

Effects of *Rubus coreanus*-Cheonggukjang on Bone Mineral Density and Bone Mineral Content in Growing Rats

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ABSTRACT: The purpose of the present study was to investigate the bone-conserving effects of *Rubus coreanus*-Cheonggukjang (RC-CGJ) supplemented with more intensified phytochemicals compared to general Cheonggukjang (CGJ) in growing rats. Eighteen rats were divided into 3 treatment groups (Control, CGJ, and RC-CGJ) and were given experimental diets for 9 weeks. All of the rats in this study were fed a AIN-93G-based diet. Both CGJ groups were fed with 33.1% CGJ and RC-CGJ powder, respectively. The results of this study indicate that weight gain, mean food intake, and food efficiency ratio were not significantly different by the experimental diets among all groups. Spine bone mineral density (BMD) and femur BMD were not significantly different by the experimental diets. Spine bone mineral content (BMC) was significantly higher in the RC-CGJ and CGJ groups than in the control group, regardless of CGJ type. The femur BMC of the CGJ supplemented group was significantly higher compared with the control group and the RC-CGJ group. Compared with the control group, spine BMD and femur BMD per weight were markedly increased in the RC-CGJ and CGJ group regardless of CGJ type. Also, spine BMC per weight was significantly higher in the RC-CGJ group than in the CGJ group. However, femur BMC per weight was significantly higher in the CGJ group than in the RC-CGJ group. It can be concluded that RC-CGJ and CGJ supplemented diets have more beneficial effects on spine and femur peak bone mass in growing rats.

Keywords: *Rubus coreanus*-Cheonggukjang, Cheonggukjang, bone mineral density, bone mineral content, growing rats

INTRODUCTION

In Korea, the osteoporosis prevalence rates by age are 0.8%, 1.6%, 5.4%, 14.2%, and 14.4% among those in their 30s, 40s, 50s, 60s, and over 70s, respectively. Similar trends are shown when those are stratified by sex: 15.4%, 32.8%, and 65.2% for women, and 4.5%, 5.5%, and 20.0% for men in their 50s, 60s, and over 70s, respectively. It is also reported that women especially over the age of 50 have about 4.5 times higher osteoporosis prevalence rate than men of the same age (men: 7.8%, women: 34.9%), while exhibiting a constant increase in the rate by age (1). Osteoporosis is a progressive bone disease, which is easier to prevent than to treat. A good prevention method is to maximize peak bone mineral density (BMD) requiring bone metabolism-related nutrients including calcium and vitamin D (2). Recent studies have reported that intake of phytochemical-rich foods is beneficial to bone metabolism by protecting bone from oxidative stress (3).

Cheonggukjang (CGJ), a Korean traditional food ingre-

dient, is made by natural a fermentation process of boiled beans using *Bacillus subtilis* (4). CGJ has distinctive flavor and scent, and the isoflavones within the beans are especially known to improve menopausal symptoms and prevent one from cardiovascular disease and osteoporosis. Moreover, fermentation or digestion of CGJ beans is beneficial to the body as it can result in high conversion of isoflavone glycosides to isoflavone aglycones. In previous studies, the intake of CGJ promoted human bioactive function by reducing blood sugar levels and by promoting antioxidant effects (5,6). Vitamin K₂, an essential nutrient for γ -carboxylation of osteocalcin, is produced during the fermentation process of CGJ (7) and is assumed to play an important role in bone metabolism.

As the utility of CGJ is being studied worldwide, several studies are being conducted to promote its functions and flavors by changing the bean types or by mixing with natural foods that have specific functions (8,9). The preference of CGJ is not good due to the formation of volatile ammonium-type nitrogen compounds during

Received 3 November 2015; Accepted 14 December 2015; Published online 31 December 2015

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the fermentation process (10).

Rubus coreanus has functional ingredients including minerals, organic acids, vitamin C, and polyphenols (11) that promote antioxidant (12) and antimicrobial activities (13). Previous studies have reported the positive effects of *Rubus coreanus* extracts on the activation and differentiation of MC3T3-E1 osteoblastic cells (14) and on diabetes-related osteoporosis by proliferation of osteoblasts (11). In this study, we evaluated the effects of CGJ and *Rubus coreanus*-Cheonggukjang (RC-CGJ) on BMD and bone mineral content (BMC) of growing rats using antioxidant-rich *Rubus coreanus* and CGJ high in bioavailable isoflavones.

MATERIALS AND METHODS

Preparation of Cheonggukjang

We used soybeans from Sangju, Gyeongbuk, Korea to produce CGJ, and the *Rubus coreanus* powder used in RC-CGJ was prepared by freeze-drying the ingredients obtained from Gochang, Jeonbuk, Korea. Selected beans were washed, submerged in 15°C water for 48 h, steamed at 121°C for 40 min, and inoculated with *Bacillus subtilis* after cooling them down to 40°C. The inoculated beans were fermented for 24 h, freeze-dried, and then grinded for consumption. The beans for RC-CGJ were steamed the same way as for regular CGJ, except for addition of *Rubus coreanus* powder at 5% of the entire sample during the fermentation process. The *Rubus coreanus* powder amount used in this study was referred to the previous study on CGJ production methods using different ingredients (15).

Experimental animals and diets

Eighteen male Sprague-Dawley rats (3 week old) with an average initial body weight of 50 g were obtained from Hyochang Science (Daegu, Korea). After arrival, the rats were housed in stainless steel cages in a room controlled for temperature (25±2°C), humidity (63±5%), light (12-h light/dark cycle) and allowed to acclimate for 1 week fed on a nonpurified pelleted stock diet (Samyangsa, Seoul, Korea). Experimental groups were divided into 3 groups: Control, CGJ, and RC-CGJ group. All animal procedures described followed established guidelines for the care and handling of laboratory animals (16).

Rats had free access to distilled deionized water throughout the experimental period. The basal diet mix (g/kg final diet) contained: casein (Maeil Daires Co., Ltd., Gyeonggi, Korea), 200; α-cellulose nondigestible fiber (Sigma Chemical Co., St. Louis, MO, USA), 50; sucrose (Samyangsa), 100; mineral mix, 35; vitamin mix, 10; L-cystine (Sigma Chemical Co.), 3.0; choline bitartrate (Sigma Chemical Co.), 2.5; soybean oil, 70. The

composition of the mineral and vitamin mixtures for the AIN-93G (Teklad Test Diets, Madison, WI, USA) based diet were as previously reported (17). The compositions of the experimental diets are shown in Table 1. For 9 weeks, the rats were fed on experimental powdered diets with 33.1% CGJ (33.1 g of CGJ and RC-CGJ powder/100 g diet) (18).

Body weight was measured one time a week at the same time, and the food efficiency ratio (FER) was calculated from the body weight gained divided by food intake over the 9 week experimental period (FER=g weight gain/g food intake).

Bone marker measurement

Blood samples were collected from the abdominal aorta, and serums were separated at 3,000 rpm for 20 min. Serums were stored at -70°C until analysis. Serum calcium and phosphate concentrations were determined using a commercially available kit (Daiichi Sankyo, Tokyo, Japan). Urine samples were collected from each rat that was individually housed for 12 h in metabolic cages without food 12 h before sacrificing. Urinary excretions of calcium and phosphorus were analyzed by an automatic biochemical analyzer (Hitachi, Ltd., Tokyo, Japan).

BMD and BMC measurement

BMD and BMC have been regarded as important determinants of osteoporotic fractures. BMD is the ratio of BMC to bone size. BMD and BMC of the spine and femur were measured using PIXImus (GE Lunar Co., Madison, WI, USA) by Dual-energy X-ray absorptiometry after 9 weeks of feeding.

Statistics analysis

All data was processed by common statistical analyzing software. IBM SPSS statistical package (SPSS 19.0, IBM Corp., Armonk, NY, USA) was used for all statistical

Table 1. Composition of control and experimental diets (unit: g/kg diet)

Ingredient	Control	CGJ ¹⁾	RC-CGJ ²⁾
Casein	200	—	—
Corn starch	529.5	478.5	478.5
Sucrose	100	100	100
Soybean oil	70	—	—
α-Cellulose	50	40	40
AIN-93G-mineral-mix	35	35	35
AIN-93-vitamin-mix	10	10	10
L-Cystine	3	3	3
Choline-bitartrate	2.5	2.5	2.5
Tert-butyl hydroquinone	0.014	0.014	0.014
Cheonggukjang	—	331	—
<i>Rubus coreanus</i> -Cheonggukjang	—	—	331

¹⁾CGJ: Cheonggukjang group.

²⁾RC-CGJ: *Rubus coreanus*-Cheonggukjang group.

analyses. Analysis of variance (ANOVA) was performed as a means to determine whether there were significant differences among the groups. When ANOVA indicated statistical significances, the Duncan's multiple range test was used to determine which means were significantly different. Results are expressed as means \pm SD. Values were reported as significant at P -values <0.05 .

RESULTS

Weight gain, food intake, and FER

Table 2 shows the effect of RC-CGJ on weight gain, mean food intake, and FER in growing rats. Body weight gain, mean food intake, and FER of rats fed the experimental diets did not differ from those of rats fed the control diet.

Previous studies on the reducing sugar content of CGJ have reported the highest reducing sugar content among RC-CGJ groups compared to others (RC-CGJ: 1,774.5 mg/100 g, CGJ: 1,656.7 mg/100 g, and steamed soybeans: 138.7 mg/100 g) (19). When *Bacillus subtilis* produces α - or β -amylase, it catalyzes the breakdown of the majority of the fermenting bean starch into sugars and that plays a pivotal role in producing sweetness of CGJ (20). Also, RC-CGJ is reported to have the highest glutamic acid content of 440.7 mg/100 g, which intensifies the delicate and deep flavor. While RC-CGJ exhibits such tastes due to reducing sugars and glutamic acid, the amounts of food intake was not significantly different by intake or types of CGJ. Similar to the previous results, the amounts of weight gain in our study were not significantly different by *Rubus coreanus* extract intake or *Rubus*

Table 2. Effects of Cheonggukjang and *Rubus coreanus*-Cheonggukjang on weight gain and food intake in growing rats

Variables	Control	CGJ ²⁾	RC-CGJ ³⁾
Weight gain (g)	315.6 \pm 44.7	330.0 \pm 56.5	317.8 \pm 43.6
Food intake (g/d)	19.4 \pm 1.2	20.9 \pm 4.3	19.1 \pm 4.1
FER ¹⁾	0.30 \pm 0.02	0.29 \pm 0.01	0.30 \pm 0.04

Mean \pm SD.

¹⁾FER: Food efficiency ratio.

²⁾CGJ: Cheonggukjang group.

³⁾RC-CGJ: *Rubus coreanus*-Cheonggukjang group.

Table 3. Effects of Cheonggukjang and *Rubus coreanus*-Cheonggukjang on serum calcium (Ca) and phosphorus (P) concentrations in growing rats (unit: mg/dL)

Variables	Control	CGJ ¹⁾	RC-CGJ ²⁾
Ca	9.87 \pm 1.49	9.80 \pm 0.68	10.02 \pm 0.35
P	7.05 \pm 0.46	8.10 \pm 0.37	6.56 \pm 0.43

Mean \pm SD.

¹⁾CGJ: Cheonggukjang group.

²⁾RC-CGJ: *Rubus coreanus*-Cheonggukjang group.

coreanus intake levels (100 mg, 200 mg, and 400 mg per kg for 10 weeks) in rats with ovary removal (21).

Serum calcium and phosphorus concentrations

Table 3 shows the effect of RC-CGJ on serum calcium and phosphorus concentrations in growing rats. The concentration of serum calcium was not significantly different among the experimental groups. Mean serum calcium was 9.87 \pm 1.49 mg/dL, 9.80 \pm 0.68 mg/dL, and 10.02 \pm 0.35 mg/dL for the control, CGJ, and RC-CGJ, respectively. Mean serum phosphorus was 7.05 \pm 0.46, 8.10 \pm 0.37, and 6.56 \pm 0.43 mg/dL for the control, CGJ, and RC-CGJ, respectively. The mean serum calcium (7.2 ~ 12.6 mg/dL) and phosphorus (3 ~ 11 mg/dL) concentrations of experimental groups were within the normal range (22).

Urinary calcium and phosphorus excretion

Table 4 shows the effect of RC-CGJ on urinary calcium and phosphorus excretion in growing rats. CGJ and RC-CGJ supplementation did not have a measurable effect on urinary calcium and phosphate excretion.

BMD and BMC

BMD and BMC in spine and femur: Table 5 shows the effect of RC-CGJ on bone mineral density and bone mineral content in growing rats. Mean spine BMD was 0.150 g/cm², 0.154 g/cm², and 0.158 g/cm² for the control, CGJ, and RC-CGJ, respectively. Mean spine BMC was 0.510 g, 0.582 g, and 0.583 g for the control, CGJ, and RC-CGJ, respectively. Mean femur BMD was 0.210 g/cm², 0.213 g/cm², and 0.218 g/cm² for the control, CGJ, and RC-

Table 4. Effects of Cheonggukjang and *Rubus coreanus*-Cheonggukjang on urinary calcium (Ca) and phosphorus (P) excretion in growing rats (unit: mg/d)

Variables	Control	CGJ ¹⁾	RC-CGJ ²⁾
Urinary Ca	0.79 \pm 0.02	0.65 \pm 0.09	0.78 \pm 0.02
Urinary P	13.8 \pm 6.7	12.0 \pm 2.5	11.7 \pm 2.8

Mean \pm SD.

¹⁾CGJ: Cheonggukjang group.

²⁾RC-CGJ: *Rubus coreanus*-Cheonggukjang group.

Table 5. Effects of Cheonggukjang and *Rubus coreanus*-Cheonggukjang on BMD and BMC in growing rats

Variables	Control	CGJ ¹⁾	RC-CGJ ²⁾
Spine BMD (g/cm ²)	0.150 \pm 0.012	0.154 \pm 0.011	0.158 \pm 0.016
Spine BMC (g)	0.510 \pm 0.051 ^b	0.582 \pm 0.286 ^a	0.583 \pm 0.078 ^a
Femur BMD (g/cm ²)	0.210 \pm 0.010	0.213 \pm 0.010	0.218 \pm 0.014
Femur BMC (g)	0.448 \pm 0.034 ^b	0.577 \pm 0.019 ^a	0.451 \pm 0.050 ^b

Values with different letters (a,b) within the row are significantly different at $P < 0.05$ by Duncan's multiple range test (Mean \pm SD).

¹⁾CGJ: Cheonggukjang group.

²⁾RC-CGJ: *Rubus coreanus*-Cheonggukjang group.

CGJ, respectively. Mean femur BMC was 0.448 g, 0.577 g, and 0.451 g for the control, CGJ, and RC-CGJ, respectively. RC-CGJ supplementation did not significantly affect the spine and femur bone mineral density. However, spine BMC was significantly higher in the CGJ and RC-CGJ groups than in the control group. Femur BMC was significantly higher in the CGJ groups than in the control and RC-CGJ group.

BMD and BMC per weight: Bone mineral density and bone mineral content per weight results are shown in Table 6. Spine BMD per weight was 0.365 g/cm²/kg, 0.400 g/cm²/kg, and 0.428 g/cm²/kg for the control, CGJ, and RC-CGJ, respectively. Spine BMC per weight was 1.388 g/kg, 1.406 g/kg, and 1.571 g/kg for the control, CGJ, and RC-CGJ, respectively. The CGJ and RC-CGJ groups showed significantly higher spine BMD per weight than the control group regardless of CGJ type. Spine BMC per weight of the RC-CGJ group was significantly higher than in the control and CGJ group.

Femur BMD per weight was 0.501 g/cm²/kg, 0.561 g/cm²/kg, and 0.588 g/cm²/kg for the control, CGJ, and RC-CGJ, respectively. Femur BMC per weight was 1.198 g/kg, 1.361 g/kg, and 1.214 g/kg for the control, CGJ, and RC-CGJ, respectively. The CGJ and RC-CGJ groups showed significantly higher femur BMD per weight than the control group regardless of CGJ type. Femur BMC per weight of the CGJ group was significantly higher than in the control and RC-CGJ group.

DISCUSSION

The effects of various ingredients within CGJ and *Rubus coreanus* have been previously reported in animal and *in vitro* studies (23,24). While the mixture of CGJ and *Rubus coreanus* may exhibit synergistic effects on bone and, thus, help prevent osteoporosis, no experimental studies on this topic have been conducted to date. Therefore, the aim of this study was to examine the effects of CGJ and RC-CGJ on spine and femur BMD and BMC of growing rats. The intake of CGJ and RC-CGJ was significantly associated with increased spine and femur BMD and BMC of growing rats. While the CGJ group showed a significantly higher spine and fe-

mur BMC than the control, those with RC-CGJ showed a significantly higher BMC for spine only. The group with regular CGJ showed a significantly higher femur BMC than those with RC-CGJ, showing results differed by body parts. Also, when BMD was analyzed in different parts, the spine BMD per weight (spine BMD/wt) and femur BMD per weight (femur BMD/wt) were significantly higher among groups with CGJ or RC-CGJ than the control; therefore, no significant differences were shown by types of CGJ. The spine BMC per weight was significantly higher among the RC-CGJ group compared to the other two groups, but the CGJ group showed a significantly higher femur BMC per weight than the other groups, thereby showing different results by body parts as well.

In this study, CGJ was produced way as described in the previous study consisting of 8.24% carbohydrates, 54.78% crude protein, 24.66% crude fats, 5.70% ash, 2.88% fiber, and 3.74% moisture (18). The amount of isoflavones after fermentation of CGJ was 49.69 mg%. With consideration of the average amount of diet per day (20 g/d), the average amount of isoflavones intake per day during the 9 weeks of feeding is approximately 3.29 mg. Such results indicate the optimal amount of isoflavones for the formation of spine and femur in growing rats.

In a previous experimental study of rats with ovary removal, groups with constant MK-7 high in CGJ (0.063, 0.125, and 0.250 g/d) for 8 weeks had a significantly increase in minerals, decancellate BMD, and bone mass factions than those with one-day MK-7 administration. The controlling effect of MK-7 high in CGJ was dependent on intake amount, and the group with maximum intake (0.250 g/d) had a significantly increase in bone mineral content (31.8%) and BMD (47.6%) (23). Such results indicate the positive effects of MK-7 high in CGJ on bone, meaning a potential for the prevention or improvement of osteoporosis. BMD and BMC showed a prominent increase when relatively high vitamin K was applied directly to CGJ. In fact, there was a proportional relationship between vitamin K amount and BMD. In our study, the spine BMC per weight was 11.7% higher in rats with RC-CGJ than with regular CGJ. However, as femur BMC of the regular CGJ group was significantly

Table 6. Effects of Cheonggukjang and *Rubus coreanus*-Cheonggukjang on BMD and BMC per weight in growing rats

Variables	Control	CGJ ¹⁾	RC-CGJ ²⁾
Spine BMD (g/cm ²)/wt (kg)	0.365±0.028 ^b	0.400±0.018 ^a	0.429±0.027 ^a
Spine BMC [g/wt (kg)]	1.388±0.145 ^b	1.406±0.095 ^b	1.571±0.091 ^a
Femur BMD (g/cm ²)/wt (kg)	0.501±0.039 ^b	0.561±0.035 ^a	0.588±0.050 ^a
Femur BMC [g/wt (kg)]	1.198±0.008 ^b	1.361±0.120 ^a	1.214±0.053 ^b

Values with different letters (a,b) within the row are significantly different at *P*<0.05 by Duncan's multiple range test (Mean±SD).

¹⁾CGJ: Cheonggukjang group.

²⁾RC-CGJ: *Rubus coreanus*-Cheonggukjang group.

higher than that of the RC-CGJ group. Further studies should focus on setting guidelines to determine the amount of *Rubus coreanus* addition during RC-CGJ production. In this study, approximately 5% of *Rubus coreanus* was contained in RC-CGJ, and a total amount of polyphenol content was 20.12 mg/g (data not shown). In other words, about 66.60 mg polyphenols were contained per 100 g of RC-CGJ intake, meaning the amount of addition should be controlled based on the threshold value for improving bone condition. As this study provides evidence that the spine BMC of growing rats increases more significantly with RC-CGJ than with regular CGJ, a proper addition amount of *Rubus coreanus*, which also prevents one from osteoporosis, should be further discussed.

A previous study in Japan has shown a positive association between the habitual intake of natto, fermented soybeans, similar to CGJ, and BMD for hip joint and femoral neck among Japanese men aged 65 years or older. Vitamin K₁ and K₂ of natto were determined to act as positive factors for bone (25). It is also documented that approximately 775 µg of K₂ are contained in 100 g of natto, and this number could rise to 1,298 ~ 1,765 µg if *Bacillus subtilis* is added during the fermentation process (26). As such, formation of vitamin K during the fermentation process is assumed to exert essential effects on the association between BMD and intake of CGJ or natto. Since CGJ has comparably a larger amount of K₂ than natto due to *Bacillus subtilis* in the fermentation process, it may be more effective to consume CGJ rather than natto for promotion of bone formation.

Moreover, while the amount of isoflavones in CGJ (1,045 µg/g) is found to be similar to that of Japanese miso (1,067 µg/g), it still dominates over Doenjang, a popular soybean paste in Korea (781 µg/g), suggesting a beneficial functionality of CGJ over Doenjang (27). It is also assumed that giving consideration to isoflavones, phytoestrogens in CGJ, and the utilization efficiency of inner body altogether may help promote formation of bone during the growth period.

No studies to date have examined the effects of RC-CGJ on bone metabolism. However, previous studies on the mixture of extracts and osteoblasts have reported a high proliferation of osteoblasts, increased ALP activation, and increased osteocalcin secretion when *Rubus coreanus* and milkvetch root extracts were mixed at 7 to 3; such mixture shows its potential as a factor against osteoporosis, and that it helps in the formation of bone rather than the delay in disease progression (28).

Isoflavonoids, the most abundant polyphenols within the *Rubus coreanus* extract, are tannic acid and quercetin, which contain about 2,568.7 mg/100 g. In the previous study, rats were treated with *Rubus coreanus* extracts or streptozotocin or ovariectomized. As a result, the ex-

pression levels of cannabinoid receptors and receptor activator for nuclear factor-κB ligand were increased in rats that were ovariectomized and treated with streptozotocin. In addition, these effects may be partially influenced by alterations in the cannabinoid receptors due to the estrogenic effects of *Rubus coreanus* (11). A similar preventive effect was shown in aged male rats as *Rubus coreanus* extracts controlled the activation of osteoblast by stimulating cannabinoid receptors (24).

As a result of applying different amounts of *Rubus coreanus* (100 mg/kg, 200 mg/kg, and 400 mg/kg) to rats with ovary removal, the ALP levels in blood increased significantly in those which consumed 400 mg/kg of *Rubus coreanus*; pyridinoline concentrations in blood significantly decreased among the group with 200 mg/kg of *Rubus coreanus* intake; and an increase in differentiation of osteoblasts and in apoptosis of osteoclasts in MC3T3-E1 cells were observed from the addition of *Rubus coreanus* extract. A previous animal study has reported that *Rubus coreanus* extract exhibits bone-protecting effects when estrogen deficiency occurs, and it acts positively on bones by inducing the promotion of osteoblast function and apoptosis of osteoclast; a quercetin-rich *Rubus coreanus* then reduces age-related bone damage by antioxidative activity (21). Our study is unique from the majority of other studies as it directly measures the effects of freeze-dried *Rubus coreanus* powder on bone metabolism by examining BMD and BMC; previous studies have used solid *Rubus coreanus* extracts in high concentrations and reported their activation at the cellular level (11,21, 24). Also, unlike Doenjang, which takes up to 12 months until full production (29) and is high in sodium, RC-CGJ could be more applicable to our daily diets as its production takes about 3 days only.

The present study examined the effects of CGJ and RC-CGJ on BMD and BMC in growing rats by adding antioxidant-rich *Rubus coreanus* to the CGJ containing high isoflavone contents. After 9 weeks of experimentation, the intake of CGJ caused a significant increase in spine and femur BMC, while spine BMC was significantly higher among the RC-CGJ group. The spine BMC per weight in RC-CGJ was relatively higher than that of regular CGJ or the control, while the regular CGJ group showed a significantly higher femur BMC per weight than the other two groups. Therefore, the intakes of CGJ and RC-CGJ in growing rats may exhibit a favorable role in the growing skeleton of spine and femur.

ACKNOWLEDGEMENTS

We thank Maeil Daires Co., Ltd. for providing the casein sample.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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