test and triglyceride G-test; Wako Pure Chemical, Osaka, Japan). HDL-cholesterol level in the serum was measured by an enzymatic method (HDL-cholesterol-test; Wako Pure Chemical, Osaka, Japan). Serum leptin and adiponectin levels were measured with commercially available rat ELISA kits according to the manufacturer's instructions (leptin: rat leptin ELISA kit; B-Bridge International, CA, USA and adiponectin: mouse/rat adiponectin; ELISA kit from Otsuka Pharmaceuticals, Tokyo, Japan).

Analysis of Gene Expression Related to Lipid Metabolism

Fatty acid synthase (FAS), hormone-sensitive lipase (HSL), peroxisome proliferator-activated receptor alpha (PPARα) and peroxisome proliferator-activated receptor gamma (PPARγ) were analyzed.

Real-time-polymerase Chain Reaction

Total mRNA (500 ng) was extracted from the liver and fat tissue using a mRNA extraction kit (Qiagen, Tokyo, Japan) and reverse-transcribed into cDNA in 10 μL of reaction mixture using a reverse transcription kit (Takara Bio. Co., Tokyo, Japan). The reaction mixture was heated to 42℃ for 15 min and denatured at 95 ℃ for 2 min. The primer sequences are shown in Table 1. Real-time-polymerase chain reaction (RT-PCR) was used to quantify the mRNA expression by SYBR Green technology. Briefly, 40 cycles of PCR were performed to amplify cDNA with the forward and reverse primers for the gene of interest using SYBR Premix Ex Taq™ (Perfect Real Time Kit; Takara Bio., Tokyo, Japan) and the ABI PRISM® 7000 sequence detection system (Applied Biosystems Ltd., CA, USA). The direct detection of PCR products was monitored by measuring the increased fluorescence caused by the binding of SYBR Green to double-stranded DNA. The expression of mRNA was quantified using a critical threshold at which the emission increased above a threshold level. Non-specific changes in gene expression were normalized by the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, and the results were normalized with reference to the amount of GAPDH mRNA. The end products were checked for contamination and 'mispriming' by verifying the denaturing peaks and ensuring the presence of only 1 product.

Statistical Analysis

All data are expressed as $\bar{x} \pm s$. Statistical analyses