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Effects of *Cheonggukjang* and *Doenjang* on Bone Loss in Ovariectomized Rats

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Abstract The effects of *cheonggukjang* and *doenjang* on bone mineral density, trabecular area and cortical thickness of the tibia, and serum osteocalcin level in ovariectomized rats were investigated. After 4 weeks, bone mineral density, bone trabecular area, the cortical thickness index, and serum osteocalcin level were analyzed. The *cheonggukjang* and *doenjang* diet groups showed significant prevention of ovariectomized (OVX)-related body weight gain. Whole body bone mineral density of the OVX group was significantly lower than that of the sham group, whereas the *cheonggukjang* and *doenjang* diets resulted in complete restoration of bone mineral density. Trabecular area in the proximal diaphysis and cortical thickness in the distal diaphysis of the tibia were increased significantly in the *cheonggukjang* and *doenjang* diet fed groups. The *cheonggukjang* and *doenjang* diets significantly reduced serum osteocalcin level in the OVX rats. These results suggest that *cheonggukjang* and *doenjang* might have inhibitory effects on osteoporosis, by showing accelerated bone formation in OVX rats.

Keywords: *cheonggukjang*, *doenjang*, bone mineral density, bone trabecular area, serum osteocalcin

Introduction

Osteoporosis is a serious bone disease attributed to increases in osteoclastic bone resorption and decreases in osteoblastic bone formation (1), and is prevalent in postmenopausal women. It is characterized by a significant decrease in bone mineral density per bone volume, resulting in increased bone fragility and fracture. The risk factors for osteoporosis are recognized as genetics, deficient calcium intake, cigarette smoking, and excessive alcohol intake, as well as reduction in estrogen level.

Hormone (estrogen) replacement therapy has been used for the prevention and treatment of postmenopausal osteoporosis. However, this therapy might increase undesirable side effects such as breast, ovarian, and endometrial cancers in postmenopausal women (2-4). Therefore, researchers have begun to investigate functional foods containing natural substances as alternatives to hormone replacement therapy for the prevention of postmenopausal osteoporosis.

Soybean, which is rich in isoflavones, has been used as a primary ingredient in both fermented and unfermented food products, and have received considerable attention due to their positive effect on the prevention of menopausal symptoms. The positive effects of soybeans are attributed to their high content of isoflavones, which have similar structure and functionality to estrogen (5,6). Previous studies have indicated that bone loss induced by ovariectomy (OVX) can be prevented by feeding rats diets that contain soybean protein or its isoflavones (7-9). In addition, epidemiological studies have suggested that the dietary habits of Asians, who consume a high amount of soy-related foods, can reduce osteoporotic fractures and postmenopausal symptoms (10,11).

Cheonggukjang and *doenjang*, traditional soybean-based fermented foods in Korea, are made from cooked and fermented soybeans, and contain many biologically active compounds that are produced during fermentation. Thus, recent studies on *cheonggukjang* and *doenjang* have received much attention for illustrating their various physiological properties (12-18), including antioxidant, fibrinolytic, anticancer, antihypertensive, hypocholesterolemic, and antimutagenic activities. In the present study, we examined the effects of *cheonggukjang* and *doenjang* on OVX-induced bone loss, where bone mineral density, bone trabecular area, and biochemical marker of bone formation were measured in OVX rats.

Materials and Methods

Materials Traditionally manufactured *cheonggukjang* and *doenjang* were purchased in Sunchang, Korea, freeze-dried to prepare a powder, and then added to the experimental diets.

Animals and osteoporotic model Female Sprague-Dawley rats (6 weeks old, Daehan Experimental Animals Inc., Eumsung, Korea) weighing 200-250 g were intraperitoneally anaesthetized with 7% chloral hydrate (0.5 mL/100 g of BW) and ovariectomized by ligation and excision of the ovaries. However, in sham operation, the ovaries were exteriorized, but not removed. The OVX rats were randomly assigned to one of 3 treatment groups: OVX control group (fed a calcium free basal diet), OVX+*cheonggukjang* group (fed a calcium free 10% *cheonggukjang* diet), and the OVX+*doenjang* group (fed a calcium free

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10% *doenjang* diet). The basal diet used in this study consisted of the following ingredients, in g/100 g of the diet: 20 g of casein, 0.3 g of DL-methionine, 55 g of sucrose, 10 g of corn starch, 5 g of corn oil, 3.5 g of AIN-76 mineral (American Institute of Nutrition), 1 g of AIN-76 vitamin mix, 0.2 g of choline bitartate, 5 g of cellulose; and in the experimental groups, 10 g of the test substance was substituted for casein. The test substances included 10% *cheonggukjang* and 10% *doenjang* powders. The week following surgery, the OVX rats were fed experimental diets for 4 weeks. Rats from the same treatment group were pair-housed in standard plastic cages, and maintained at a 22±2°C and 60±5% relative humidity, in a room with a 12 hr light/dark cycle with free access to food and water *ad libitum*.

Measurements of body weight and bone mineral density Body weight was measured weekly and changes were determined by comparison with previous results during the experimental period. Bone mineral density (BMD) of the whole body was measured using dual energy X-ray absorptiometry (DEXA) scans (DPX-α; Lunar Co., Madison, WI, USA) immediately before necropsy. For scanning, the rats were intraperitoneally anaesthetized with 7% chloral hydrate (0.5 mL/100g of BW) and positioned prone on the DEXA scanning table. Whole body scans were performed using Hologic Regional High Resolution software (DPX-α; Lunar Co.) and analyzed using the small animal software version.

Tissue processing Six rats were sacrificed after being fed the experimental diets for 4 weeks. At necropsy, the left tibia was removed to clean the soft tissues such as the muscle, ligament, and tendon. The cleaned tibias were fixed in 10% neutral buffered formalin for 2 days. The tibias were then decalcified in formic acid-sodium citrate solution for 9 days and washed in tap water for 4 hr. The tissues were embedded with paraffin and cut into a thickness of 7 μm (longitudinal and transverse section) using a microtome. Hematoxylin and eosin (H&E) staining was performed after mounting on the slides.

Measurement of bone trabecular area and the cortical thickness index Bone trabecular area (19), as appearing in the spongy bone of the tibial proximal diaphysis, was measured area (246×10^4 μm^2) below and parallel to growth plate at a magnification of ×31 using a microscope and image analyzer (Analysis Pro version 3.2, Soft imaging System GmbH, Munster, Germany). The data were used as the mean trabecular area. The cortical thickness index (Fig. 1) was calculated by dividing the combined cortical thickness (AB+CD) by the bone diameter (AD). AB and CD represent the anteroposterior and mediolateral diameters of the transverse section of the tibia, respectively, while AD represents the cortical thickness of the transverse section of the tibia.

Combined cortical thickness: AB+CD
Cortical thickness index = (AB+CD)/AD

Analysis of serum osteocalcin The rats were killed at the end of the experimental period under anaesthesia. Blood samples were collected by cardiac puncture, transferred to a refrigerator for 6 hr, and then centrifuged at 1,000×g for 15 min at 4°C. Serum was removed to measure the serum osteocalcin activity, which was determined by a rat osteocalcin enzyme-linked immunosorbent assay (ELISA) kit (Biomedical Technology Inc, Stoughton, MA, USA) using an ELISA reader (Sunrise, Tecan, Grodig/Salzburg, Austria).

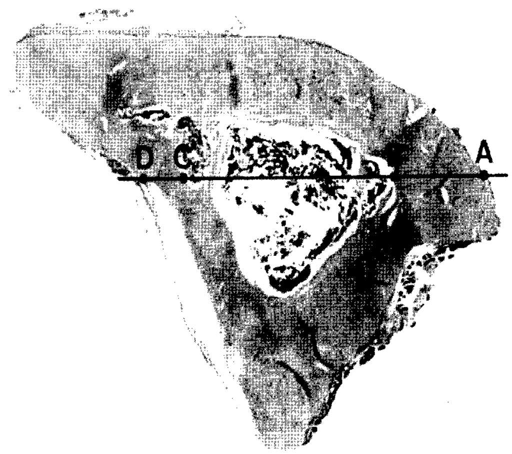

Fig. 1. Transverse section of the tibia for the calculation of the cortical thickness index. AB, CD, and AD represent the anteroposterior, mediolateral diameters, and cortical thickness of the transverse section of the tibia, respectively.

Statistical analysis The data were analyzed by one-way analysis of variance (ANOVA) with PROC MIXED in PC SAS (version 8.2, SAS Institute Inc., Cary, NC, USA). The results are expressed as mean±SE, and the differences among the groups were analyzed using Duncan's multiple range tests. Significance was considered at $p<0.05$.

Results and Discussion

Changes in body weight The initial body weights in the sham, OVX, OVX+*cheonggukjang*, and OVX+*doenjang* diet groups were 211.8±7.5, 240.4±10.2, 235.2±12.3, and 223.0±9.5 g, respectively, and the final body weights after feeding the study diets for 4 weeks were 246.3±11.1, 323.2±12.6, 298.0±10.5, and 294.0±12.3 g, respectively (Table 1). The weight increases between the initial and final body weights of all groups ranged from 16.1% for the sham group to 34.4% for the OVX group, and the 2 experimental groups (OVX+*cheonggukjang* and OVX+*doenjang*) had weight increases (26.7 and 31.8%, respectively) compared to the sham group. However, the *cheonggukjang* and *doenjang* diets prevented OVX-related body weight gain. The observed weight loss after feeding the *cheonggukjang* and/or *doenjang* diets may be due to the isoflavone content of the soybeans used as raw material, since isoflavones can act as a source of proestrogenic compounds (7).

BMD, trabecular area, and the cortical thickness index Table 2 shows BMD of the whole body for the experimental groups. The OVX rats had a significant reduction (256.8± 0.6 mg/cm²) in BMD of the whole body over the 4 weeks as compared to the sham rats (261.2±0.4 mg/cm², $p<0.01$). BMD was completely restored by the *cheonggukjang*

Table 1. Changes in body weight for the 4 experimental groups

Group	Initial body weight (g)	Final body weight (g)	Increase rate of body weight (%)
Sham	211.8±7.5[1)]	246.3±11.1	16.1
OVX	240.4±10.2	323.2±12.6	34.4
OVX+*cheonggukjang*	235.2±12.3	298.0±10.5	26.7
OVX+*doenjang*	223.0±9.5	294.0±12.3	31.8

[1)]Values are mean±SE; n=6.

Table 2. The effects of the *cheonggukjang* and *doenjang* diets on whole body bone mineral density

Group	Bone mineral density (mg/cm^2)**
Sham	261.2±0.4[1)b]
OVX	256.8±0.6[c]
OVX+*cheonggukjang*	266.6±0.5[a]
OVX+*doenjang*	265.8±0.6[a]

[1)]Values are mean±SE; n=6; **p<0.01.

(266.5±0.5 mg/cm^2) and *doenjang* (265.8±0.6 mg/cm^2) diets, indicating these fermented foods may have preventive affects on bone loss. This is supported by results showing increased trabecular area in the proximal diaphysis of the tibia (Fig. 2). Figure 2 shows the trabecular areas in the proximal diaphysis of the tibia collected from the sham, OVX, and OVX rats fed diets containing *cheonggukjang* or *doenjang* for 4 weeks. In the sham group, the trabeculae in the proximal diaphysis of the tibia were well developed (Fig. 2.1), whereas those in the OVX rats disappeared and showed widening of the marrow spaces (Fig. 2.2). For the OVX + *cheonggukjang* (Fig. 2.3) and *doenjang* (Fig. 2.4) groups, the trabecular bone volume and trabecular number in the proximal diaphysis of the tibia increased as compared to the OVX group.

Table 3. The effects of the *cheonggukjang* and *doenjang* diets on trabecular area in the proximal diaphysis, and the cortical thickness index of the tibia

Group	Trabecular area (%)**	Cortical thickness index**
Sham	46.4±3.2[1)a]	0.284±0.001[b]
OVX	26.3±2.5[c]	0.264±0.003[c]
OVX+*cheonggukjang*	36.2±2.8[b]	0.298±0.001[a]
OVX+*doenjang*	35.8±3.1[b]	0.305±0.002[a]

[1)]Values are mean±SE; n=6; **p<0.01.

The results show that the trabecular area in the proximal diaphysis of the tibia was significantly higher in the sham group (46.4±3.2%) than in the OVX group (26.3±2.5%), whereas it increased significantly by feeding the *cheonggukjang* (36.2±2.8%) and *doenjang* (35.8±3.1%) diets (Table 3). The cortical thickness index, which was measured in the distal portion of the tibia, was significantly reduced in the OVX group (0.264±0.003) as compared to the sham group (0.284±0.001), whereas it significantly increased by feeding the *cheonggukjang* (0.298±0.001) and *doenjang* (0.305±0.002) diets (Table 3). These results indicate that *cheonggukjang* and/or *doenjang* consumption might be useful in preventing OVX-induced trabecular and cortical

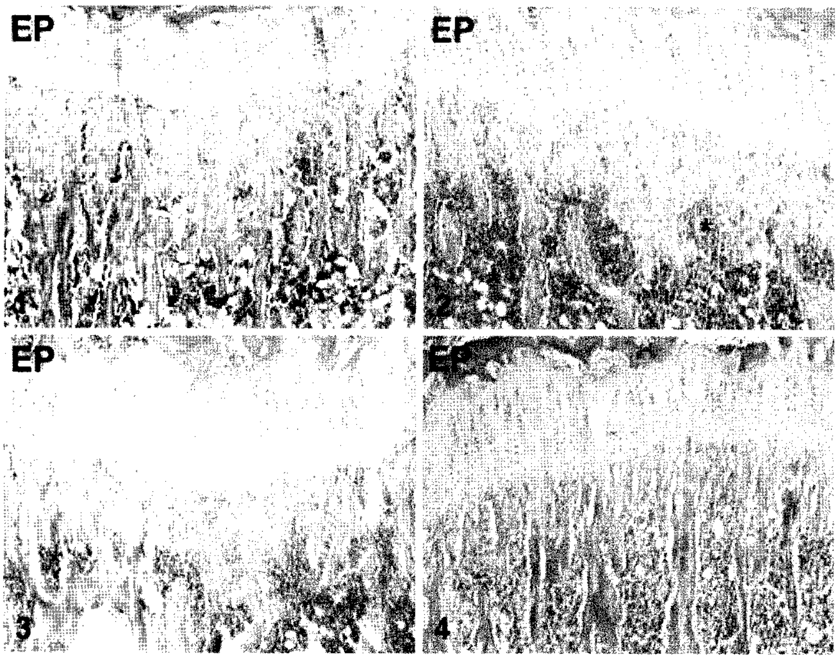

Fig. 2. Trabecular area in the proximal diaphysis of the tibia from different diet groups (H&E stain, ×50). 1, Sham group; 2, OVX group (*disappearance and widening of the marrow spaces); 3, OVX+*cheonggukjang* group; 4, OVX+*doenjang* group.

Table 4. The effects of the *cheonggukjang* and *doenjang* diets on serum osteocalcin level

Group	Osteocalcin (ng/mL)**
Sham	46.8±3.2[1)c]
OVX	134.0±3.2[a]
OVX+*cheonggukjang*	65.5±2.6[b]
OVX+*doenjang*	61.9±2.1[b]

[1)]Values are mean±SE; n=6; ** $p<0.01$.

bone loss in rats.

The restoration of BMD as well as the trabecular area in the proximal diaphysis and the cortical thickness to the levels in the sham group by dietary intake of *cheonggukjang* and/or *doenjang*, might be attributed to the isoflavones in these fermented soybean-based foods. *Cheonggukjang* and *doenjang*, which are representative soybean-based fermented foods in Korea, are manufactured by the fermentation of cooked soybeans. Markedly increased isoflavone contents, particularly aglycones, have been reported in fermented soybean products as compared to unfermented soybeans (20-23). Most isoflavones are hydrolyzed by the action of microorganisms during the fermentation process, resulting in large amounts of aglycones, which have better bioavailability than their glycoside forms in human bodies (21,22). These aglycones that are produced may be related to maintaining bone health, due to their positive effects on both bone mineral density and bone loss in OVX-induced rats (24,25).

Several animal studies (7-9,26,27) have shown that soybean isoflavones such as daidzein and genistein have preventive effects on the bone loss induced by estrogen deficiency. Arjmandi *et al.* (7) reported that soybean protein isolate containing isoflavones prevented bone loss in OVX rats and had a greater effect on trabecular bone than on cortical bone. Moreover, Fanti *et al.* (5) demonstrated that genistein had a bone sparing effect for both distal femur cancellous bone and whole tibia BMD in OVX rats. There have also been reports that daidzein prevents cancellous and cortical bone loss through the suppression of increased bone turnover in OVX rats (9,28), while genistein prevents bone loss through a similar mechanism with estrogen in OVX mice (25). Therefore, based on these results, we expect that *cheonggukjang* and/or *doenjang* consumption might be helpful to maintain bone health.

Serum osteocalcin level Serum osteocalcin, an important biochemical marker for bone formation, is secreted by mature osteoblasts and released during osteoclastic degradation (29). The serum osteocalcin level in the OVX group was significantly higher than that of the sham group (Table 4), resulting in occurring bone loss in the OVX rats. This result is in accord with previous reports showing significantly increased serum osteocalcin levels in OVX rats (5,30,31). Moreover, although the *cheonggukjang* and *doenjang* diet groups had significantly higher serum osteocalcin levels than the sham group, the diets caused a significant suppression in the rise of serum osteocalcin in the OVX group, suggesting that these soybean-based fermented foods might inhibit OVX-induced increases in bone turnover. The serum osteocalcin pattern found by feeding *cheonggukjang* and *doenjang* diets in this study was different from previously reported patterns by feeding soy protein with isoflavones, which mostly produced an increase in bone formation in the OVX rats (1,5,27). In contrast, this pattern seems to be similar to that by estrogen, which is known to suppress the increase of bone formation in OVX rats (27,30,31). Therefore, reductions in serum osteocalcin, when compared with the OVX group, by the intake of *cheonggukjang* and/or *doenjang*, might prevent the bone turnover induced by OVX.

In conclusion, the ingestion of *cheonggukjang* and/or *doenjang* prevented bone loss in OVX-induced rats, suggesting the consumption of these soybean-based fermented foods could be a good source of prevention for postmenopausal osteoporosis.

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