

# Comparative Study on Reduction of Bone Loss and Lipid Metabolism Abnormality in Ovariectomized Rats by Soy Isoflavones, Daidzin, Genistin, and Glycitin

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The effects of the soy isoflavone glycoside, daidzin, genistin, and glycitin on bone loss and lipid metabolism in ovariectomized (ovx) rats were compared with those of estrone. Thirty-six 11-week-old female Sprague–Dawley rats were assigned to six groups, sham-operated, ovx, ovx+glycitin, ovx+daidzin, ovx+genistin, and ovx+estrone and fed matched amounts of a commercial calcium-deficient diet for 4 weeks. Throughout this period, daidzin, genistin or glycitin (25, 50 or 100 mg/kg/d) was given orally using a stomach tube, or estrone (7.5 µg/kg/d) was administered subcutaneously. Daidzin, genistin and glycitin significantly prevented bone loss in ovx rats at a dose of 50 mg/kg/d, like estrone. At this dose glycitin and daidzin also prevented ovx-induced uterine atrophy and increases in body weight gain, abdominal fat, serum total cholesterol and triglyceride, and urinary excretion of pyridinoline and deoxypyridinoline with statistical significance, like estrone. On the other hand, genistin prevented ovx-induced uterine atrophy only at a dose of 100 mg/kg, but did not block any other change of ovx rats at a dose of 50 or 100 mg/kg. These findings indicate that daidzin, glycitin, and genistin are effective in preventing bone loss and the former two compounds are effective in reversing the unfavorable changes of lipid metabolism in this model. It is suggested that the preventive effect of daidzin or glycitin on bone loss in ovx rats is due to suppression of bone turnover, as in the case of estrone, but genistin has a different mechanism of action from the other compounds. Soy isoflavone glycosides may represent a potential alternative therapy in the treatment of bone loss and lipid metabolism abnormality in ovarian hormone-deficient women.

**Key words** soy isoflavone glycoside; glycitin; ovariectomy; bone loss; lipid metabolism; rat

The risk of coronary heart disease drastically increases after menopause.<sup>1)</sup> Arteriosclerosis induced by lipid metabolism abnormality after menopause has been recognized as a major risk factor for cardiovascular disease.<sup>2)</sup> Osteoporosis associated with ovarian hormone deficiency following menopause is the most common cause of age-related bone loss. A sharp decrease in estrogen production causes rapid hormone-related bone loss during the first decade after menopause.<sup>3)</sup>

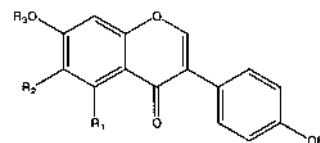
Post-menopausal estrogen replacement therapy (ERT) has shown potential for reduction or prevention of coronary heart disease,<sup>4)</sup> and is considered to be the most effective method to reduce the rate of osteoporosis in post menopausal women.<sup>5)</sup> However, ERT may be accompanied by unacceptable side effects such as endometrial and breast cancer in some women.<sup>6)</sup> Therefore, ERT is recommended only for women who have no contraindications. Thus, it would be most helpful to discover a natural and safe dietary substance that minimizes bone loss and/or improves lipid metabolism in post menopausal women.

It is well known that soybeans contain free isoflavones, daidzein, genistein and glycitein, and their glycoside derivatives. Daidzin, genistin and their acylated derivatives account for the major portion of isoflavones in soybeans, while glycitin and its acylated derivatives are minor components.<sup>7)</sup> It is not easy to prepare gram amounts of isoflavones, especially glycitein and glycitin, from soybeans or by synthesis for biological testing *in vivo*.<sup>7,8)</sup> Both *in vitro* and *in vivo* studies have shown that daidzein, genistein, and their glycosides exert a weak estrogenic effect.<sup>9)</sup> Therefore, numerous studies have been performed with daidzein and genistein, but there has been only one biological study on the estrogenic activity

of glycitein *in vitro* and *in vivo*,<sup>10)</sup> to our knowledge.

Several investigators have reported that soybean consumption may contribute to lower risk of the postmenopausal diseases, including coronary heart disease<sup>11)</sup> and osteoporosis.<sup>12)</sup> Recently, the hypocholesterolemic and osteoporosis-ameliorating properties of soy protein isolate, which is a rich source of isoflavones, have received much attention.<sup>13)</sup> It is not yet clear whether the preventive effect of soy protein on lipid and bone metabolism abnormality is due to its amino acid composition, to nonprotein constituents such as saponins, phytic acids and isoflavones, or to a combination of these factors. However, there has been increasing interest in the soy isoflavones and their biological activities in recent year.<sup>14)</sup>

Several recent reports indicate that soy isoflavones may have beneficial effects on bone. Recent studies indicate that oral administration of daidzin, genistin and their succinyl derivatives significantly prevents bone loss in an ovx model<sup>15)</sup> and genistein is also effective.<sup>16)</sup> Further, daidzin inhibits atrophy of the uterus, while genistin or genistein does not at the examined dose. The findings of these studies suggest that the action mechanism of daidzin on bone loss is similar to that of estrone, but genistin and genistein have different



	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
Daidzin	H	H	Glc
Genistin	OH	H	Glc
Glycitin	H	OCH <sub>3</sub>	Glc
Ipriflavone	H	H	CH(CH <sub>3</sub> ) <sub>2</sub>

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mechanisms of action. So, there is a great interest in the effect and action mechanism of another soy isoflavone glycitin on bone and uterine tissue in this osteoporosis model.

The hypocholesterolemic effect of soy protein has in part been linked to its isoflavone content.<sup>17)</sup> Recently Dodge *et al.* and Nogowski *et al.* reported that genistein lowers serum lipid in ovx rats.<sup>18)</sup> However, the effects of soy isoflavone glycosides on lipid metabolism in ovx animal model have not been evaluated.

The present study was conducted to compare the effects of daidzin, genistin and glycitin on bone loss, body weight gain, uterine atrophy, serum lipid profiles, and abdominal fat with those of estrone in ovx rats.

## MATERIALS AND METHODS

Melting points were determined on a Yanaco micro melting point apparatus and are uncorrected. IR spectra were taken on a JASCO IR-700 IR spectrophotometer. FAB-MS spectra were obtained on a JMS-SX 102 machine, using glycerin as matrix. NMR spectra were recorded on a Bruker DRX-500 spectrometer, using tetramethylsilane as an internal standard. UV spectra in methanol was taken on a JASCO U-30 spectrometer.

**Materials** Commercial soybeans (*Glycine max* (L.) Merrill, Canadian) were used in this study. Authentic samples of daidzin and genistin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Ipriflavone was prepared from a commercial product manufactured by Takeda Chemical Industries, Ltd. (Osaka).

**Isolation of Glycitin, Daidzin and Genistin from Soybeans** Daidzin and genistin were isolated from soybeans and glycitin from soybean hypocotyls as follows, based on the report of Kudou *et al.*<sup>7a)</sup>

Soybeans (80 kg) were pulverised and extracted with hot water (400 l) for 1 h. The water extract was applied to a Diaion SP-207 column, which was eluted with water (35 l) and then methanol (27 l). The methanol eluate (200 g) was subjected to gel filtration on a Sephadex LH-20 column (10 l) using methanol as a eluent. The eluate was separated into five fractions [fraction I (88.8 g), II (42 g), III (63 g), IV (25 g) and V (1 g)] ( $K_d=0-0.70$ ,  $0.71-1.07$ ,  $1.08-1.86$ ,  $1.87-2.19$ ,  $2.20-2.80$ ) by TLC (Kieselgel 60 Art. 5721 (Merck); mobile phase: lower layer of chloroform-methanol-water (65:35:10); visualized with Vaughans reagent). Daidzin was isolated from fraction II, and genistin from fraction III. Daidzin and genistin were recrystallized from 80% methanol to obtain colorless needles (16.5 g, mp 237–239 °C) and colorless plates (33.4 g, mp 256–257 °C), respectively.

Hypocotyls of soybeans (10 kg) were ground and extracted for 0.5 h with hot water (100 l). The hot water extract was applied to a Diaion SP-207 column (Mitsubishi Kasei Co.) (7 l), which was eluted with water (35 l) and then methanol (27 l). The methanol eluate was dissolved in water (700 ml), heated at 120 °C in an autoclave for 15 min, and then lyophilized. The lyophilized material (260 g) was dissolved in 80% methanol and allowed to stand at 4 °C for overnight. The precipitates (22 g) were recrystallized from 80% methanol twice, and then from methanol to obtain colorless fine needles (4.1 g, mp 237–239 °C).

Identification of these soy isoflavone glycosides was carried out by comparison of their physicochemical data (mp, NMR, IR, UV, and FAB-MS) with the reported data for authentic samples.<sup>7)</sup>

**Animals and Administration Procedure** Female Sprague-Dawley rats (conventional), aged 9 weeks, were purchased from Japan SLC (Hamamatsu). The animals were acclimated in an environmentally controlled animal laboratory and fed commercial food (Casein diet, CLEA Japan, Inc., Tokyo) containing 0.8% Ca and 0.7% P at a room temperature of 25 °C, with free access to distilled water for 1 week. At 10 weeks of age, bilateral ovariectomy was performed *via* a dorsal midline incision under ether anesthesia. Upon recovery from anesthesia, animals were assigned to experimental groups, intact (sham-operated), ovx, ovx+glycitin, ovx+daidzin, ovx+genistin, and ovx+estrone, with five to six animals per group, per experiment. All rats were fed matched amounts of the food described above for 1 week on basis of consumption of the rats in sham-operated and ovx+daidzin groups. From 11 weeks of age, all the rats were allowed controlled access to a commercial calcium-deficient diet (powder) containing 0.004% Ca and 0.3% P (Diet 11-Ca, Japan CLEA, Tokyo) (Table 1) and free access to deionized water for 28 d. Throughout this period, daidzin (25 and 50 mg/kg/d), genistin (50 and 100 mg/kg/d) or glycitin (25 and 50 mg/kg/d) suspended in water containing 1% hydroxypropyl cellulose (HPLC-L, Nippon Soda Co., Japan) (0.4 ml/200 g) was given orally using a stomach tube, or estrone (7.5 µg/kg/d) in sesame oil was given subcutaneously for 4 weeks. Normal (sham-operated) and control (ovx) rats received only the vehicle solution (0.4 ml of 1.5% carboxymethylcellulose/200 g body weight) orally. The food intake of all rats was measured every 3 d. At day 14 after first dosing, the urine of each rat was collected over 24 h, using a metabolic cage. On the day after the last dose, the rats were bled from the iliac artery after cardiac puncture under light anesthesia with ether. The uterus was removed and the wet weight, including the intrauterine fluid, was determined. Abdominal fat was also removed and the wet weight was determined. The femurs were also removed immediately after bleeding for bone analyses. Guidelines for the ethical care and treatment of animals from the Animal Care Committee

Table 1. Composition of Calcium-Deficient Diet

Ingredient	(g/kg diet)
Vitamine free-casein	180.0
Glucose	656.0
Microcrystalline cellulose	30.0
Vitamine mixture <sup>a)</sup>	1.0
Cotton seed <sup>b)</sup>	100.0
Choline chloride	2.0
L-Cystine	2.0
Mineral mixture <sup>c)</sup>	20.0
Potassium phosphate, monobasic	3.95
Potassium phosphate, dibasic	5.05

a) Vitamine mixture (g/kg diet): vitamin B-1, 0.005; vitamin B-2, 0.005; vitamin B-6, 0.005; vitamin B-12 (0.1% (trituration)); calcium pantothenate, 0.056; nicotinic acid, 0.005; inositol, 0.02; folic acid, 0.0002; , 0.02; biotin (2% (trituration)), 0.005; glucose, 0.68. b) Cotton seed (g/kg diet): cotton oil, 99.12; DL- $\alpha$ -tocopherol, 1.077; vitamin K-3, 0.065;  $\beta$ -calotol, 0.045. c) Mineral mixture (g/kg diet): KCl, 11.540; NaCl, 4.180;  $MgSO_4 \cdot 7H_2O$ , 3.580;  $FeSO_4 \cdot 7H_2O$ , 0.644;  $CuSO_4 \cdot 5H_2O$ , 0.0156; NaF, 0.0226;  $CoCl_2 \cdot 6H_2O$ , 0.0008; KI, 0.002;  $MnSO_4 \cdot H_2O$ , 0.008;  $ZnSO_4 \cdot 7H_2O$ , 0.088;  $(NH_4)_2MoO_4 \cdot 4H_2O$ , 0.010.

of the University of Shizuoka at Shizuoka, Japan were strictly followed.

**Analytical Procedures** Serum Lipids: Blood samples were centrifuged at  $2000 \times g$  for 15 min to obtain serum, after standing for 30 min at  $37^\circ\text{C}$ . Serum total cholesterol and triacylglycerol were determined manually by an enzymatic method, using Cholesterol E-test Wako and Triacylglycerol C-test Wako (Wako Pure Chemical Industries, Ltd., Osaka), respectively.

Serum Calcium and Phosphorus: Serum calcium and inorganic phosphate were measured spectrophotometrically, using commercial kits (Calcium C-test Wako, Phospha B-test Wako, and Creatinine-HR II-test Wako, respectively).

Bone Length and Density: The femurs were also removed immediately after bleeding for bone analyses. The right and left femurs were freed of soft tissue. The removed right femurs were freed of soft tissue using small scissors, tweezers and cotton gauze. The length of each femur was measured with a Vernier caliper. Following the same method as in the previous report,<sup>15)</sup> bone volume and density were measured by applying Archimedes' principle.<sup>19)</sup> Then the bones were dehydrated and defatted in acetone and anhydrous ether, dried for 12 h at  $110^\circ\text{C}$  and reweighed to obtain the dry bone weights.

Bone Strength (Breaking Force): Bone strength was measured according to Ezawa *et al.*<sup>20)</sup> by means of a three-point bending test on an universal test instrument of the Instron type (Rheolometer-MAX I, Techno Co., Tokyo, Japan), as reported previously.<sup>15)</sup>

Urinary Excretion of Creatinine, Pyridinoline, and Deoxypyridinoline: Urinary creatinine was measured with a commercial kit, Creatinine-test Wako (Wako Pure Chemical Industries, Ltd.). Urinary pyridinoline and deoxypyridinoline were measured according to Takahashi *et al.*,<sup>21)</sup> as reported previously.<sup>15)</sup>

**Statistical Methods** Data from the animal experiments

were expressed as means and S.E.M. for each of the groups and compared using analysis of variance and Student's *t*-test or Dunnett's multiple range test with Stat View Software (Version 4.5, Berkeley, CA). *p* values of less than 0.05 were considered to indicate significant differences.

## RESULTS

**Body Weight and Food Intake** At the end of the study, the ovx group and ovx+genistin group had significantly higher mean body weights than the sham-operated group, the ovx+daidzin (25 and 50 mg/kg) group, the ovx+glycitin (50 mg/kg) group or the ovx+estrone group (Table 2).

**Uterine and Abdominal Fat Weights** Ovariectomy caused atrophy of the uterus. This was prevented by estrone, daidzin (50 mg/kg), glycitin (50 mg/kg) and genistin (100 mg/kg) significantly, but not by 50 mg/kg of genistin (Table 2).

The rats in the sham-operated, ovx+daidzin (50 mg/kg), the ovx+glycitin (50 mg/kg) group or the ovx+estrone had significantly lower abdominal fat weights than the ovx group and ovx+genistin group, while the latter two groups had similar food intake to the former groups (Table 2).

**Serum Lipids** The concentrations of serum lipids, total cholesterol and glyceride were significantly higher in ovx rats in comparison with sham-operated rats (Table 3). The ovx-induced rise in serum lipids was prevented by daidzin (25 and 50 mg/kg), glycitin (50 mg/kg), or estrone, but not by genistin.

**Serum Calcium and Phosphorus** Serum concentrations of calcium and phosphorus were not appreciably altered by ovariectomy and were similar among all treatment groups for 4 weeks (Table 3).

**Femoral Length, Strength, Density, Ash Weight and Calcium and Phosphorus Content** None of the treatments influenced bone length (data not shown). However,

Table 2. Effect of Ovariectomy, Daidzin, Glycitin, Genistin, and Estrone on Food Intake, Body Weight, and Relative Uterus and Abdominal Fat Weights in Rats

Measure	Sham	Ovx	Ovx+daidzin		Ovx+glycitin		Ovx+genistin		Ovx+estrone
			25 mg/kg	50 mg/kg	25 mg/kg	50 mg/kg	50 mg/kg	100 mg/kg	7.5 $\mu\text{g/kg}$
Food intake, g/d	13.9 $\pm$ 0.6	14.6 $\pm$ 0.7	14.5 $\pm$ 0.7	13.8 $\pm$ 0.9	14.6 $\pm$ 0.8	14.8 $\pm$ 0.9	14.5 $\pm$ 0.6	14.7 $\pm$ 0.7	13.9 $\pm$ 1.1
Initial body weight, g	235 $\pm$ 3	234 $\pm$ 2	235 $\pm$ 3	233 $\pm$ 3	234 $\pm$ 3	234 $\pm$ 2	235 $\pm$ 3	234 $\pm$ 3	233 $\pm$ 3
Final body weight, g	269 $\pm$ 4	303 $\pm$ 9 <sup>a)</sup>	286 $\pm$ 7 <sup>b)</sup>	265 $\pm$ 7 <sup>c)</sup>	292 $\pm$ 7	270 $\pm$ 5 <sup>c)</sup>	301 $\pm$ 8	297 $\pm$ 11	267 $\pm$ 4 <sup>c)</sup>
Uterus, mg/100 body wt.	172.0 $\pm$ 7.7	41.6 $\pm$ 2.0 <sup>a)</sup>	48.0 $\pm$ 9.3	73.1 $\pm$ 7.7 <sup>b)</sup>	42.3 $\pm$ 9.0	73.0 $\pm$ 7.9 <sup>b)</sup>	42.0 $\pm$ 6.9	71.9 $\pm$ 9.2 <sup>b)</sup>	128.3 $\pm$ 7.7 <sup>c)</sup>
Abdominal fat, mg/100 body wt.	7.2 $\pm$ 0.6	9.6 $\pm$ 0.7 <sup>a)</sup>	7.7 $\pm$ 0.4 <sup>b)</sup>	5.2 $\pm$ 0.4 <sup>c)</sup>	8.4 $\pm$ 0.6	5.5 $\pm$ 0.5 <sup>c)</sup>	9.4 $\pm$ 0.7	9.3 $\pm$ 0.6	5.9 $\pm$ 0.3c

Values are means $\pm$ S.E.M., *n*=6. Within a row, values with a superscript are significantly different: a) *p*<0.01 compared with sham rats; b) *p*<0.01, c) *p*<0.001 compared with ovx rats.

Table 3. Effect of Ovariectomy, Daidzin, Glycitin, Genistin, and Estrone on Serum Concentrations of Cholesterol, Triglyceride, Calcium, and Phosphorus in Rats

Measure (mmol/l)	Sham	Ovx	Ovx+daidzin		Ovx+glycitin		Ovx+genistin		Ovx+estrone
			25 mg/kg	50 mg/kg	25 mg/kg	50 mg/kg	50 mg/kg	100 mg/kg	7.5 $\mu\text{g/kg}$
Cholesterol	2.49 $\pm$ 0.26	3.30 $\pm$ 0.23 <sup>a)</sup>	2.53 $\pm$ 0.25 <sup>b)</sup>	1.85 $\pm$ 0.25 <sup>c)</sup>	2.94 $\pm$ 0.21	2.02 $\pm$ 0.14 <sup>c)</sup>	3.51 $\pm$ 0.21	3.40 $\pm$ 0.24	2.01 $\pm$ 0.19 <sup>c)</sup>
Triglyceride	1.00 $\pm$ 0.10	1.38 $\pm$ 0.11 <sup>a)</sup>	1.05 $\pm$ 0.09 <sup>b)</sup>	0.75 $\pm$ 0.07 <sup>c)</sup>	1.25 $\pm$ 0.10	0.79 $\pm$ 0.11 <sup>c)</sup>	1.48 $\pm$ 0.10	1.44 $\pm$ 0.11	0.81 $\pm$ 0.13 <sup>c)</sup>
Calcium	3.2 $\pm$ 0.1	3.5 $\pm$ 0.1	3.5 $\pm$ 0.1	3.3 $\pm$ 0.1	3.3 $\pm$ 0.1	3.3 $\pm$ 0.1	3.3 $\pm$ 0.1	3.4 $\pm$ 0.1	3.3 $\pm$ 0.1
Phosphorus	2.1 $\pm$ 0.1	2.2 $\pm$ 0.2	2.2 $\pm$ 0.2	2.1 $\pm$ 0.2	2.1 $\pm$ 0.2	2.0 $\pm$ 0.1	2.1 $\pm$ 0.2	2.2 $\pm$ 0.1	2.0 $\pm$ 0.1

Values are means $\pm$ S.E.M., *n*=6. Within a row, values with a superscript are significantly different: a) *p*<0.01 compared with sham rats; b) *p*<0.05, c) *p*<0.01 compared with ovx rats.

Table 4. Effect of Ovariectomy, Daidzin, Glycitin, Genistin, and Estrone on Length, Density and Strength of Femur in Rats

Measure	Sham	Ovx	Ovx + daidzin 50 mg/kg	Ovx + glycitin 50 mg/kg	Ovx + genistin 50 mg/kg	Ovx + estrone 7.5 $\mu$ g/kg
Femoral length, mm	34.5 $\pm$ 0.2	35.2 $\pm$ 0.3	35.1 $\pm$ 0.3	35.4 $\pm$ 0.2	35.7 $\pm$ 0.3	35.5 $\pm$ 0.3
Density, g/cm <sup>3</sup>						
Wet	1.463 $\pm$ 0.006	1.412 $\pm$ 0.007 <sup>a)</sup>	1.438 $\pm$ 0.006 <sup>b)</sup>	1.439 $\pm$ 0.006 <sup>b)</sup>	1.441 $\pm$ 0.007 <sup>b)</sup>	1.457 $\pm$ 0.008 <sup>c)</sup>
Dry	0.956 $\pm$ 0.007	0.892 $\pm$ 0.007 <sup>a)</sup>	0.918 $\pm$ 0.008 <sup>b)</sup>	0.919 $\pm$ 0.006 <sup>b)</sup>	0.924 $\pm$ 0.009 <sup>b)</sup>	0.938 $\pm$ 0.006 <sup>c)</sup>
Breaking force, 10 <sup>6</sup> dyn	13.1 $\pm$ 0.2	11.5 $\pm$ 0.3 <sup>a)</sup>	12.6 $\pm$ 0.2 <sup>b)</sup>	12.7 $\pm$ 0.3 <sup>b)</sup>	12.5 $\pm$ 0.2 <sup>b)</sup>	12.8 $\pm$ 0.2 <sup>c)</sup>

Values are means $\pm$ S.E.M.,  $n=6$ . Within a row, values with a superscript are significantly different: a)  $p<0.01$  compared with sham rats; b)  $p<0.05$ , c)  $p<0.01$  compared with ovx rats.

Table 5. Effect of OVX, Daidzin, Genistin, and Estrone on Urinary Excretion of Creatinine, Pyridinoline, and Deoxypyridinoline in Rats

Measure	Sham	Ovx	Ovx + daidzin 50 mg/kg	Ovx + glycitin 50 mg/kg	Ovx + genistin 50 mg/kg	Ovx + estrone 7.5 $\mu$ g/kg
Creatinine, $\mu$ mol	73.4 $\pm$ 1.2	74.7 $\pm$ 1.8	74.0 $\pm$ 2.9	77.4 $\pm$ 2.8	76.3 $\pm$ 2.6	75.8 $\pm$ 2.4
Pyridinoline/creatinine, pmol/ $\mu$ mol	54.4 $\pm$ 4.9	97.6 $\pm$ 14.0 <sup>a)</sup>	56.4 $\pm$ 5.0 <sup>b)</sup>	50.9 $\pm$ 4.0 <sup>b)</sup>	98.6 $\pm$ 10.7	57.2 $\pm$ 6.3 <sup>b)</sup>
Deoxypyridinoline/creatinine, pmol/ $\mu$ mol	79.8 $\pm$ 7.0	125.0 $\pm$ 13.8 <sup>a)</sup>	70.8 $\pm$ 4.6 <sup>b)</sup>	75.3 $\pm$ 3.7 <sup>b)</sup>	110.0 $\pm$ 9.7	72.1 $\pm$ 8.7 <sup>b)</sup>

Values are means $\pm$ S.E.M.,  $n=6$ . Within a row, values with a superscript are significantly different: a)  $p<0.01$  compared with sham rats; b)  $p<0.01$  compared with ovx rats.

ovariectomy significantly reduced the other morphological indexes examined (Table 4).

Rats in the ovx group had significantly lower density and strength of the femur compared with the sham-operated group. The rats treated with daidzin, glycitin, genistin and estrone at a dose of 50 mg/kg had significantly higher femur density and strength than rats in the ovx group, but had similar or lower values compared with the sham-operated group.

**Urinary Analysis** The urine volumes and urinary creatinine excretion rates did not differ among groups (data of urine volume not shown). However, the urinary excretion ratio of pyridinoline and deoxypyridinoline to endogenous creatinine excretion was significantly increased 21 d after ovariectomy (Table 5). The increase was clearly prevented by administration of daidzin (50 mg/kg), glycitin (50 mg/kg) and estrone, but not genistin, 14 d after dosing.

## DISCUSSION

Gram scale production of the soy isoflavone glycosides daidzin and genistin from soybeans was performed using column chromatographies on Diaion SP-207 and Sephadex LH-20 and recrystallization from 80% methanol, and that of glycitin from hypocotyls was achieved by column chromatography on Diaion SP-207, followed by repeated recrystallizations from 80% methanol and methanol, as described in the experimental section, on the basis of the report by Kudou *et al.*<sup>7)</sup>

As in the previous report,<sup>15)</sup> we confirmed that daidzin and genistin prevented ovariectomy-related bone loss and bone strength reduction in ovx rats after oral administration for 4 weeks at 50 mg/kg/d, like estrone. Furthermore, daidzin suppressed uterine atrophy and increase in urinary excretion of the sensitive bone turnover markers pyridinoline and deoxypyridinoline, caused by ovariectomy, whereas genistin did not at 50 mg/kg/d. In this study, it was demonstrated that glycitin showed similar effects to daidzin, *i.e.*, prevention of ovx-induced bone loss, bone strength reduction, uterine atrophy, and increase in urinary excretion of pyridinoline and deoxypyridinoline at the same administration dose (50 mg/kg/d) as daidzin. Genistin prevented ovx-induced uterine atro-

phy at a dose of 100 mg/kg, but did not block any other change of ovx rats at a dose of 50 or 100 mg/kg. This shows that glycitin treatment inhibits bone turnover like daidzin, as estrone also does.<sup>22)</sup> The more effective preventive action of glycitin over that of genistin on uterine atrophy is partially supported by the finding of Song *et al.* that its aglycone, glycitein enlarged uterine more potently than genistein at the same dose, but was much less effective than ovarian hormone in weaning female mice.<sup>10)</sup>

Interestingly, the synthetic isoflavone, ipriflavone (7-isopropoxy-3-phenyl-4*H*-1-benzopyran-4-one), which is structurally similar to soy isoflavones, is devoid of estrogenic activity, but is effective in preventing the unfavorable changes in serum cholesterol associated with ovarian hormone deficiency in the ovx animals.<sup>23)</sup> We found in this study that daidzin and glycitin treatment effectively prevented the ovx-induced rise in serum lipids, total cholesterol and triglycerides, as estrone did. However genistin did not show any such effect. This is the first report to show that the soy isoflavone glycosides daidzin and glycitin potently suppress the ovarian hormone deficiency-induced rise in serum lipids, to our knowledge. In partial support of our observations, a recent study by Arjmandi *et al.* has shown that feeding soy protein rich in soy isoflavones to ovx rats was more effective in lowering serum cholesterol than feeding a similar diet poor in soy isoflavone content.<sup>13a,23)</sup> Soy isoflavones may account for 70 to 80% of the hypocholesterolemic effect of soy protein, judging from the report of Arjmandi *et al.*<sup>13a,23)</sup>

Rats in the ovx group had significantly greater final body weight and weight gain, together with abdominal fat accumulation, than those in the sham-operated group, as expected from previous work.<sup>15)</sup> This was prevented by estrogen, as reported previously.<sup>13a,15)</sup> Both daidzin and glycitin reduced body weight gain and abdominal fat in ovx rats and daidzin was slightly more effective than glycitin. But genistin did not show such effects. Firstly this demonstrated that daidzin and glycitin reduced the abdominal fat in ovx animals. The increased body weights in ovx rats and postmenopausal women suggest a shift in energy metabolism due to ovarian hormone deficiency.<sup>24)</sup> Our findings suggest that daidzin and glycitin may direct triglycerides to other tissues and away from adi-

pose tissue for catabolism, similar to the proposed mechanism of action of estrogen on adipose tissue.<sup>25)</sup> Thus, it appears that the lower mean final body weights of the daidzin-, glycitin- and estrone-treated ovx rats were a result of the normalization of metabolic pathways of the ovx rats by the administered compounds. We propose that glycitin and daidzin act as a source of proestrogenic compounds based on their effects on bone loss and uterine atrophy in ovx rats, compared with those of estrone. These effects may be tissue-specific.

Although the beneficial effects on bone texture were the same among daidzin, glycitin, and genistin, there were significant differences in terms of the effectiveness on uterus tissue, body weight gain, abdominal fat or serum lipid in ovx rats between the former two compounds and the last. It has already been shown that the affinity of daidzein and genistein for the estrogen receptor is weaker than that of estrogen, being approximately  $1 \times 10^{-3}$  to  $1 \times 10^{-5}$  times that of estradiol.<sup>26)</sup> It has been reported that the relative activity of the individual isoflavones varies with both the species and strain of animal used and the route of administration.<sup>27)</sup> Recent results suggest that genistin and related compounds have a selective affinity for the estrogen receptor in bone rather than those in other organs, such as uterus, ovary and lung under certain conditions.<sup>15,16b)</sup> This may be the reason why only daidzin and glycitin show distinct or more strong estrogenic activity in female Sprague-Dawley rats, while genistin or genistein does not. As regards the structure-estrogenic activity relationship of soy isoflavones and ipriflavone, it is suggested that the 4'-hydroxyl moiety of daidzin, genistin and glycitin is essential for estrogenic activity in some animals.<sup>28)</sup> The 5-hydroxyl function, which is present only in genistein and its glycosides, may be required to have more selective affinity for estrogen receptor in bone than in uterine in the rats used in this study. In any event, genistin and genistein appear to be selective modulators of the estrogen receptor in bone among soy isoflavones.<sup>16b)</sup>

In summary, we have demonstrated that oral administration of glycitin alone prevents bone loss in an ovarian hormone-deficiency rat model, as found for daidzin and genistin. Glycitin and daidzin seem to be proestrogenic compound, which suppress bone resorption to prevent bone loss after ovariectomy by directly acting on bone sites, like estrone. Interestingly, genistin has a different mechanism of action from that of daidzin and glycitin. Daidzin and glycitin appear to suppress ovx-induced lipid metabolism abnormality. Soy isoflavones are well tolerated by humans, as soybeans have long been a part of the human diet. Consumption of such soy isoflavone glycosides may offer a potential alternative therapy for the treatment of health problems such as cardiovascular disease and osteoporosis in ovarian hormone-deficient women. Their action mechanisms on lipid metabolism in ovx rats appear to be similar to that of estrone. Additional studies are needed to demonstrate their efficacy in humans and to elucidate their mode of action in animals.

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