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

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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.

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 aclimation 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 Π). 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.

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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) 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), and a combination of 0.2% isoflavone glycoside diet and 7.5% 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, acetylaidzin 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 glycinit and glycitine together account for around 13%) was obtained from Fujifco Co. Ltd., Japan. FOS (Meioligo-P, Meiji Seika Kaisha Ltd., Tokyo, Japan) is a mixture around 13%) was obtained from Fujicco Co. Ltd., Japan. FOS and blood were immediately obtained. After laparotomy, whole blood was collected by abdominal vein puncture, and the rats were killed. The samples of the femora and bone area (BA; mm²) of the right femur of each rat were measured parameters were BMC (mg), BMD (mg/cm³), cortical and subcortical mineral density (mg/cm³), and area of the medullary cavity (trabecular bone; mm³).

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/mm³), and bone area (BA; mm²) 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 femur 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 metaphysis, 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, peak mode 2). The measured parameters were total BMC (mg), total BMD (mg/cm³), cortical and subcortical mineral density (mg/cm³), total BA (mm²), cortical area (mm²) and area of the medullary cavity (trabecular bone; mm²).

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; Iodienki 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 fluoroimmunosassay to measure serum genistein, daidzein, and equol. Serum genistein, daidzein, and equol were analyzed by the time-resolved fluoroimmunosassay (TR-FIA) methods of Wang,(21) Uehara(22) and Brouwers 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 (Table 2).

Apparent absorption of Ca. Absorption (%) of Ca appeared to decrease after GX; however, the absorption in rats calculated by the following formula: apparent absorption (%) = (intake – fecal excretion)/intake.

Table 1. Composition of experimental diets

| Ingredients (g/kg) | C | I | F | IF |
|-------------------|---|---|---|---|
| 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 Mixture⁴ | 3.5 | 3.5 | 3.5 | 3.5 |
| Vitamin Mixture⁴ | 1 | 1 | 1 | 1 |
| L-cystine         | 0.3 | 0.3 | 0.3 | 0.3 |
| Fructooligosaccharides | 7.5 | 7.5 | 7.5 | 7.5 |
| Fujiflavone P-40  | 0.5 | 0.5 | 0.5 | 0.5 |

C: control diet, ISO: 0.2% isoflavone diet, FOS: 7.5% fructooligosaccharides diet, IF: 0.2% ISO and 7.5% FOS diet. ⁴Prepared according to AIN-93G formulation.

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 ± 4.6⁶ | 365.0 ± 14.9⁶ | 316.2 ± 20.0⁶ | 374.2 ± 13.9⁶ | 343.7 ± 11.3³⁶ |
| Apparent Ca absorption (%) | 39.7 ± 5.0⁶ | 23.4 ± 3.5⁶ | 31.1 ± 5.2⁶ | 40.1 ± 2.5⁶ | 44.3 ± 1.5⁶ |

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). ⁶²Values 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...
postgastrectomy (post-GX) osteopenia in rats; however, the combination of isoflavone glycosides 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. In the previous study, which involved the feeding of growing mice, isoflavone glycosides 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. However, GX induces mineral absorption disorder in the intestinal tract. Ca malabsorption has been reported to be a cause of osteopenia attributable to GX. 

It is thought that gastric acid dissolves insoluble Ca in the diet, and thereby facilitates the absorption of Ca in the small intestine. However, it is also known that polyphenols, including isoflavones, inhibit mineral absorption by forming insoluble chelate compounds with minerals from the small intestine. 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. 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. 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. 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 concentration 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. The concentration of equol observed in the present study (Fig. 2C) was about 2-fold higher than that observed in the previous study. 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. 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 secretions. 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 isoflavone daidzein on bone loss might be due to its ability to produce equol in the gut. We previously reported that the administration of equol (0.5 mg/day subcutaneously) inhibited bone loss in the whole body and the femur in OVX mice without uterine hypertrophy. 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.

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Abbreviations

| Abbreviation | Description   |
|--------------|---------------|
| BA           | bone area     |
| BMC          | bone mineral content |
| BMD          | bone mineral density |
| Ca           | calcium       |

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% fructooligosaccharide diet (GIF) for 6 weeks. Values are expressed as means ± SE for each group. Bars not sharing a letter differ, p<0.05.
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DXA dual-energy X-ray absorptiometry
ER estrogen receptor
FOS Fructooligosaccharides
GX gastrectomized
OVX ovariectomized
pQCT peripheral quantitative computed tomography
TR-FIA Time-resolved fluoroimmuno assay

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