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Differential effects of isoflavones on bone formation in growing male and female mice

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Abstract

Few studies have examined the effects of isoflavones on bone formation during growth period in male and female animals. In this study, the effects of daidzein or genistein on bone formation were assessed in immature male and female mice. Five-week-old male and female mice were divided respectively into 3 groups (n = 8 per group) as follows: control group (C) fed a control diet (AIN-93G), daidzein group (D) fed a control diet containing 0.08% pure daidzein, and genistein group (G) fed a control diet containing 0.08% pure genistein. After 4 weeks, the male D group had a significantly higher bone mineral density (BMD) in whole body, lumbar spine, and femur than did the C group. On the contrary, BMD of the whole body and femur in the female D group was significantly lower than that in the C group. The BMD of the whole femur in the male G group also was significantly higher than for the C group. Histologic analysis revealed that the bone formation rate was significantly higher in the male D and G groups, and lower in the female D group compared with the C group. These results suggest that daidzein has a specific, sexually dimorphic effect on bone formation and BMD during growth period in mice.

1. Introduction

Soybean isoflavones show structural similarity to 17β-estradiol and therefore exhibit affinity for the estrogen receptor and estrogenic activity, and compete with estrogen for this receptor [1,2]. Recently, isoflavones have received a great deal of attention for their preventive role against hormone-dependent diseases including postmenopausal osteoporosis, hyperlipidemia, cancer [3,4], and its antioxidant activity [5]. Particularly, a number of studies have reported that soybean isoflavones dose-dependently inhibited bone loss in both female and male osteoporotic animal models without causing notable effects on the reproductive organs [6]. We previously reported that the oral or subcutaneous injection of genistein or daidzein inhibited bone loss in ovariectomized or orchidectomized mice [7-11]. Several observational clinical studies also revealed that there was a correlation between isoflavone intake and bone mineral density (BMD), and intervention studies showed that isoflavone prevented bone loss in postmenopausal women [6].

However, a few studies have assessed the effects of isoflavones on bone formation. Bone development mostly advances until puberty, and 50% of peak bone mass is obtained during this period in humans [12]. Many factors, such as genetics, nutrition, mechanical stress, and hormonal status, play an important role in bone development and the attainment of peak bone mass [13]. In particular, hormonal secretion influences bone development during growth period. Estrogen affects bone metabolism and regulates calcium metabolism regulating hormone, cytokines, and growth factors; and estrogen also abolishes

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growth hormone-dependent periosteum bone acquisition in females. In males, although attention has traditionally focused on the role of androgens in bone metabolism in men, reports of severe osteopenia in men with a mutated gene of the estrogen receptor or aromatase deficiency have raised the question of the role of estrogens in the male skeleton [14,15]. Furthermore, a study was designed to examine the relative contributions of androgen vs estrogen in regulating bone turnover in normal elderly men, which demonstrated that estrogen was the dominant sex steroid regulating bone resorption [16]. These reports suggest that estrogen also plays an important role in regulating the male skeleton.

Although Ohta et al [17] reported that isoflavones increased BMD in intact mature mice, no study examined in detail the influence of the effect of isoflavonoid on bone formation. In humans, no changes were observed in bone growth and biomarkers of bone metabolism in children fed with soy protein formulas for 6 months [18] and in adolescent males fed 50 mg/d of isoflavones for 6 weeks [19]. Recently, however, because extracted isoflavones are available in numerous forms as dietary supplements, it might be necessary to assess the effects of isoflavones on bone growth during growth period. The present study examined the effects of isoflavone intake on bone formation and BMD in growing male and female mice. The purpose was to assess whether isoflavones including genistein and daidzein have specific, sexually dimorphic effects on bone formation and BMD during growth period.

2. Materials and methods

2.1. Animals and chemicals

Five-week-old male and female ddY strain mice were purchased from the Shizuoka Laboratory Animal Center (Shizuoka, Japan), and the mice were housed in individual cages in a temperature- and humidity-controlled room (23°C ± 1°C and 60% ± 5% relative humidity, respectively) under a 12-hour light/dark cycle. Male and female mice were divided respectively into 3 groups as follows: control diet (C), 0.08% genistein diet (G), and 0.08% daidzein (D) groups (n = 8 per group). The control diet was prepared according to the AIN-93G formulation where corn oil was used instead of soybean oil [20]. Dry powdered genistein and daidzein (purity, >98%; Nagara Science, Gifu, Japan) were added to the diet at 0.08% (wt/wt) instead of sugar (Table 1). This dose is almost half of that which has been shown to increase BMD in intact adult mice [17]. The mice were pair-fed and given free access to distilled water. Bone labeling of mice with a subcutaneous injection of calcein (1.6 mg/kg body weight) (Sigma, St Louis, MO) was performed 6 and 2 days before death.

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

Table 1
Composition of experimental diets

Ingredient	Control	Daidzein	Genistein
	g/kg diet		
Cornstarch	529.5	529.5	529.5
Casein milk	200.0	200.0	200.0
Sucrose	100.0	99.2	99.2
Corn oil	70.0	70.0	70.0
Cellulose	50.0	50.0	50.0
Mineral mixture[a]	35.0	35.0	35.0
Vitamin mixture[a]	10.0	10.0	10.0
L-Cystine	3.0	3.0	3.0
Choline bitartrate	2.5	2.5	2.5
tert-Butylhydroquinone	0.014	0.014	0.014
Daidzein	–	0.8	–
Genistein	–	–	0.8

[a] Prepared according to the AIN-93G formulation [19].

In each 4 weeks of experiment, body weight and weights of reproductive organ were measured, and the right and left femora were removed to measure BMD or to carry out histomorphologic analysis, respectively.

2.2. Radiographic analysis of whole body and lumbar BMD and bone mineral content

The BMD of the entire body and lumbar spine were measured using a PIXImus densitometer (software version 1.4x, Lunar, Madison, WI). Through whole-body scanning, the BMD of the entire body and the L4 through L6 vertebrae were analyzed. The coefficient of variation of BMD of the entire body and lumbar vertebrae were 0.8% and 0.7%, respectively.

2.3. Radiographic analysis of the femur

The BMD of the femur was measured by dual-energy x-ray absorptiometry (model DCS-600EX-R, Aloka, Tokyo, Japan) and BMD was calculated using the bone mineral content (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 of 5.3 mm each: the proximal femur, the midshaft, and the distal femur.

2.4. Histomorphometry

An undecalcified section was obtained from the site of the femoral midshaft. The specimen was then embedded in methyl methacrylate without staining to yield a 40-μm-thick crosscut ground section. Measurements were made using a semiautomatic image analyzing system (OsteoplanII; Carl Zeiss, Thornwood, NY). Dynamic parameters such as the mineral apposition rate (MAR; interlabel width per day), mineralizing surface/bone surface [MS/BS = (double-labeled surface + single-labeled surface/2)/BS], and bone formation rate/bone surface [BFR/BS = MAR × (MS/BS)/100] on the periosteal surface were measured by calcein double labeling. Nomenclature and units used were those recommended by

Table 2
Body and organ weight, and food intake in growing male and female mice fed with control, daidzein, and genistein diets

	Control group	Daidzein group	Genistein group
Male			
Body weight (g)			
Initial	25.3 ± 0.3	25.3 ± 0.3	25.3 ± 0.2
Final	37.8 ± 1.1[a]	34.4 ± 0.6[b]	34.7 ± 0.4[b]
Food intake (g/4 wk)	111.1 ± 1.7	105.0 ± 2.7	112.3 ± 1.8
Organs weight (mg)			
Testis	249.2 ± 10.9	242.5 ± 6.5	254.3 ± 13.9
Thymus	45.0 ± 4.0[a]	30.2 ± 1.3[b]	29.5 ± 2.8[b]
Liver	1,595.0 ± 48.0	1,517.8 ± 62.0	1,507.3 ± 46.4
Kidney	512.5 ± 18.6	506.3 ± 22.3	481.3 ± 11.7
Spleen	107.5 ± 10.0	104.5 ± 6.6	101.0 ± 4.6
Female			
Body weight (g)			
Initial	23.0 ± 0.4	23.1 ± 0.3	23.2 ± 0.5
Final	29.9 ± 0.4	27.7 ± 0.8	28.0 ± 1.3
Food intake (g/4 wk)	112.3 ± 1.8	113.0 ± 2.0	109.7 ± 2.5
Organs weight (mg)			
Uterus	137.8 ± 25.0	148.3 ± 15.7	142.5 ± 19.6
Thymus	52.3 ± 2.7[a]	42.2 ± 4.6[b]	42.5 ± 5.8[b]
Liver	1,424.5 ± 45.1[a]	1,499.2 ± 31.1[a]	1,227.0 ± 33.6[b]
Kidney	352.2 ± 6.5	379.5 ± 3.6	369.5 ± 14.7
Spleen	119.2 ± 9.2	134.8 ± 8.2	126.7 ± 6.3

Values are means ± SEM (n = 8); a,b, the means without a common letter are different at $P < .05$.

nomenclature committee of the American Society for Bone and Mineral Research [21].

2.5. Time-resolved fluoroimmunoassay for measuring plasma isoflavones

Plasma genistein, daidzein, and equol were analyzed using the time-resolved fluoroimmunoassay (TR-FIA) method of Brouwers et al [22]. 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, and 100 μL of antiserum in the assay buffer for equol and 100 μL of europium-labeled equol was added to each well. The strips were slowly shaken at room temperature for 90 minutes and then washed. A 200-μL volume of DELFIA enhancement solution was next added to each well. After an additional 5 minutes of shaking, fluorescence was determined using a DELFIA Victor 1420 multilabel counter (Wallac, Oy Turku, Finland). The final results were calculated using the following formula: final results = concentration (read) × 1/recovery × dilution factor (nmol/L).

2.6. Statistical analysis

The data were expressed as means ± SEM. The significance of the differences was determined by 1-factor analysis of covariance and Fisher protected least significant difference test (StatView 5.0, Abacus Concepts, Calabasas, CA). Body weight was used as covariate in the analysis of tissue weight and BMD to adjust for possible confounding. Differences were considered significant at $P < .05$.

3. Results

3.1. Body and tissue weights

The initial body weight of the male mice did not differ among the groups, but the final body weight in the D and G groups were significantly lower than that in the C group ($P < .05$) (Table 2).

There were no significant differences in the initial and final body weight among the groups in female mice. No difference was observed in lean body mass and fat mass between D and C groups in male and female mice. Fat mass in the G group in female mice was lower compared with that in the C group (data not shown). The amount of the food

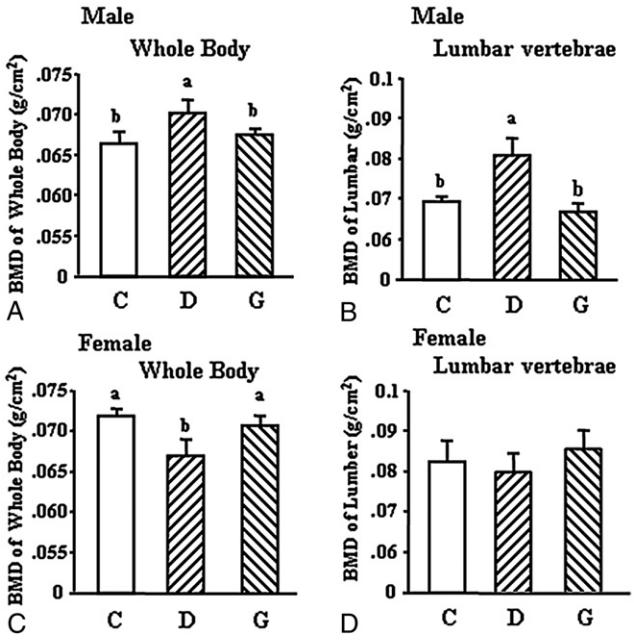

Fig. 1. The BMD of whole body and lumbar spine in male and female mice fed a 0.08% daidzein or genistein diet during the course of 4 weeks. The BMD was measured by dual-energy x-ray absorptiometry analysis. Values are means ± SEM (n = 8 per group). The means without a common letter are different at $P < .05$.

Table 3
Bone mineral content of whole body, lumbar spine, and femur (divided into proximal, middle, and distal regions) in growing male and female mice fed with control, daidzein, and genistein diets

	Male			Female		
	Control group	Daidzein group	Genistein group	Control group	Daidzein group	Genistein group
BMC (g)						
Whole body	0.850 ± 0.025	0.890 ± 0.021	0.862 ± 0.016	0.883 ± 0.002	0.819 ± 0.026	0.906 ± 0.021
Lumbar spine	0.030 ± 0.002	0.036 ± 0.003	0.026 ± 0.001	0.032 ± 0.003	0.033 ± 0.002	0.035 ± 0.003
Femur						
Whole femur	0.0259 ± 0.0008	0.0272 ± 0.0005	0.0263 ± 0.0008	0.0276 ± 0.0008	0.0270 ± 0.0009	0.0274 ± 0.0003
Proximal region	0.0092 ± 0.0003	0.0094 ± 0.0003	0.0091 ± 0.0003	0.0092 ± 0.0003	0.0092 ± 0.0003	0.0092 ± 0.0001
Middle region	0.0067 ± 0.0003	0.0071 ± 0.0002	0.0070 ± 0.0002	0.0068 ± 0.0003	0.0068 ± 0.0003	0.0070 ± 0.0001
Distal region	0.0088 ± 0.0002	0.0097 ± 0.0002*	0.0091 ± 0.0004	0.0105 ± 0.0004	0.0102 ± 0.0004	0.0106 ± 0.0002

Values are means ± SEM (n = 8).
* $P < .05$, significantly different from control diet group.

intake for 4 weeks did not differ among the groups for males and females. The intake of daidzein and genistein did not affect the testicular weight of the male mice and the uterine weight of the female mice. However, the thymus weight in both the G and D groups for males and females was significantly lower than for the C groups. The spleen weight did not differ among the groups in male and female mice.

3.2. Bone mass of the whole body and lumbar vertebrae

In male mice, the intake of daidzein affected the BMD and BMC in the whole body and lumbar spine. The BMD of the whole body and lumbar spine in the D group was significantly higher than that in the C group ($P < .05$) (Fig. 1A and B). The results of BMC of the whole body and lumbar spine showed similar tendency to those of BMD (Table 3). The intake of genistein did not affect the BMD and BMC of the whole body.

In the female mice, the BMD of the whole body in the D group was significantly lower than that in the C mice ($P < .05$) (Fig. 1C). Bone mineral content of the whole body in the D group also tended to be lower than that in C mice ($P = .06$, Table 3). However, the intake of genistein did not affect the BMD and BMC of the whole body in the female mice. The bone area of the whole body of the 3 groups also did not differ. In the lumbar spine, no significant differences were observed in the BMD (Fig. 1D), BMC (Table 3), and bone area among the 3 groups.

3.3. Bone mineral density of the femur

The BMD of the whole and distal femur of the male mice in the D and the G groups were significantly higher than of the C group ($P < .05$) (Fig. 2A). The BMD of the middle femur of the male mice in the G group was significantly higher than of the C group ($P < .05$). The femoral distal BMC in the D group was significantly higher than that in the C group ($P < .05$, Table 3). No difference in BMD was observed in proximal femur in different male mice groups. In the female mice, the BMD of the whole, proximal, and distal femur in the D group were significantly lower than those in the C group ($P < .05$) (Fig. 2B). Bone mineral content of the whole and distal femur in D group in the female mice tended to be lower than that in the C group (Table 3).

3.4. Histomorphometry of the femur

Fig. 3 shows the histologic parameters for bone formation in the cortical bone of the femoral diaphysis. The periosteal

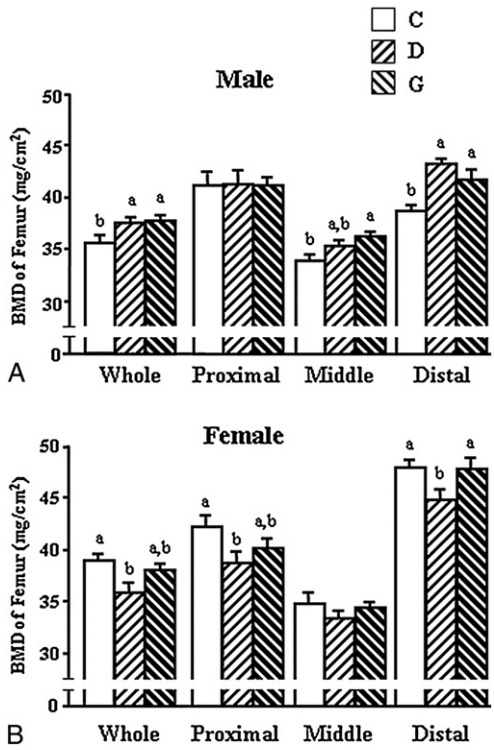

Fig. 2. The BMD of the femur measured by dual-energy x-ray absorptiometry. Femora were collected from male and female mice fed a 0.08% daidzein or genistein diet during the course of 4 weeks. The BMD was measured by dual-energy x-ray absorptiometry analysis. The scanned area of the mouse femur was equally divided into 3 parts 5.3 mm each: the proximal femur, the midshaft, and the distal femur. Values are means ± SEM (n = 8 per group). The means without a common letter are different at $P < .05$.

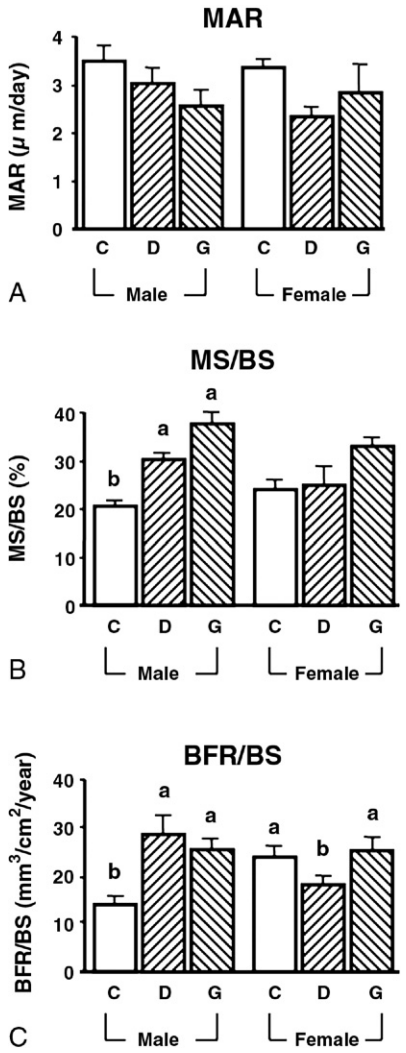

Fig. 3. Histologic analysis of cortical bone of the midshaft collected from male and female mice fed a 0.08% daidzein or genistein diet. Femora were collected 4 weeks later and sections of the diaphysis were prepared. MAR (interlabel width per day), MS/BS [double-labeled surface + single-labeled surface/2)/BS], and BFR/BS [MAR × [MS/BS]/100] were measured by calcein double labeling on the periosteal surface. Values are means ± SEM (n = 8 per group). The means without a common letter are different at $P < .05$.

MAR did not significantly differ among the groups for male and female mice (Fig. 3A). Treatment of the male mice with daidzein and genistein increased their MS/BS and BFR/BS ($P < .05$) (Fig. 3B and C). In the female mice, MAR and MS/BS did not differ among the groups (Fig. 3A and B); however, BFR/BS in the daidzein-treated group was significantly lower compared with that in the control mice ($P < .05$) (Fig. 3C).

3.5. Plasma isoflavone concentration

The concentration of genistein in the male and female mice treated with genistein was significantly increased compared with that in the untreated mice (Fig. 4). Also, the concentrations of daidzein were higher in the male and female mice treated with daidzein than that in other groups. Feeding the mice a daidzein diet increased the level of plasma equol, a metabolite of daidzein. The plasma equol concentration in the male mice was significantly higher than that in the female mice ($P < .05$). There was no significant difference in the testosterone and 17β-estradiol concentrations among the groups for males and females, respectively (data not shown).

4. Discussion

The present study clearly reveals that daidzein and genistein increase the BMD in male mice by stimulating bone formation, although daidzein inhibits bone growth by decreasing the BFR in female mice. This indicates that the effects of isoflavones on bone metabolism in growing mice depend on sex. Furthermore, we found that high isoflavone intake during growth resulted in lower thymus weight.

Male mice fed with daidzein or genistein had a higher BMD in whole body, lumbar spine, and femur than those in control mice. Data are scarce on the anabolic effects of isoflavones on bone so far, although we previously reported that treatment of mature mice with isoflavones increases their BMD [17]. Gao and Yamaguchi [23] reported that genistein and daidzein induced an increase in alkaline phosphatase activity, DNA, and calcium content in rat femoral bone in organ cultures, suggesting that isoflavones may have a bone anabolic effect. The present histologic data in growing male mice clearly showed that the increase in the BMD in the femur induced by isoflavones resulted from the increase in MS/BS and BFR. These results suggest that the isoflavones daidzein and genistein have the ability to stimulate osteoblastic recruitment in young male mice. On the other hand, hormone secretion influences bone development during growth. Growth hormones induce longitudinal bone development [24] and periosteum bone expansion;

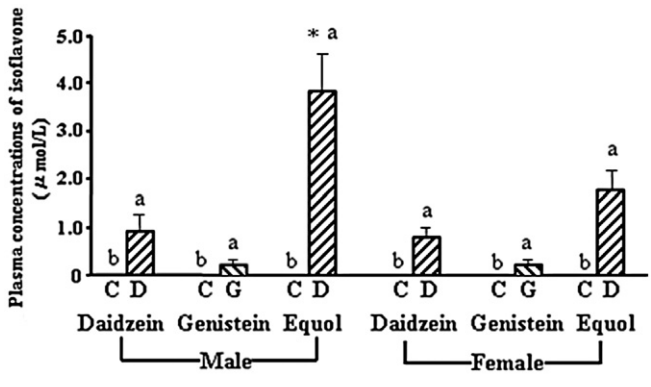

Fig. 4. Plasma concentrations of daidzein, genistein, and equol in male and female mice with or without 0.08% daidzein or genistein diet. Plasma isoflavone was measured by TR-FIA at the period of the experiments. Values are means ± SEM (n = 8 per group). The means without a common letter are different at $P < .05$. *$P < .05$, significantly different from females.

androgens stimulate periosteal bone formation, whereas estrogen stimulates endocortical apposition. Taken together, growth hormones, androgens, and isoflavones with weak estrogenic activity may cooperatively stimulate bone formation in growing male mice.

Soybean isoflavone daidzein, on the contrary, exhibited different behavior on bone metabolism in growing female mice that may have been caused by the difference in hormonal status from the male mice. In the female mice, the whole body, femoral BMD, and BFR/BS at the periosteal of the femur were lower in the D group, but not in the G group, compared with the C group. Daidzein is metabolized to equol in the gastrointestinal tract by gut microflora. Equol possesses a stronger affinity for estrogen receptors than does daidzein [25] and exhibits the strongest transcriptional activity among soybean isoflavonoids in yeast 2-hybrid systems [26]. It is also suggested that equol increases steroid hormone–binding globulin in the serum, resulting in a reduction in free estrogen in female mice. Loukovaara et al [27] reported that equol and daidzein, but not genistein, increase steroid hormone–binding globulin secretion in HepG2 cells. Thus, equol as well as daidzein may compete with endogenous estrogen at estrogen receptor and masks its functions in female animals. In this study, we found that the plasma concentration of equol was dramatically increased after daidzein feeding in males and females. This may explain the significant change in the BMD in whole body and spine in male and female mice (Fig. 1). Further studies are needed to clarify the mechanism of the inhibitory effects of daidzein on bone formation in immature female mice. However, in the estrogen-deficient mice, we have reported that equol directly prevented bone loss induced by ovariectomy [28]. Furthermore, several studies suggested that the clinical effectiveness of isoflavones might be because of their ability to produce equol [29]. No difference was observed in lean body mass and fat mass between D and C groups in male and female mice (data not shown), suggesting that BMD in D and C groups was not affected by body composition in growing male and female mice.

Few studies have examined the safety of isoflavones on growth in animals. Soybean isoflavone has attracted much attention because of its health benefits, and it can be easily taken as a supplement. As a phytoestrogen, isoflavones have hormonal functions; some adverse effects can be expected during immaturity. In this study, we investigated the influence of relatively high doses of isoflavones on safety as well as on bone metabolism during immaturity in mice. The intake of 4 g/d including 0.08% isoflavone diet, at approximately 100 mg/kg body weight per day of isoflavone intake, resulted in lower final body weight and thymus weight in growing male and female mice, although it did not affect their reproductive organs at this dosage (Table 2). It has been reported that subcutaneous treatment with genistein at 20 to 200 mg/kg body weight reduces the thymus weight and influences the population of thymic T cells in ovariectomized mice [30]. These results suggest that high isoflavone consumption may exhibit potential immune effects during maturity. We previously reported that the plasma concentration of genistein induces uterine hypertrophy is approximately 20.4 μmol/L, whereas bone effects were observed at 1.3 μmol/L genistein in ovariectomized mice [8]. In this study, the plasma concentrations of daidzein and genistein were less than 1.3 μmol/L in the male and female mice (Fig. 4). This suggests that isoflavones affect the thymus and bone at doses less than one tenth of the plasma concentration that induces uterine hypertrophy. Further studies are necessary to assess the effects of isoflavones on immune function in detail during immaturity in rodents.

The plasma concentrations of genistein, daidzein, and equol ranged 0.21 to 0.22, 0.87 to 0.94, and 1.7 to 3.8 μmol/L, respectively, in the male and the female mice. These concentrations of the isoflavones except for genistein were several times higher than those in Asian people [31,32]. Although the dose used in this study may not apply well to humans, our results suggest that the excessive intake of isoflavones during growth should be assessed carefully in further studies. The safety and efficacy of isoflavones that relate to the widespread use of soy-based formula by infants has been argued [33]. Isoflavones from soy formula are well absorbed by infants and circulate at concentrations 13 000- to 22 000-fold higher than those for estradiol [34], generating considerable concern regarding their long-term health effects. Strom et al [35] examined the association between infant exposure to soy formula and numerous endocrinologic and reproductive outcomes as adults aged 20 to 34 years by a retrospective cohort study design. The results showed no statistically significant differences between exposed and nonexposed groups for more than 30 health outcomes. One of the possible explanations for the discrepancy between the findings in animal research and subsequent lack of confirmation in human trials may be found in species differences in the metabolism of the isoflavones [36]. One of the main metabolites of the isoflavone daidzein is equol, which is thought to be the most potent modifier of the effects of isoflavones. In humans, the production of equol depends on the individual's intestinal microflora, whereas rodents are primarily equol producers. The continual growth of knowledge concerning the safety and efficacy of soy products fortified with isoflavones and extracted supplements remains a research priority, particularly because of their widespread availability and growing use [37].

In conclusion, the effects of isoflavones on bone metabolism during growth depend on sex. Consumption of a diet with 0.08% isoflavones stimulates bone formation in immature male mice and exerts the opposite effect in female mice. These results suggest that the endogenous hormonal status influences the efficacy of isoflavone, especially daidzein, on bone metabolism during immaturity in mice.

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