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Effects of Calcium Lactate and *Chungkukjang* on Calcium Status in Rat

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Abstract

Effects of dietary calcium lactate (CaL-A) and *Chungkukjang* (Korean native fermented soybean) on bone mass, calcium status, body weight, serum glucose and cholesterol levels in young male rats were investigated. *Chungkukjang* was fermented by mixing 4 types of *Bacillus* sp., and then dried at 45°C. Calcium lactate was prepared from the ash of black snail. The rats were fed a commercial rat diet for 1 week and then the experimental diets for 4 weeks. Animals were divided into four dietary groups: one calcium-deficient diet (Ca-De) and one of three with calcium supplemented diets (5 g calcium/kg diet) with either calcium phosphate (Ca-P), CaL-A, or CaL-A + *Chungkukjang* (CaL-AC). Calcium supplemented diets contained 39 g Ca-P/kg diet and 28 g/kg of calcium lactate in the CaL-A and CaL-AC diets. Body weight gains during the 4 weeks in the Ca-P, CaL-A, CaL-AC and Ca-De groups were 130.45 g, 112.50 g, 143.40 g and 10.20 g, respectively. Feed consumption of the groups from high to low was CaL-AC > Ca-P > CaL-A > Ca-De. The Ca-De group had low femur weights and low serum calcium concentrations, while they were comparatively high in CaL-AC, Ca-P and CaL-A groups. The Ca-De groups excreted less calcium in urine than did the other rats, probably due to increased absorption of the mineral in Ca-P, CaL-A and CaL-AC groups. Microscopic observations revealed that there were many regularly spaced holes in the femur of Ca-De group, while there were much smaller regularly spaced holes in Ca-P group. However, no holes in femur were observed in the CaL-A and CaL-AC groups. Bone surfaces were especially smooth and clean in the CaL-AC group. Serum concentrations of glucose and total cholesterol were remarkably lower in the CaL-AC group than in the other supplemented groups. These results suggest that calcium from CaL-A has higher bioavailability than from Ca-P, and dietary *Chungkukjang* may have a beneficial effect on calcium metabolism.

Key words: *Chungkukjang*, calcium lactate, black snail, calcium bioavailability

INTRODUCTION

Calcium is the most plentiful mineral in the human body. An adult contains about 1,200 g of calcium, 99% of which forms bones and teeth. The remaining 1% is present in the cell sap and implements a variety of functions including neural transmission, muscle contraction and relaxation, myocardial contraction, cellular metabolism, villus movement, blood coagulation, bacterium engulfment of lymphocyte, excitement, and metabolism of hormone and nutrients (1). Excretion of internal calcium may be incrementally increased by increases in animal protein intake, thereby increasing osteoporosis (2). Therefore, the development of a highly bioavailable calcium supplement is needed for preventing hypertension, diabetes and brain disease as well as osteoporosis (3). Calcium lactate is a nontoxic water soluble food additive used as a calcium supplement (4) in making bread (5), soy milk (6), orange juice (7), and fermented foods such as yogurt (8), fermented soybean paste, soybean sauce and pickles (9). Calcium lactate is also reported to have functions that include anti-microbial activity (10), enhancement of bone mineral density (11), anti-caries (12), and anti-carcinogen (13).

Chungkukjang is a Korean traditional fermented food, a kind of paste. It is prepared as follows: boiled beans are placed into a container with straw on the bottom and fermented at 40°C for two days and then seasoned with garlic, red pepper powder, salt, etc. Compared to other traditional fermented foods such as red pepper paste, soybean sauce and soybean paste, the fermentation period is relatively short. Due to its functional properties, *Chungkukjang* use has been rapidly increasing in recent years. Besides the functionalities of soybean, *Chungkukjang* contains polyglutamate, which is involved in adjusting cell osmosis. The material is sticky like spun

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thread, produced by bacilli microbes (14). In addition, it has substantial nattokinase activity, which is effective in preventing epilepsy by dissolving thrombus (15). Other functional properties of *Chungkukjang* include: anti-cancer effects, the inhibition of cholesterol production, and suppression of the activation of angiotensin converting enzyme which is involved in the development of hypertension (16). It is widely believed to prevent osteoporosis by enhancing calcium metabolism, but that has not been confirmed by scientific studies.

This study investigated the effect of black snail-derived calcium lactate and *Chungkukjang* on calcium status in rats in order to evaluate the utilization of the shell as a high quality value-added calcium resource. Black snail broth has a long history of use as a traditional remedy because the blue-colored substances soaking out in boiling water are known to be good for liver diseases such as hepatitis, cirrhosis, and liver cancer.

MATERIALS AND METHODS

Materials

The black snails (*Semisulcopira bensoni*) had an average length of 35 mm and diameter of 15 mm, and originated in North Korea. A 72% ultrapure lactic acid (Daejung Co. Ltd., Korea) was used for preparing calcium lactate. Korean grown soybeans (*Glycine max* Eunha) were used for making *Chungkukjang*. The initial animal diet was a commercial pellet feed (Jinyang Co., Korea). Ingredients for the experimental diets were: choline bitartrate (ICM Co., Japan), zinc carbonate (Yakuri Co., Japan), sodium selenite (Acros Co., Japan), chromium potassium sulfate (Kanto Co., Japan), calcium phosphate (Daejung Co., Korea), and a vitamin mixture (AIN-76, Teklad, USA) used. All other chemicals were of analytical grade.

Preparation of dehydrated calcium lactate

Calcium lactate was prepared from the ash of ABS according to the method of Lee and Kim (9). One hundred milliliter of 10% lactic acid solution and ABS were added to a 500 mL Erlenmeyer flask with a cooling device attached, and heated at 70°C with constant stirring on a magnetic stirrer/hot plate (Misung, MS-300, Korea), and then neutralized to pH 7.0. The neutralized lactate solution was then filtered through a glass filter to eliminate the residue and was dehydrated at 120°C to obtain the anhydrous calcium lactate.

Preparation of *Chungkukjang*

Carefully selected soybeans were soaked in water for 8 hours, steamed at 121°C for 30 minutes, and then cooled down to 40°C. *Bacillus lichenifomis, Bacillus subtilis, Bacillus circulans* and *Bacillus pumilus*, were isolated from traditional *Chungkukjang*, transplanted to 3% BactoTM Tryptic Soybroth (Becton, Dickinson & Co., USA), and cultivated at 37°C for 24 hours. A mixed strain that combined each culture solution at a ratio of 1:1:1:1 (v/v) was inoculated into the steamed soybeans at a 1% (v/w) concentration, and was cultivated for 40 hours. The beans were then dried at low temperatures, below 55°C, and pulverized to a granularity of 100 mesh and used as an dietary ingredient for animal tests.

Animal and diets

Forty 4 week old male (mean body weight: 90.5 g) Sprague-Dawley (Korean Chemical Institutes Daejun, Korea) rats were obtained and individually housed in stainless steel cages in a temperature (22 ± 2°C), humidity (60 ± 5%) and light controlled room with a 12 hour light-dark cycle. The animals were fed normal chow (Jeiljedang Suwon, Korea) for 1 week, and then, randomly divided into 4 groups: one normal group (Ca-P) and three experimental groups, Ca-deficient group (Ca-De), black snail-derived calcium lactate group (CaL-A), CaL-A+ *Chungkukjang* group (CaL-AC). Calcium content of all of the groups, except Ca-DE group, was adjusted to 5 g/kg. Experimental diets as shown in Table 1, were fed for 4 weeks. All animals had free access to water and feed. Feed intake was measured daily and body weight was measured weekly. Weight gain was expressed as the weight increase during the experimental period. The food efficiency rate was calculated as weight gain/mean food intake.

Feces and urine were collected for 24 hours prior to sacrificing the animals; food was withheld for about 15 hours. Each animal was anesthetized by injection with Ketamin-HCl. Blood was collected from the inferior vena cava, and plasma prepared by centrifuging at 4°C, 3000 rpm for 20 min was used for analysis. Liver, kidney and femur were removed, rinsed with phosphate buffered saline solution, wiped with a paper towel, and weighed.

Measurements and microscopic observation

Serum glucose and total cholesterol concentrations were determined using Gluco-Tester (Life Scan, USA) and an enzymatic assay kit (Nissui Pharm. Co. Ltd., Japan), respectively. Calcium concentrations in blood and urine were determined using the Cobas Integra 400/700/800 System in which the calcium ions react with *o*-cresolphthalein complex under alkaline conditions to form a violet colored complex. The addition of 8-hydroxy-quinoline prevents interference by magnesium and iron. The color intensity of the complex formed is directly proportional to the calcium concentration, which

Table 1. Composition of experimental diets (g/kg)

Ingredient	Ca-De[1)]	Ca-P (Normal)[2)]	CaL-A[3)]	CaL-AC[4)]
Corn starch	712	684	684	614
Casein	150	150	150	121
Corn oil	100	100	100	99
Mineral mixture[5)]	12	12	12	12
Vitamin mixture[6)]	10	10	10	10
Choline	2	2	2	2
DL-Methionine	3	3	3	3
Neutralized phosphoric acid[7)]	11	-	11	11
Calcium phosphate	-	39(5)[10)]	-	-
Calcium lactate (CaL-A)[8)]	-	-	28(5)[10)]	28(5)[10)]
Chungkukjang[9)]	-	-	-	100
Total	1000	1000	1000	1000

[1-4)]Abbreviations: Ca-De, calcium deficiency group; Ca-P, calcium phosphate group; CaL-A, calcium lactate group; CaL-AC, calcium lactate with Chungkukjang group.
[5)]Mineral mixture: sodium chloride 2560 mg, potassium phosphate (dibasic) 7200 mg, potassium sulfate 1200 mg, magnessium oxide 560 mg, manganous carbonate 140 mg, ferric citrate 240 mg, zinc carbonate 64 mg, cupric carbonate 12 mg, potassium iodate 2 mg, sodium selenite 2 mg, chromium potassium sulfate 20 mg.
[6)]Vitamin mixture was prepared according to AIN-76 (Teklad, USA).
[7)]Phosphoric acid was neutralized with NaOH.
[8)]Calcium lactate (CaL-A) was prepared from ash of black snail.
[9)]*Chungkukjang* was fermented for 48 hrs at 40°C by culturing with 4 *Bacillus* sp. separated from Korean native *Chungkukjang* and dried below 55°C. The products contained 70% carbohydrate, 29% protein and 1% lipid.
[10)]Parenthesis denotes the calcium content (g).

is determined by measuring the increase in absorbance at 552 nm. The calcium contents of feces, fumur, liver and kidney were determined by using ICP-AES (JY 38 Plus, France) after ashing at 600°C and solubilizing in 6 N HCl. Microscopic observation of femur surfaces (× 50 and ×300) was performed with an Ion Sputtering Device (E-1030, Hitachi, Japan) and a Scanning Election Microscope (JSM-5410, Jeol, Japan).

RESULTS AND DISCUSSION

Feed intake, body weight and excretion weight of feces

Feed intake, feed efficiency and the weight of waste of the dietary groups are shown in Table 2 and Fig. 1. Feed intake for four weeks was 125.70 g in the Ca-De group, and 177.55 ~ 178.10 g in the CaL-A and the CaL-AC groups, which was higher than that of the Ca-De group. Weight gain for the four weeks was as little as 10.20 g in the Ca-DE group, while it was 143.40 g in the CaL-AC group, which was heavier than 134.45 g and 112.50 g in the Ca-P and CaL-A groups respectively, but there were no significant differences among the calcium supplemented groups. The weight of waste per day was heaviest (1.10 g) in the CaL-AC group and ranged

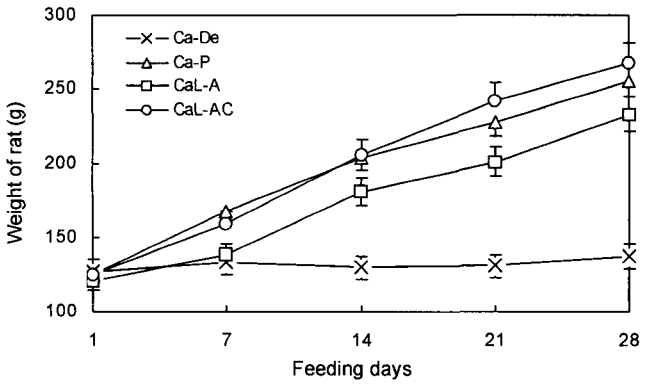

Fig. 1. Changes in body weights of rats fed black snail calcium lactate with and without *Chungkukjang*. Abbreviations: See Table 1. Values are mean ± SD of 10 rats.

Table 2. Feed intake, weight change and feed efficiency

Groups[1)]	Feed intake (g/week)	Weight gain (g/week)	Feed efficiency	Weight of feces (g/day)
Ca-De	125.70 ± 1.76[b2)]	10.20 ± 5.38[d]	0.08 ± 0.01[d]	0.62 ± 0.02[c]
Ca-P	177.55 ± 9.99[a]	130.45 ± 6.43[b]	0.73 ± 0.01[b]	1.04 ± 0.06[b]
CaL-A	177.55 ± 9.70[a]	112.50 ± 5.41[c]	0.63 ± 0.01[c]	1.05 ± 0.08[b]
CaL-AC	178.10 ± 9.32[a]	143.40 ± 6.46[a]	0.80 ± 0.01[a]	1.10 ± 0.09[a]

[1)]Abbreviations: See Table 1.
[2)]Values are mean ± standard deviations (SD) of 10 rats, different superscripts within a column indicate significant differences at $p < 0.05$.

from 1.044~1.048 g in the other calcium supplemented groups, but was only 0.615 g/day in the Ca-De group. Chung et al. (17) added calcium phosphate (dibasic), calcium lactate, calcium gluconate or calcium carbonate as the sources of calcium in feed and performed a dietary test on the four groups. They found no significant differences in dietary intake, but the calcium gluconate group gained less weight than the other three groups, and feed efficiencies were higher in the calcium lactate and phosphate groups than in calcium carbonate and calcium gluconate groups. Lee and O (18) fed calcium-deficient diets to osteoporosis model white mice for three weeks, resulting in a decrease in body weight. The test group fed with sufficient calcium showed significant increases in dietary intake and weight, and the test group fed with the mixture of soybean protein and calcium phosphate showed the highest dietary intake but significantly lower increases in weight, which means low efficiency of the feed.

Weight of liver, kidney and femur

The mean weights of liver, kidney and femur for the dietary groups are shown in Fig. 2~4. Liver weights were 9.21 g in CaL-AC group, 8.47 g in the CaL-A group, 8.26 g in the Ca-P group and 4.84 g in the Ca-De, but only the Ca-De group differed significantly from other groups. Kidney weights were the heaviest at 1.90 g in the CaL-AC group, and were 1.77 g and 1.73 g in the CaL-A and Ca-P groups respectively, which were significantly heavier than in the Ca-De group. The femur weight was 1.10 g in the Ca-De group, 1.60 g in the Ca-P group, 1.70 g in the CaL-A group and 1.89 g in the CaL-AC group, so the group fed the mixture of CaL-A and *Chungkukjang* had the heaviest femur. The average weight of femur of the group fed with CaL-AC was heavier than that of the groups fed Ca-P or CaL-A, but there were no significant differences ($p<0.05$) between the Ca-P and CaL-A groups.

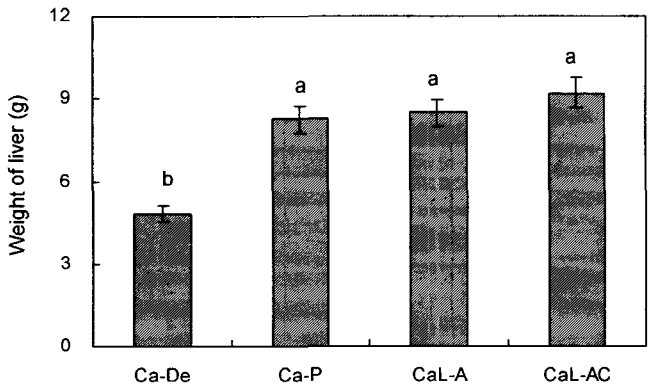

Fig. 2. Weight of liver of rats fed black snail calcium lactate with and without *Chungkukjang*. Abbreviations: See Table 1. Values are mean±SD of 10 rats, different letters indicate significant differences at $p<0.05$.

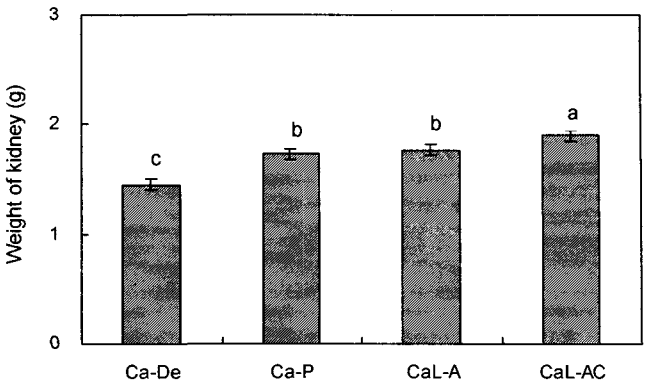

Fig. 3. Weight of kidney of rats fed black snail calcium lactate with and without *Chungkukjang*. Abbreviations: See Table 1. Values are mean±SD of 10 rats, different letter indicate significant differences at $p<0.05$.

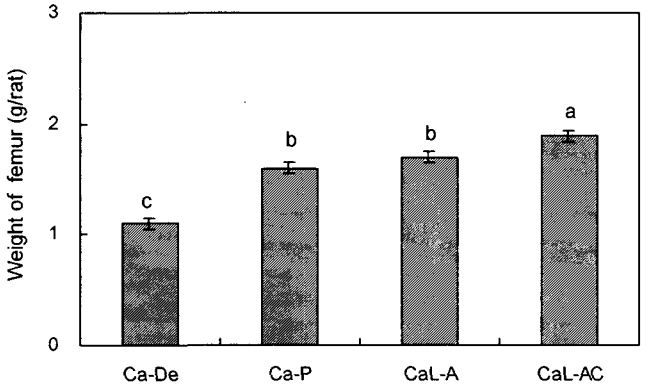

Fig. 4. Weight of femur of rats fed black snail calcium lactate with and without *Chungkukjang*. Abbreviations: See Table 1. Values are mean±SD of 10 rats, different letters indicate significant differences at $p<0.05$.

In rats weighing approximately 100 g, liver weights were between 3.24~3.63 g, varying insignificantly. The weight of kidney was 1.05 g in the Ca-De group, but as light as 0.68~0.76 g in the Ca-P, CaL-A and CaL-AC groups, and did not vary significantly.

Chung et al. (17) reported that liver weights were heavier in rats fed calcium lactate than calcium phosphate, but not significantly. Contrary to the results of Chung et al. (17), Greger et al. (19) reported that there was no difference in the weight of liver but kidney was heavier in the calcium phosphate group. On the other hand, Kim et al. (20) investigated changes in femur weights when osteoporosis occurred as a result of ovary surgery. According to their results, the femurs in the surgery group were light, but rather heavy in relation to the body weight, which is similar to the result of the present experiment and implies that change in the weight of organs resulting from the occurrence of osteoporosis is not very significant.

Calcium levels of femur, blood, liver, kidney, urine and feces

Calcium concentrations of femur, organs, blood and waste are shown in Table 3. The calcium content of the femur was the highest with 28.29% in the CaL-AC group, and was 26.87% in the CaL-A group, 27.06% in the Ca-P group, and significantly lower at 16.51% in the Ca-De group. The calcium content of the blood was also the highest, 10.20 mg%, in the CaL-AC group, 9.09 ~9.15 mg% in the CaL-A and Ca-P groups, and was significantly lower at 7.61 mg% in the CaL-AC group.

The calcium content of the liver was 128.40 mg% in the CaL-A group, higher than 112.50 mg% in the CaL-AC group, and 85 mg% in the Ca-De groups, which was higher than 49.90 mg% in the Ca-P group. The calcium content of the kidney was the highest 99.60 mg% in the CaL-AC group and 17.60~32.40 mg% in the other groups. The calcium content of urine was significantly lower at 2.00 mg% in the Ca-De group than the 6.07~6.78 mg% of the other groups. The calcium content of feces was 6.96~7.31 mg% in the calcium treated groups CaL-AC, and CaL-A groups, but 1.80 mg% and 0.83 mg% in the Ca-P and Ca-De groups, respectively. The restriction of calcium intake reduced the calcium levels of femur, blood, liver, kidney and waste. On the other hand, supplemental calcium increased the calcium content significantly although the increases varied according to the source of calcium. The diet combining *Chungkukjang* and calcium accelerated the increase in calcium content. These results are consistent with a report that the level of calcium intake has a great influence on calcium metabolism in blood and organs including bones. In their research on the use of calcium in the body of osteoporosis model white mice (21), O and Lee (22) reported that the calcium levels of femur, serum, liver and kidney were significantly reduced by a low calcium diet, but no significant difference was found when cow bone and calcium phosphate were fed, although organic calcium is more effective than inorganic. According to their reports, however, not only calcium content but also the stiffness of bone increased significantly when calcium and soybean protein were fed together. Jeong et al. (23) said that calcium homeostasis of blood was maintained despite internal and external change in osteoporosis, and was within a normal range despite changes in experimental factors.

In this experiment, however, the calcium content of the blood in the Ca-De group was low, and calcium content varied significantly according to the source of calcium. Such results show that, compared to calcium phosphate, black snail-derived calcium lactate is a highly bioavailable calcium that is well absorbed by the body. In addition, it appeared that the diet of the mixture of black snail-derived calcium lactate and *Chungkukjang* further accelerates calcium metabolism.

Microscopic observation of femur

SEM micrographs of femur surfaces, used to visualize the effect of black snail-derived calcium lactate and *Chungkukjang* on bone structure, are shown in Fig. 5. Regular holes observed on the femur tissue of the Ca-De group show that osteoporosis occurred due to the calcium-deficient diet. Though not as bad as that of the Ca-De group, the femur tissue of the Ca-P group also had a large number of small holes that were distributed regularly. No holes were observed in the CaL-A group, even though the amount of elemental calcium was the same as in the Ca-P group (Table 3). In the CaL-A group, however, white spots that regarded as the deposition of calcium on the surface of femur appeared. These spots were not observed in the CaL-AC group, which implied that *Chungkukjang* had a beneficial influence on calcium metabolism. Osteoporosis is a metabolic disease, resulting from a quantitative decrease in the elements of the marrow, namely, from an imbalance of marrow absorption (24). Lack of calcium, vitamin D and estrogen prompts such a phenomenon, and a decrease in bone density reflects a decreased calcium and hydroxyproline deposition in the bone (25). However, such problems are corrected by normal diets containing calcium. Kim et

Table 3. Calcium concentrations in femur, blood, liver, kidney, urine and feces of rat fed black snail calcium lactate with or without *Chungkukjang*

	Ca-De[1)]	Ca-P[2)]	CaL-A[3)]	CaL-AC[4)]
Femur (%)	16.51±0.24[c5)]	27.06±0.39[ab]	26.87±0.31[b]	28.29±0.36[a]
Blood (mg%)	7.61±0.16[c]	9.09±0.27[b]	9.15±0.22[b]	10.20±0.31[a]
Liver (mg%)	85.10±1.48[c]	49.90±0.18[d]	128.40±4.20[a]	112.50±1.67[b]
Kidney (mg%)	29.00±0.65[c]	32.40±0.61[b]	17.60±0.64[d]	99.60±1.80[a]
Urine (mg%)	2.00±0.13[c]	6.07±0.20[b]	6.69±0.32[a]	6.78±0.28[a]
Feces (%, dry basis)	0.83±0.01[c]	6.80±0.03[b]	6.96±0.13[ab]	7.31±0.09[a]

[1-4)]Abbreviations: See Table 1.

[5)]Values are mean±Sd of 10 rats, different letters within a row indicate significant differences at $p<0.05$.

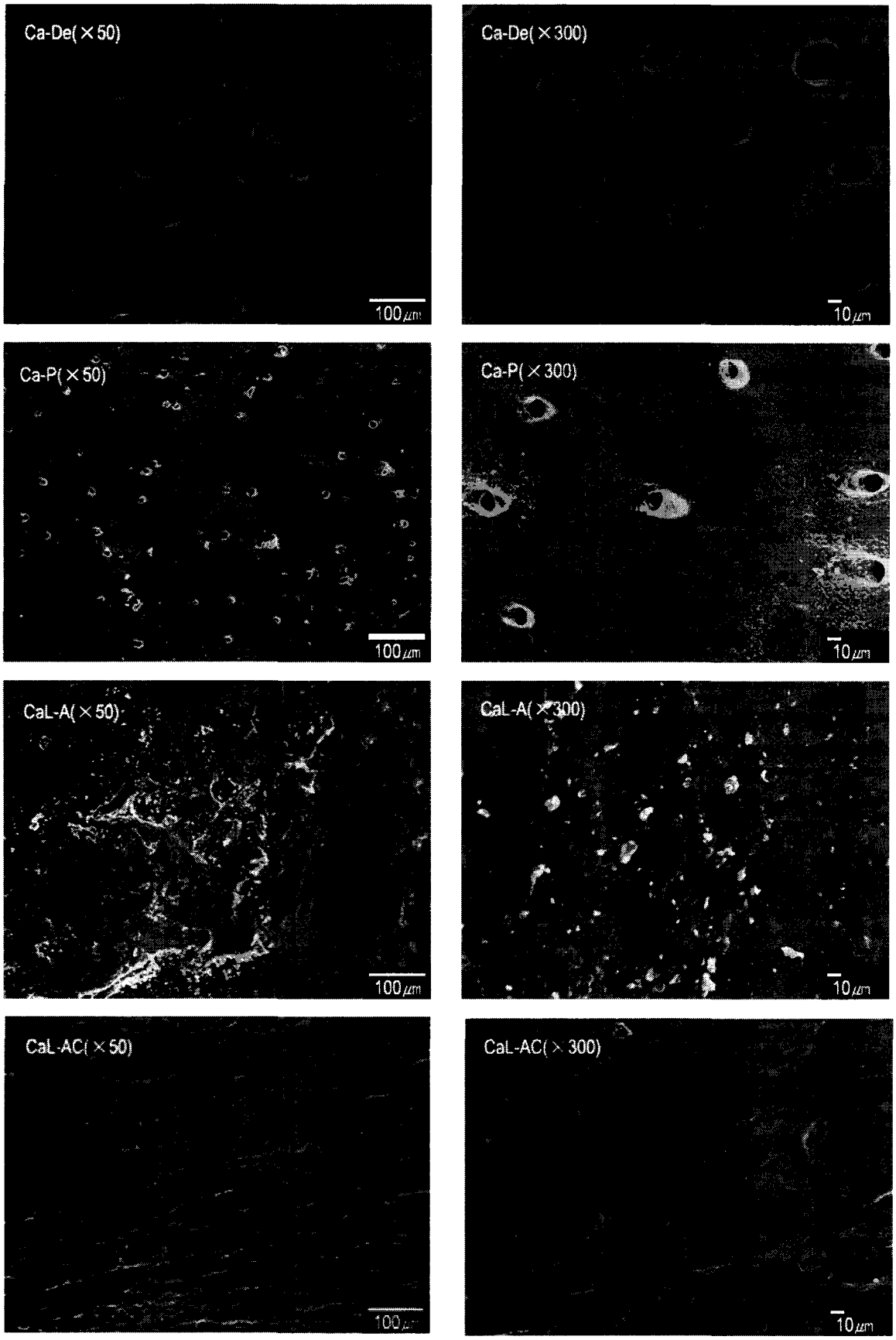

Fig. 5. Scanning electron microphotographs of bone surface of femur of rats fed black snail calcium lactate with and without *Chungkukjang* (×50 and ×300). Abbreviations: See Table 1.

al. (26) reported that a diet including oyster shell calcium bonded with gelatin peptide from fish skin raised the calcium content of bone. On the other hand, Lee and Lee (27) suggested that the excessive intake of calcium caused the calcification of bone. Considering that the deposition of calcium on the surface of bone in the CaL-A group in this experiment occurred even though that group received the same quantity of calcium as the other calcium supplemented groups, we can hypothesize that an increased absorption of calcium caused excessive calcification. Furthermore, the absence of either osteoporosis or calcium surface deposition in the group fed with the mixture of CaL-A and *Chungkukjang* shows that *Chungkukjang* plays a role in regulating calcium metabolism. Furthermore, the beneficial effect of *Chungkukjang* is supported by a report by O and Lee (22) that feeding the combination of calcium and soybean protein significantly increased both calcium content in bone and bone strength.

Content of blood glucose and cholesterol

The effects of calcium mixed with *Chungkukjang* on serum glucose and cholesterol concentrations are shown in Fig. 6 and 7. Serum glucose was the highest, at 156.00 mg/dL, in the Ca-De group, was 115.75 and 114.55 mg/dL in the Ca-P and CaL-A groups, respectively, and was the lowest, at 92.05 mg/dL, in the Ca-AC group. Serum cholesterol was 119.91 mg/dL in the Ca-De group, 111.82 mg/dL in the Ca-P group, 107.40 mg/dL in the CaL-A group and 93.52 mg/dL in the CaL-AC, so it was highest in the Ca-De group, and lowest in the group fed the mixture of black snail-derived calcium lactate and *Chungkukjang*. The result suggests that calcium and *Chungkukjang* influence the metabolism of glucose and cholesterol. In general, serum glucose homeostasis is maintained through the actions of insulin and glucagon. However, lack of calcium and decreased bone mass bring

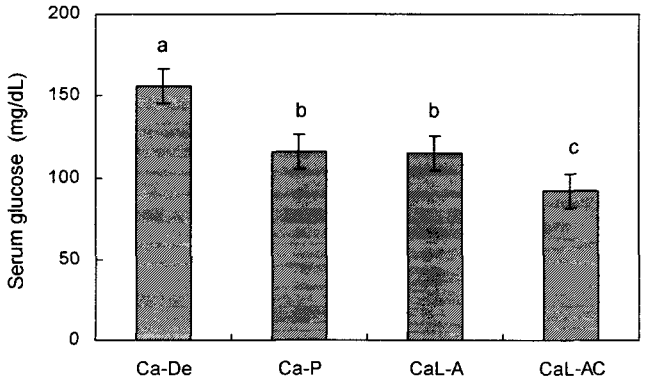

Fig. 6. Serum glucose concentrations in rats fed black snail calcium lactate with and without *Chungkukjang*. Abbreviations: See Table 1. Values are mean±SD of 10 rats, different letters indicate significant differences at $p<0.05$.

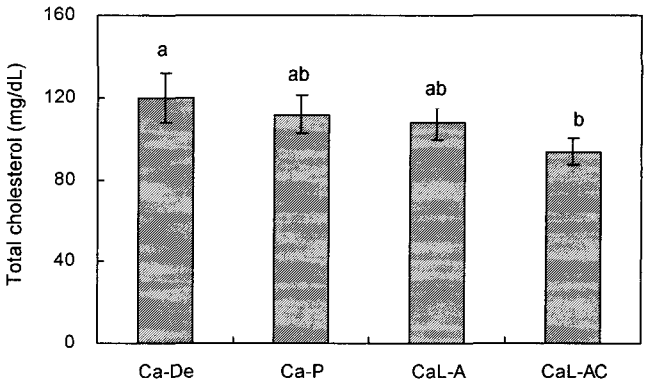

Fig. 7. Total serum cholesterol of rats fed black snail calcium lactate with or without *Chungkukjang*. Abbreviations: See Table 1. Values are mean±SD of 10 rats, different letters indicate significant differences at $p<0.05$.

about an increase in blood sugar. Many studies on type I and type II diabetics have reported a correlation between osteoporosis and high blood glucose (28). Nagasaka et al. (29) reported that high blood glucose in non-insulin-dependent diabetes mellitus caused the excessive discharge of calcium through urine, which in turn hinders the development of bone. This was consistent with the results of this experiment. However, Thalassinos et al. (30) reported that decreases in bone mass lowered the level of blood glucose in diabetics, which is different from the results of this study. There are insufficient data on the direct effect of calcium on lipid metabolism. Usually, diabetics have both high blood glucose and hyperlipidemia as a result of the hypofunction of insulin, which also raises the concentration of cholesterol in the blood. The concentrations of both glucose and cholesterol in blood were lower in the CaL-AC groups than in the CaL-P and CaL-A groups, which may suggest that *Chungkukjang* affects the action of insulin and calcium metabolism directly and indirectly.

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