Synergistic effect of isoflavone glycosides and fructooligosaccharides on postgastrectomy osteopenia in rats

Yoshifumi Kimira,[1] Kiyono Tajima,[1] Atsutane Ohta,[2] Yoshiko Ishimi,[3] Shin-ichi Katsumata,[1] Kazuharu Suzuki,[1] Herman Adlercreutz[4] and Mariko Uehara[1,]*

[1]Department of Nutritional Science, Tokyo University of Agriculture, Tokyo 156-8502, Japan
[2]Department of Pharmaceutical Science, Josai International University, Chiba 283-8555, Japan
[3]Food Function and Labeling Program, National Institute of Health and Nutrition, Tokyo 162-8636, Japan
[4]Institute for Preventive Medicine, Nutrition and Cancer, Folkhälsan Research Center, and Division of Clinical Chemistry, FIN-00014, University of Helsinki, Helsinki, Finland

Fructooligosaccharides stimulate the growth of *Bifidobacteria*, which cleave isoflavone glycosides to yield corresponding aglycones, and convert metabolites by enhancing enterohepatic recirculation of isoflavones in rats. In the present study, we determined the synergistic effect of dietary isoflavone glycosides and fructooligosaccharides on postgastrectomy osteopenia in rats. Nine-week-old male Sprague-Dawley rats were gastrectomized (*n* = 20) or sham operated, (control, *n* = 5) and then randomly assigned to 5 diet groups: sham-a purified diet control, gastrectomized-control, gastrectomized-isoflavone (0.2% isoflavone glycosides), gastrectomized-fructooligosaccharides (7.5% fructooligosaccharides), and isoflavone and fructooligosaccharides (0.2% isoflavone glycosides + 7.5% fructooligosaccharides). After 6 weeks, the rats were killed and biological samples were collected. In gastrectomized rats, fructooligosaccharides prevented femoral bone fragility, but isoflavone without fructooligosaccharides did not inhibit postgastrectomy osteopenia. Isoflavone and fructooligosaccharides exhibited a synergistic in the distal metaphyseal trabecular bone, indicated by peripheral quantitative computed tomography. Moreover, fructooligosaccharides increased calcium absorption and equol production from daidzein in gastrectomized rats. These results indicate that isoflavone alone did not inhibit postgastrectomy osteopenia, but the combination of isoflavone and fructooligosaccharides improved the inhibition of trabecular bone loss by increasing calcium absorption and equol production through fructooligosaccharides supplementation.

Key Words: equol, daidzein, genistein, fructooligosaccharides, postgastrectomy osteopenia

Two major soy isoflavones, genistein and daidzein, have attracted attention because their possible effectiveness toward preventing Western diseases.[1,2] They occur in plant foods such as glycosides that, after consumption, are cleaved by intestinal microflora to yield corresponding aglycones, in addition to metabolites available for intestinal absorption. It has been suggested that recent clinical effectiveness of soy isoflavones in humans might be attributable to their ability to produce equol, a metabolite of daidzein, in the intestinal tract, and the production of equol might lead to beneficial effects for the prevention of hormone-related cancers, cardiovascular diseases and osteoporosis.[3–5]

Fructooligosaccharides (FOS) are known to stimulate the growth of *Bifidobacteria* and their enhanced hydrolytic activity may result in the increased absorption and enterohepatic recirculation of soy isoflavones and production of equol.[6–8] Equol has also been subject to recent research focus because of its possible role in the prevention of life-related diseases such as cancer, menopausal symptoms and osteoporosis.[3–5,9]

A high prevalence of bone disorders has been reported in gastrectomized (GX) patients,[10] and osteopenia after total GX in animals were initially reported in 1938.[11] Common findings in GX rats include decreased bone mass,[12–14] anemia,[15] and decreased intestinal calcium (Ca) absorption.[16]

FOS stimulates mineral absorption in the large intestine.[17,18] Our research group and coworkers have previously shown that dietary FOS improved Ca and iron absorption, bone loss, and anemia in GX rats.[15,16]

Since the main animal model of osteoporosis is ovariectomized (OVX) rodents, we determined the effects of a combination of isoflavone and FOS on bone loss in OVX mice.[8] However, to our knowledge, there has been no investigation on the effects of isoflavone in the presence and absence of FOS in GX animals. In the present study, we clarified the sole effect of isoflavone glycosides and the synergistic effect of isoflavone glycosides and FOS on post GX osteopenia in rats.

Materials and Methods

Animals and surgical procedure.   Male Sprague-Dawley rats (9-week-old; *n* = 25, Clea Japan, Tokyo, Japan) were housed in individual stainless-steel wire-mesh cages in a room at 22°C and 55% relative humidity with a 12-h light/dark cycle. The rats were fed a diet of pellets (MF, Oriental Yeast, Tokyo, Japan) for a 1-week acclimation period before the operation. All rats were anesthetized by intraperitoneal injection of Nembutal (sodium pentobarbital, 35 mg/kg of body weight; Abbot Laboratories, North Chicago, IL). The 25 rats were randomly assigned to 5 groups. In 4 groups (total, 20 rats), the stomach was surgically removed (Biloth II).[19] Another group of rats was subjected to a sham operation as a surgical control; the abdominal cavity was opened for approximately 45 min, which is the same length of time required for a gastrectomy procedure. This study was approved by the Animal Studies Committee of Tokyo University of Agriculture, and the animals were maintained in accordance with the guidelines for the care and use of laboratory animals of Tokyo University of Agriculture.

*To whom correspondence should be addressed.
E-mail: mari@nodai.ac.jp

Table 1. Composition of experimental diets

	C	I	F	IF
Ingredients (g/kg)				
Dextrin	53.2	53.2	53.2	53.2
Casein	20	20	20	20
Sucrose	10	9.5	2.5	2
Corn oil	7	7	7	7
Cellulose powder	5	5	5	5
Mineral Mixture[#]	3.5	3.5	3.5	3.5
Vitamin Mixture[#]	1	1	1	1
L-cystine	0.3	0.3	0.3	0.3
Fructooligosaccharides			7.5	7.5
Fujiflavone P-40		0.5		0.5

C: control diet, ISO: 0.2% isoflavone diet, FOS: 7.5% fructooligo-saccharides diet, IF: 0.2% ISO and 7.5% FOS diet. [#]Prepared according to AIN-93G formulation.

Experimental groups and diets. After these operations, the rats were deprived of food for 24 h and then were allowed free access to homogenized, pasteurized cow's milk (Meiji Milk Products Co. Ltd., Tokyo, Japan) for 48 h. The rats were then fed a purified control diet (modified AIN-93G diet[20]) at 15 g/day for the next for 4 days. Dietary treatments began on day 7 postoperation, at which time the GX surgically treated rats were randomly assigned into 4 diet groups (of 5 individuals each); a control diet (GC), an isoflavone glycoside diet (GI), a FOS diet (GF), an isoflavone glycoside and FOS diet (GIF). The 5 rats from the sham operation were fed the control diet (Sham). The composition of each diet is shown in Table 1. Fujiflavone P40 (isoflavone content: 40% daidzin, malonyldaidzin, acetyldaidzin and daidzein account for 20.4, 0.1, 1.1, and 0.3%, respectively; genistin, acetylgenistin and genistein account for 4.6, 0.3, and 0.1%, respectively; and glycitin and glycitein together account for around 13%) was obtained from Fujicco Co. Ltd., Japan. FOS (Meioligo-P, Meiji Seika Kaisha Ltd., Tokyo, Japan) is a mixture of 42% 1-kestose, 46% nystose and 9% 1F-beta-fructofuranosyl-nystose. The rats were fed their respective diets for 6 weeks (20 g/day). All rats received an intramuscular injection of vitamin B-12 (Wako Pure Chem. Ind., Ltd., Tokyo, Japan) at 0.5 mg/kg every other week to prevent pernicious anemia, with the injection starting on the initial day of the feeding period. The rats were allowed free access to deionized water throughout the experiment. Feces were collected during the last 3 days. On the final day of the experiment, all rats were anesthetized by exposure to diethyl ether. After laparotomy, whole blood was collected by abdominal vein puncture, and the rats were killed. The samples of the femora and blood were immediately obtained.

Determination of Ca content in diets and feces. The amount of Ca in diet and feces was quantified using an atomic absorption spectrophotometer (A-2000, Hitachi, Ltd., Tokyo, Japan). The diets and dried feces were ashed at 550°C for 48 h. The ashed samples were dissolved in 4 mL of HCl solution (2 mol/L) and diluted appropriately with deionized water and lanthanum chloride for atomization. The apparent absorption of Ca was calculated by the following formula: apparent absorption (%) = (intake – fecal excretion)/intake.

Bone mineral content, bone area, and bone mineral density in femora by dual energy X-ray absorptiometry. Bone mineral content (BMC; mg), bone mineral density (BMD; mg/mm^2), and bone area (BA; mm^2) of the right femur of each rat were measured using dual energy X-ray absorptiometry (DXA; DCS-600A, Aloca, Tokyo, Japan). The BMD was calculated by BMC of the measured BA. The scanned area of each rat femur was divided into 3 equal parts (5.3 mm each): proximal, middle, and distal femur.

Assessment of BMD of the femora by peripheral quantitative computed tomography. Various parameters were assessed in cross section of the femur using peripheral quantitative computed tomography (pQCT; XCT-960A, Norland Stratec, Birkenfeld, Germany). The measurement in the right femur was started at the metaphysic, 3 mm below the articular surface, and visualized with the aid of the scout-view. The cross section was made at a distance of 1 mm. The section for analysis was defined with a clearly complete cortical ring. The voxel size was 0.08 mm and the threshold was 0.464 (at contour mode 2, peel mode 2). The measured parameters were total BMC (mg), total BMD (mg/cm^3), cortical and subcortical mineral density (mg/cm^3), trabecular mineral density (mg/cm^3), total BA (mm^2), cortical area (mm^2) and area of the medullary cavity (trabecular bone; mm^2).

Bone strength and radiography. Bone mechanical strength was tested by using Bone Strength Tester DYN-1255 (plunger speed 0.5 mm/s; full scale, 20 kg; Iiodenki INC, Ehime, Japan). Radiographic analysis of the femur was performed using a soft x-ray system (SOFRON SOR-M50LSOKEN, Co. Ltd., Tokyo, Japan).

Time-resolved fluoroimmunoassay to measure serum genistein, daidzein, and equol. Serum genistein, daidzein, and equol were analyzed by the time-resolved fluoroimmunoassay (TR-FIA) methods of Wang,[21] Uehara[22] and Brouwerset et al.,[23] respectively. After enzymatic hydrolysis and extraction by diethyl ether, the concentrations of serum genistein, daidzein, and equol concentrations were determined using a DELFIA Victor1420 multilabel counter (Perkin Elmer, Wellesley, MA). The final results were calculated using the following formula: final results = concentration (read) × 1/recovery × dilution factor (nmol/L).

Statistical analysis. Data were expressed as mean and standard error (SE) values. The significance of the differences among the groups was determined by 1-way analysis of variance and Fisher's protected least-significant difference. The differences were considered significant when $p < 0.05$.

Results

Food intake and body weight. Mean food intake (20 g/day) during the feeding period and initial body weight did not differ among the 5 groups; however, the final body weight decreased after the GX procedure (Table 2). Among the 4 GX groups, the final body weight was significantly lower in the GI and GIF groups than in the GC and GF groups (Table 2).

Apparent absorption of Ca. Absorption (%) of Ca appeared to decrease after GX; however, the absorption in rats

Table 2. Body weights and apparent calcium (Ca) absorption

	Sham	GC	GI	GF	GIF
Initial body weight (g)	221.8 ± 6.8	215.0 ± 7.8	212.3 ± 6.4	213.7 ± 5.7	216.0 ± 4.3
Final body weight (g)	427.0 ± 4.6[a]	365.0 ± 14.9[b]	316.2 ± 20.0[c]	374.2 ± 13.9[b]	343.7 ± 11.3[b,c]
Apparent Ca absorption (%)	39.7 ± 5.0[a]	23.4 ± 3.5[b]	31.1 ± 5.2[a,b]	40.1 ± 2.5[a]	44.3 ± 1.5[a]

Values are expressed as means ± SE for each group. sham-operated (sham), and gastrectomized rats fed a control diet (GC), a 0.2% isoflavone glycoside diet (GI), a 7.5% fructooligosaccharides (FOS) diet (GF), or a combination of 0.2% isoflavone glycoside diet and 7.5% FOS diet (GIF). [a,b,c]Values with different superscript letters are significantly different, $p < 0.05$.
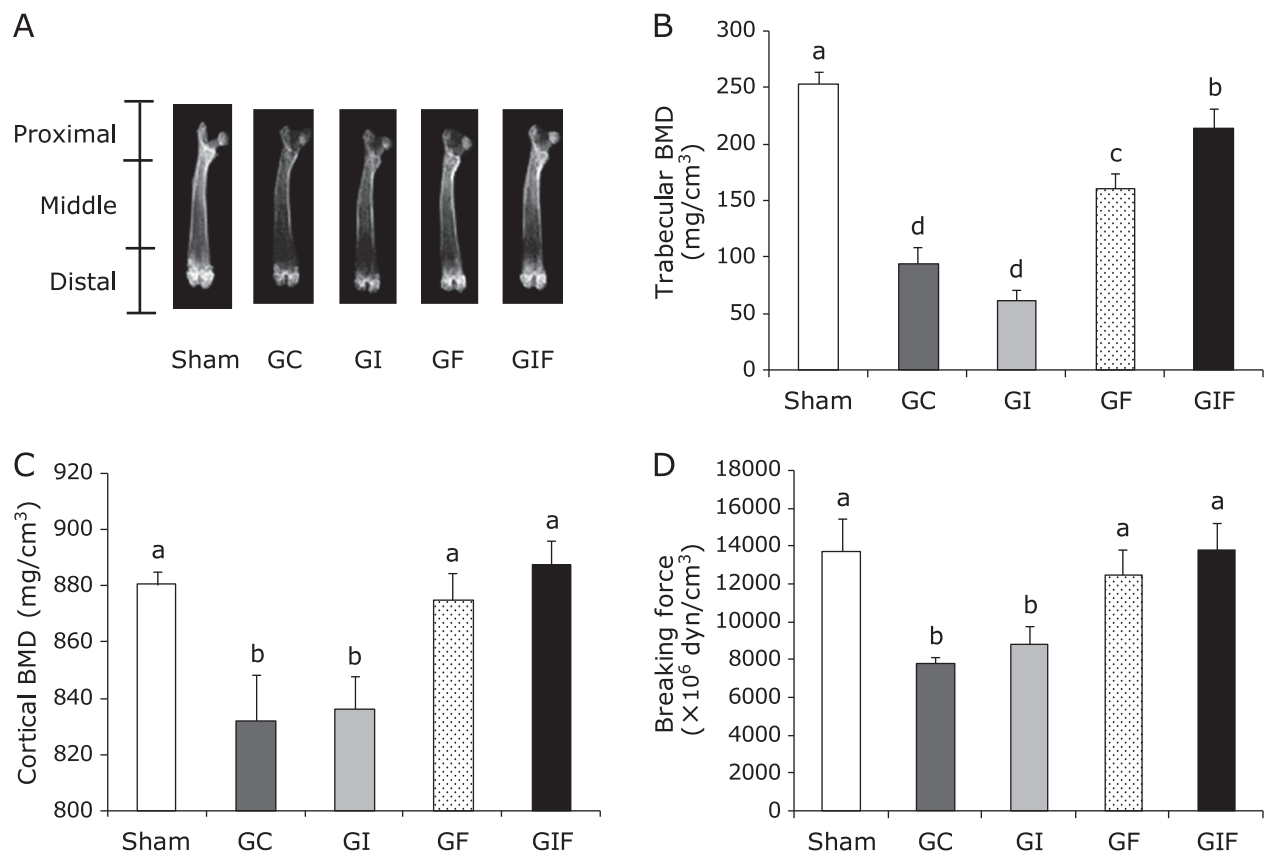
Fig. 1. Radiography (A), distal metaphyseal trabecular bone mineral density (BMD), dyaphyseal cortical BMD using pQCT (B), and breaking force (C) of the femur collected from sham-operated (sham), gastrectomized (GX) rats fed a control diet (GC), a 0.2% isoflavone glycoside diet (GI), a 7.5% fructooligosaccharides (FOS) diet (GF), or a combination of 0.2% isoflavone glycoside diet and 7.5% FOS diet (GIF) for 6 weeks. [a,b,c]Values are expressed as means ± SE for each group. Bars not sharing a letter differ, $p<0.05$.

Table 3. Femoral bone mineral density (BMD) by dual-energy X-ray absorptiometry (DXA)

	Sham	GC	GI	GF	GIF
Whole BMD (mg/cm²)	159.7 ± 1.4[a]	109.5 ± 0.8[c]	109.1 ± 1.1[c]	139.2 ± 4.6[b]	140.5 ± 2.3[b]
Proximal BMD (mg/cm²)	178.5 ± 1.5[a]	118.7 ± 2.2[c]	120.8 ± 1.1[c]	155.8 ± 5.4[b]	158.8 ± 2.0[b]
Middle BMD (mg/cm²)	137.2 ± 3.2[a]	99 ± 0.4[c]	98.8 ± 1.2[c]	122.3 ± 4.1[b]	117.5 ± 1.9[b]
Distal BMD (mg/cm²)	192.3 ± 2.2[a]	125.8 ± 1.7[c]	123.7 ± 2.7[c]	158.6 ± 5.1[b]	165.4 ± 2.8[b]

Values are expressed as means ± SE for each group. [a,b,c]Values with different superscript letters are significantly different, $p<0.05$.

fed the FOS diet (GF and GIF) was significantly higher than that in rats fed the control diet (GC) ($p<0.05$), although there was no significant difference in the Ca absorption between the GC and GI groups (Table 2).

Femur BMD by DXA and by pQCT. The BMD in the whole femur and all regions (proximal, middle, and distal) of the femur decreased after GX; however, decreased BMD was inhibited by treatment with the FOS diets (GF and GIF), as shown by the DXA and the radiography results (Fig. 1A and Table 3; $p<0.05$). The BMD in GX rats treated with only isoflavone glycosides did not differ from that in GX control rats (GC). The distal metaphyseal trabecular and diaphyseal cortical BMD values also decreased after GX; however the GF and GIF diets inhibited post-GX bone loss (Fig. 1 B and C; $p<0.05$). Although the diet with isoflavone glycosides alone did not inhibit bone loss caused by GX in both the trabecular and cortical bones (Fig. 1 A and B), the trabecular BMD in rats fed the GIF diet (treated with the combination of isoflavone glycosides and FOS diet was greater than that in the GF

rats (treated with the FOS diet) (Fig. 1 A and B; $p<0.05$).

Femoral bone strength. Bone breaking force significantly decreased after GX; however, the 2 FOS diets (GF and GIF) prevented bone fragility (Fig. 2C; $p<0.05$). Isoflavone did not have any effect on the decreased breaking force.

Serum genistein, daidzein and equol concentrations. The diets containing isoflavone glycosides significantly increased genistein and daidzein concentrations (Fig. 2 A and B). There was no difference in genistein concentration between rats fed the GI and GIF diets (Fig. 2A). Daidzein concentrations were significantly lower in rats fed the GIF diet than in those fed the GI diet ($p<0.05$). Concentrations of equol, a metabolite of daidzein, were significantly higher in rats fed the GIF diet than in those fed GI diet (Fig. 2C; $p<0.05$).

Discussion

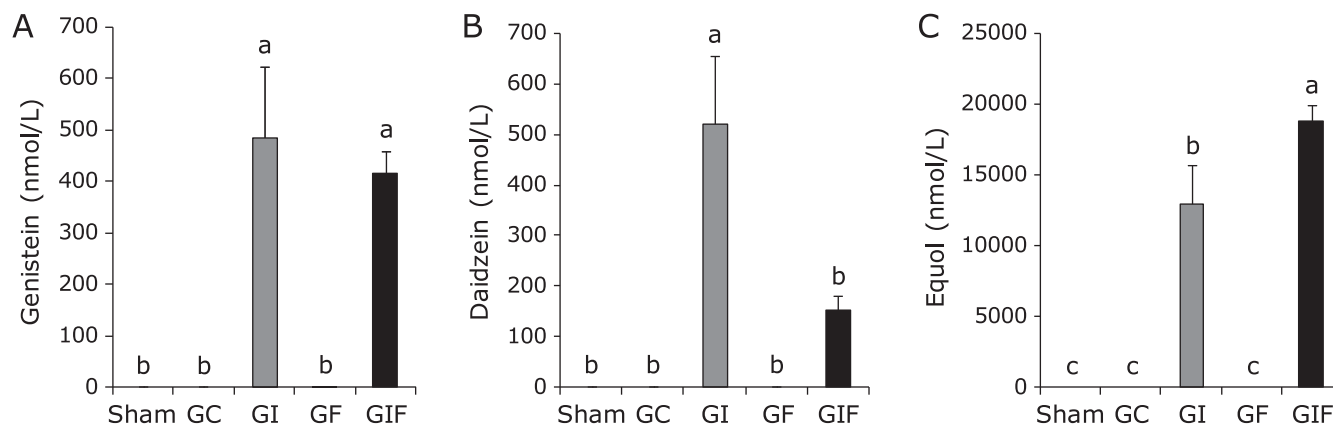The present study demonstrates that isoflavone does not inhibit

Fig. 2. Serum genistein (A), daidzein (B) and equol (C) concentrations in sham-operated (Sham) and gastrectomized rats fed a control diet (GC), a 0.2% isoflavone glycoside diet (GI), a 7.5% fructooligosaccharide diet (GF), or a combination of 0.2% isoflavone glycoside diet and 7.5% fructool-igosaccharide diet (GIF) for 6 weeks. [a,b,c]Values are expressed as means ± SE for each group. Bars not sharing a letter differ, $p<0.05$.

postgastrectomy (post-GX) osteopenia in rats; however, the combination of isoflavone glycoside and FOS improve trabecular bone loss, indicating that the effect of isoflavones on post-GX bone metabolism is attributable, at least in part, to equol, which is a metabolite of daidzein.

We previously reported that the combination of dietary FOS and isoflavone glycosides increases femoral BMD and equol production from daidzein in OVX mice.[8] In the previous study, which involved the feeding of growing mice, isoflavone glyco-sides markedly prevented post-OVX bone loss, both with and without FOS. It is well-known that isoflavone has a major effect on bone loss attributable to OVX. This is because isoflavone has a similar structure to estrogen, allowing it to bind to the estrogen receptor (ER) and stimulate bone metabolism via ER.[24,25] However, GX induces mineral absorption disorder in the intestinal tract. Ca malabsorption has been reported to be a cause of osteo-penia attributable to GX.[15,16] It is thought that gastric acid dissolves insoluble Ca in the diet, and thereby facilitates the absorption of Ca in the small intestine.[26,27] However, it is also known that polyphenols, including isoflavones, inhibit mineral absorption by forming insoluble chelate compounds with minerals from the small intestine.[28] Therefore, in the present study, isoflavone glycosides did not inhibit post-GX osteopenia. In contrast, dietary FOS enhanced Ca absorption and prevented changes indicative of post-GX osteopenia, such as decrease in femoral BMD value or bone breaking force, in GX rats. These results support those of the previous studies.[15,16] In addition, our coworkers visibly demonstrated the ability of FOS in post-GX osteopenia through the use of backscattered electron images, μCT scanning, and cross-sectional images.[29,30] In the present study, we observed that the cortical BMD results were similar to those of bone breaking force. We assessed the breaking force in the cortical area. It is possible that cortical bone loss influence bone breaking force. Furthermore, GX induces mineral malabsorption of not only Ca but also iron and copper, which are associated with type I collagen synthesis as cofactors of prolyl hydroxylase or lysyl oxidase.[31,32] Therefore, GX might induce bone fragility due to changes in collagen metabolism.

Although it was clearly shown that only isoflavone glycosides had no effect on bone loss and bone fragility in GX rats in the present study, the combination of isoflavone glycosides and FOS inhibited trabecular bone loss in the distal metaphysis of the femur. This combined ability was greater when equol was enhanced to produce from daidzein than the ability of FOS alone (Fig. 1A, 1B, and 2C). FOS decreased serum daidzein concentra-tion and increased equol concentration in GX rats (Fig. 2 B and C).

We previously indicated that FOS increased cecal β-glucosidase activity and equol production from daidzein in both OVX and surgical control (Sham) mice fed isoflavone glycosides.[8] The concentration of equol observed in the present study (Fig. 2C) was about 2-hold higher than that observed in the previous study.[8] This difference might be due to (1) a difference in isoflavone metabolism between mice and rats, and/or (2) GX causing a change in the condition of the gastrointestinal tract. Piskura et al.[33,34] had reported that there is probably a decrease in the solubility of the administered epicatechin and isoflavones in the gastrointestinal tract, that strongly influence the extent of absorption, because the stomach produces acidic secreta. Since GX rats had no stomach, it seems that no effect of acidic pH on absorption of isoflavones and enterohepatic recirculation of their metabolites was observed in the gut.

It has also been suggested that the beneficial effects of isofla-vone daidzein on bone loss might be due to its ability to produce equol in the gut.[5,35] We previously reported that the administra-tion of equol (0.5 mg/day subcutaneously) inhibited bone loss in the whole body and the femur in OVX mice without uterine hypertrophy.[35] Equol might also be effective in inhibiting bone loss caused by GX after normalizing mineral absorption in the large intestine through FOS feeding.

In conclusion, we clearly showed that isoflavone glycosides alone did not inhibit post-GX osteopenia, but the combination of isoflavone and FOS improved the distal metaphyseal trabecular bone loss, leading to increase Ca absorption and equol production by FOS supplementation.

Conflict of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Abbreviations

BA	bone area
BMC	bone mineral content
BMD	bone mineral density
Ca	calcium

DXA	dual-energy X-ray absorptiometry	OVX	ovariectomized
ER	estrogen receptor	post-GX	post gastrectomy
FOS	Fructooligosaccharides	pQCT	peripheral quantitative computed tomography
GX	gastrectomized	TR-FIA	Time-resolved fluoroimmuno assay

References

1 Adlercreutz H, Mazur W. Phyto-oestrogens and Western diseases. *Ann Med* 1997; **29**: 95–120.

2 Cassidy A. Potential risks and benefits of phytoestrogen-rich diets. *Int J Vitam Nutr Res* 2003; **73**: 120–126.

3 Setchell KD, Brown NM, Lydeking-Olsen E. The clinical importance of the metabolite equol-a clue to the effectiveness of soy and its isoflavones. *J Nutr* 2002; **132**: 3577–3584.

4 Setchell KD, Clerici C. Equol: pharmacokinetics and biological actions. *J Nutr* 2010; **140**: 1363S–1368S.

5 Wu J, Oka J, Ezaki J, *et al*. Possible role of equol status in the effects of isoflavone on bone and fat mass in postmenopausal Japanese women: a double-blind, randomized, controlled trial. *Menopause* 2007; **14**: 866–874.

6 Hidaka H, Eida T, Takizawa T, Tokunaga T, Tashiro Y. Effects of fructooligosaccharides on intestinal flora and human health. *Bifidrobact Microflora* 1986; **5**: 37–50.

7 Uehara M, Ohta A, Sakai K, Suzuki K, Watanabe S, Adlercreutz H. Dietary fructooligosaccharides modify intestinal bioavailability of a single dose of genistein and daidzein and affect their urinary excretion and kinetics in blood of rats. *J Nutr* 2001; **131**: 787–795.

8 Ohta A, Uehara M, Sakai K, *et al*. A combination of dietary fructooligo-saccharides and isoflavone conjugates increases femoral bone mineral density and equol production in ovariectomized mice. *J Nutr* 2002; **132**: 2048–2054.

9 Aso T, Uchiyama S, Matsumura Y, *et al*. A natural s-equol supplement alleviates hot flushes and other menopausal symptoms in equol nonproducing postmenopausal Japanese women. *J Womens Health (Larchmt)* 2012; **21**: 92–100.

10 Zittel TT, Zeeb B, Maier GW, *et al*. High prevalence of bone disorders after gastrectomy. *Am J Surg* 1997; **174**: 431–438.

11 Bussabarger RA, Freeman S, Ivy AC. Experimental production of severe homogeneous osteoporosis by gastrectomy in puppies. *Am J Physiol* 1938; **121**: 137–148.

12 Klinge B, Lehto-Axtelius D, Akerman M, Håkanson R. Structure of calvaria after gastrectomy. An experimental study in the rat. *Scand J Gastroenterol* 1995; **30**: 952–957.

13 Mühlbauer RC, Schenk RK, Chen D, Lehto-Axtelius D, Håkanson R. Morphometric analysis of gastrectomy-evoked osteopenia. *Calcif Tissue Int* 1998; **62**: 323–326.

14 Rümenapf G, Schwille PO, Wagner W, Tiecks FP, Fries W, Galewski D. Highly selective vagotomy in the rat: effects on bone and mineral metabolism. *Scand J Gastroenterol* 1994; **29**: 232–237.

15 Ohta A, Ohtsuki M, Uehara M, *et al*. Dietary fructooligosaccharides prevent postgastrectomy anemia and osteopenia in rats. *J Nutr* 1998; **128**: 485–490.

16 Ohta A, Ohtsuki M, Hosono A, Adachi T, Hara H, Sakata T. Dietary fructooligosaccharides prevent osteopenia after gastrectomy in rats. *J Nutr* 1998; **128**: 106–110.

17 Ohta A, Ohtsuki M, Baba S, Adachi T, Sakata T, Sakaguchi E. Calcium and magnesium absorption from the colon and rectum are increased in rats fed fructooligosaccharides. *J Nutr* 1995; **125**: 2417–2424.

18 Ohta A, Ohtsuki M, Baba S, Takizawa T, Adachi T, Kimura S. Effects of fructooligosaccharides on the absorption of iron, calcium and magnesium in iron-deficient anemic rats. *J Nutr Sci Vitaminol (Tokyo)* 1995; **41**: 281–291.

19 Oscarson J, Håkanson R, Liedberg G, Lundqvist G, Sundler F, Thorell J. Variated serum gastrin concentration: trophic effects on the gastrointestinal tract of the rat. *Acta Physiol Scand Suppl* 1979; **475**: 2–27.

20 Reeves PG, Nielsen FH, Fahey GC, Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* 1993; **123**: 1939–1951.

21 Wang GJ, Lapcík O, Hampl R, *et al*. Time-resolved fluoroimmunoassay of plasma daidzein and genistein. *Steroids* 2000; **65**: 339–348.

22 Uehara M, Arai Y, Watanabe S, Adlercreutz H. Comparison of plasma and urinary phytoestrogens in Japanese and Finnish women by time-resolved fluoroimmunoassay. *Biofactors* 2000; **12**: 217–225.

23 Brouwers E, L'homme R, Al-Maharik N, *et al*. Time-resolved fluoro-immunoassay for equol in plasma and urine. *J Steroid Biochem Mol Biol* 2003; **84**: 577–588.

24 Morito K, Hirose T, Kinjo J, *et al*. Interaction of phytoestrogens with estrogen receptors alpha and beta. *Biol Pharm Bull* 2001; **24**: 351–356.

25 Coxam V. Phyto-oestrogens and bone health. *Proc Nutr Soc* 2008; **67**: 184–195.

26 Allen LH. Calcium bioavailability and absorption: a review. *Am J Clin Nutr* 1982; **35**: 783–808.

27 Recker RR. Calcium absorption and achlorhydria. *N Engl J Med* 1985; **313**: 70–73.

28 Sandberg AS. Bioavailability of minerals in legumes. *Br J Nutr* 2002; **88**: S281–S285.

29 Morohashi T, Ohta A, Yamada S. Dietary fructooligosaccharides prevent a reduction of cortical and trabecular bone following total gastrectomy in rats. *Jpn J Pharmacol* 2000; **82**: 54–58.

30 Hirama Y, Morohashi T, Sano T, *et al*. Fructooligosaccharides prevent disorders of the femoral neck following gastrectomy in growing rats. *J Bone Miner Metab* 2003; **21**: 294–298.

31 Tuderman L, Myllylä R, Kivirikko KI. Mechanism of the prolyl hydroxylase reaction. 1. Role of co-substrates. *Eur J Biochem* 1977; **80**: 341–348.

32 Harris ED, Gonnerman WA, Savage JE, O'Dell BL. Connective tissue amine oxidase. II. Purification and partial characterization of lysyl oxidase from chick aorta. *Biochim Biophys Acta* 1974; **341**: 332–344.

33 Piskula MK, Terao J. Accumulation of (−)-epicatechin metabolites in rat plasma after oral administration and distribution of conjugation enzymes in rat tissues. *J Nutr* 1998; **128**: 1172–1178.

34 Piskula MK, Yamakoshi J, Iwai Y. Daidzein and genistein but not their glucosides are absorbed from the rat stomach. *FEBS Lett* 1999; **447**: 287–291.

35 Fujioka M, Uehara M, Wu J, *et al*. Equol, a metabolite of daidzein, inhibits bone loss in ovariectomized mice. *J Nutr* 2004; **134**: 2623–2627.