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Abstract

Within the elderly population, the use of calcium supplements and the intake of calcium from food are on the rise in order to maintain health. Calcium is absorbed as an ion *in vivo*, leading to speculation that absorption efficiency is affected by the solubility of the calcium consumed. In our study, the bioavailability of two types of calcium supplements with different solubilities was evaluated. Experimental animals were fed water-soluble or insoluble calcium supplements for 6 weeks. We found that blood alkali phosphatase activity, osteocalcin content, and urine crosslinks values were not different between the groups. Similarly, the degree of apparent calcium absorption between the two calcium supplements was not significantly different. The bone mineral density and bone mineral content of the femur and the tibia increased in the group that consumed insoluble calcium compared with those of the water-soluble calcium supplemented group. However, when considering body weight, the bone mineral density value for all areas, including the spine, was significantly higher in the group that consumed the water-soluble calcium supplement.

Key words: calcium supplement, water-soluble calcium, calcium absorption, bone mineral density, calcium carbonate

INTRODUCTION

With the prolongation of the average life-span, as society ages the intake of sufficient calcium is often emphasized. Calcium is the most abundantly distributed mineral in the human body, present primarily in the bones and teeth, but also in body fluids for performing functions of physiological signaling and control (1). Long-term calcium deficiency can lead to osteoporosis, and may also have mediating effects on the development of hypertension, colorectal cancer, etc. (2,3). As a result, there is great interest in calcium supplement use, as well as the intake of calcium in foods.

Calcium supplements are classified as natural supplements derived from shells, eggshells, etc., or as synthetic supplements such as calcium carbonate, calcium citrate, etc. The use of inexpensive calcium supplements is common, and the representative calcium supplement in this category, calcium carbonate, has a very low solubility. Previously, interest in calcium was focused on increasing the level of calcium intake, and most studies looked at physiological changes based on a level of calcium intake (4-6). It has now been recognized that the bioavailability of calcium is low compared to other minerals. Recently,

it was reported that calcium citrate supplements have a high solubility and can have excellent effects (7-9). Therefore, there is speculation that calcium's efficacy may be influenced by its solubility and the degradation of the supplement in the body. In other words, calcium absorption may depend on its dissociation to the ion form, and as a supplement's solubility becomes higher, its ionization may be accelerated allowing calcium to be absorbed faster. On the other hand, some researchers have reported that the absorption rate of calcium is not associated with the supplement's properties (10,11). Therefore, studies on the bioavailability of calcium supplements must focus on a variety of aspects.

In this study, we evaluated the physiological efficiency of calcium supplements based on their solubility. Here, experimental animals were supplemented with a water-soluble calcium supplement or insoluble calcium supplement, and their biochemical markers and changes in bone mineral density examined.

MATERIALS AND METHODS

Experimental animals and diet
In this study, growing female rats, for which the ad-

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ministration of liquid calcium supplement was feasible and calcium metabolism was active, were selected as experimental animals. Thirty female Sprague-Dawley rats weighing an average of 90 g were purchased (Danhan Biolink Co.), and after adaptation of one week on rat chow (Sam Yang Co.), were randomly divided to 3 groups of 10 according to the body weight, and fed experimental diets for 6 weeks. During the experiment period, the animal room was maintained 22 ± 2^oC and $55 \pm 5\%$ relative humidity with a 12 hr light/dark cycle. Experimental diets and deionized water were offered ad libitum. A calcium free AIN-93G (Ca free) diet was prepared (Table 1). Experiment groups were divided to the control group supplied only calcium free diet (F-Ca), the group fed water-soluble calcium supplement in addition to the calcium free diet (S-Ca), and the group fed insoluble calcium supplement in addition to the calcium free diet (I-Ca).

Administration of calcium supplements

Water-soluble calcium supplement (70 mg/mL, over 90% ionizing ratio) was provided by Keimyung Foodex Co., and insoluble calcium supplement was supplied as the suspension of calcium carbonate in deionized water (70 mg/mL). Each calcium supplements was administered once a day at a constant time, 10 mL/kg body weight, and the control group (F-Ca) was exposed to the identical stress by administering deionized water orally through a gavage needle.

Dietary consumption rate and weight measurement

Dietary consumption rate was measured once a day, and body weight was measured two times a week at the same times, and the diet efficiency was calculated by dividing the weight gain during the experimental period by the diet consumption amount.

Sample collection

After 6 weeks of feeding, rats were fasted overnight and the urine and feces were collected, anesthetized with ether and killed. The blood collected from the aorta was centrifuged at 3000 rpm for 20 min, the serum was separated, and stored frozen at -70^oC. In addition, the amount of calcium intake during the last 3 days of feeding and the amount of calcium excreted in the feces were measured, and the apparent calcium absorption rate was calculated.

Biochemical analysis

Serum calcium content was measured by an automatic absorption analyzer applying the TECHNICON CHEM^{TM} system, and alkaline phosphatase (ALP) activity was analyzed using a kit based on the Kind and King method (12). Serum osteocalcin content was analyzed by an osteocalcin radioimmuno-assay kit (Brahams Co.). Deoxypyridinoline and creatinine content were analyzed using a collagen crosslinks kit (Metra biosystems Inc. USA) by ELISA. The crosslinks value was the value obtained by dividing deoxypyridinoline content by creatinine content. Feces were ashed and the calcium content was analyzed by atomic absorption spectrophotometer (Hitachi Inc., Japan).

Bone mineral density and bone mineral content measurement

At 6 weeks of feeding, the anesthetic pentobabobital sod. (Han Lim Pharmaceuticals) was injected intraperitoneally, at 1 mL/kg dose, and using the dual energy x-ray absorptionmetry (DEXA) FIXImus (LUNAR, Madison, WI, USA), the bone mineral density (BMD) and bone mineral content (BMC) of the spine, the femur, and the tibia were measured.

Statistical analysis

Experiment results were presented as a mean and standard deviation using the SPSS program (12.0), the comparison among experiment groups was performed by one way ANOVA, and their significance was validated by Duncan's multiple range test ($p < 0.05$), and the comparison of the absorption rate of calcium supplements and calcium efficiency was analyzed by independent sample t-test.

RESULTS AND DISCUSSION

Diet and calcium intake, weight gain, and food efficiency ratio

Dietary intake, calcium intake, and the amount of weight gain according to the types of calcium supplements are shown in Table 2. For the insoluble calcium supplemented group, weight gain and average dietary intakes were significantly higher. Similarly, the calcium consumption rate showed a trend towards being higher,

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

Ingredient	Calcium free diet
Casein	200
Corn starch	592.486
Sucrose	100
Soybean oil	70
Cellulose	50
t-Butylhydroquinone	0.014
Min-mix[1]	35
Vit-mix[2]	10
L-Cystine	3
Choline bitartrate	2.50

[1] AIN-93G mineral mixture (Ca free).
[2] AIN-93VX vitamin mixture.

Table 2. Diet and calcium intake, weight gain and food efficiency ratio of rats fed different calcium supplements

Group[1]	Dietary intake (g/day)	Calcium intake (g/day)	Weight gain (g/day)	Food efficiency ratio
F-Ca	16.10 ± 0.77^{b}	–	$4.19 \pm 0.27^{2)b3)}$	$0.26 \pm 0.01^{NS4)}$
S-Ca	15.87 ± 0.23^{b}	0.165 ± 0.013^{NS}	4.06 ± 0.55^{b}	0.26 ± 0.04
I-Ca	17.60 ± 0.74^{a}	0.175 ± 0.009	4.67 ± 0.26^{a}	0.27 ± 0.01

[1]F-Ca: rats fed only calcium free diet, S-Ca: rats fed Ca free diet and soluble calcium supplement, I-Ca: rats fed Ca free diet and insoluble calcium supplement. [2]Mean $\pm$ SD. [3]Values with different superscripts within the column are significantly different at $p < 0.05$. [4]Not significantly different.

but a significant difference was not detected. The results for the control group (calcium free diet) were similar to those of a study by Lee and Oh (13) that restricted calcium intake, and where the diet consumption rate and weight gain both decreased. In the water-soluble calcium supplemented group, we speculate that the calcium ionized easily due to its high solubility, causing it to taste bitter and reducing the diet consumption rate.

Serum and urine composition

The results for the serum calcium content and bone formation markers, alkaline phosphatase and osteocalcin, are shown in Table 3. Serum calcium in the insoluble calcium supplemented group was 9.95 mg/dL and significantly higher than in the control group. The water-soluble calcium supplemented group was not significantly different from the other groups. The serum alkaline phosphatase in the control group was 183.90 IU/L, which was considered high. There was no difference detected between the two calcium supplemented groups for the alkaline phosphatase levels. The level of osteocalcin among the groups was not significantly different. Changes in the serum calcium showed a similar trend to results reported by Lee and Oh (13) where serum calcium was significantly low in the low calcium consumption group. However, our results were different from those of others who reported that serum calcium

concentrations were maintained at a constant level regardless of the calcium content of the diet (14,15). In our study, the calcium free diet was considered the basic diet, and hence, different from a report on the homeostasis of serum calcium concentrations (16). In addition, the weight gain of the insoluble calcium supplemented group was high. Hence, it was considered that, although the difference was not significant, calcium supplements that were administered according to body weight mediated effects on serum calcium.

Serum alkaline phosphatase and osteocalcin are markers of bone formation, and their serum concentrations have been shown to increase when osteoblast activity is increased (17). Here, the alkaline phosphatase level in the control group was high, which was attributed to a calcium free diet where the skeleton could not grow normally causing an increased *in vivo* demand for bone formation and enhanced osteoblast activity. This is similar to results reported by Hamalainen (18) where, during calcium deficiency, alkaline phosphatase activity was increased.

Table 4 reveals that there was no significant difference in the urine deoxypyridinoline levels among the experimental groups. The crosslinks value was significantly higher in the control group than those in the calcium supplemented groups, but no difference was observed between the water-soluble and insoluble calcium

Table 3. Serum calcium, alkaline phosphatase (ALP) and osteocalcin of rats fed different calcium supplements

Group[1]	Serum calcium (mg/dL)	ALP (IU/L)	Osteocalcin (ng/mL)
F-Ca	$9.23 \pm 0.33^{2)b3)}$	183.90 ± 41.19^{a}	$1.80 \pm 0.46^{NS4)}$
S-Ca	9.70 ± 0.60^{ab}	120.00 ± 30.97^{b}	1.66 ± 0.21
I-Ca	9.95 ± 0.48^{a}	106.10 ± 19.57^{b}	1.40 ± 0.25

[1]Refer to Table 1. [2]Mean $\pm$ SD. [3]Values with different superscripts within the column are significantly different at $p < 0.05$ by Duncan's multiple range test. [4]Not significantly different.

Table 4. Urine deoxypyridinoline, creatinine and crosslinks value of rats fed different calcium supplements

Group[1]	Deoxypyridinoline (nM)	Creatinine (mM)	Crosslinks value[4] (nM/mM)
F-Ca	$1608.00 \pm 293.43^{2)NS}$	$6.32 \pm 2.76^{NS3)}$	$258.30 \pm 32.08^{a5)}$
S-Ca	1266.80 ± 238.91	8.17 ± 1.29	154.54 ± 47.07^{b}
I-Ca	1301.25 ± 135.91	7.70 ± 2.22	168.95 ± 16.23^{b}

[1]Refer to Table 1. [2]Mean $\pm$ SD. [3]Not significantly different. [4]Crosslinks value = Deoxypyridinoline/Creatinine. [5]Values with different superscripts within the column are significantly different at $p < 0.05$ by Duncan's multiple range test.

Table 5. Calcium intake, fecal calcium excretion, and calcium absorption in rats fed different calcium supplements

Group[1]	Calcium intake (mg/day)	Fecal calcium excretion (mg/day)	Apparent calcium absorption (%)
S-Ca	229.46±17.29[2]NS	15.65±3.20[NS3]	93.17±1.89[NS]
I-Ca	239.66±10.25	20.80±5.93	91.30±1.76

[1]Refer to Table 1. [2]Mean±SD. [3]Not significantly different.

supplemented groups. Deoxypyridinoline is a cross-linked collagen present in the skeleton, and excreted in the urine with collagen byproducts as a result of osteoclast action (19,20). We speculate that in the control group, where calcium intake was restricted, the bone resorption rate was increased and the crosslinks value comparable to rats whose bone resorption rate was increased to 202.6~280.4 nM/mM due to oophorectomy (19). Considering the results for the blood bone formation markers and urine bone resorption markers, no significant difference was detected between the supplemented groups based on the calcium solubility.

The apparent calcium absorption rate

The calcium absorption rates were calculated from the dietary calcium intake rates and calcium excretion rates in feces for 3 days, prior to completion of the experiment (Table 5). A trend toward high calcium intake and excretion was observed in the insoluble calcium group, and a higher absorption rate observed for the water-soluble group, but a significant difference was not detected. These results were similar to those reported by Benson et al. (21) where an increased calcium intake resulted in a decreased endogenous calcium reabsoprtion rate.

Bone mineral density (BMD) and bone mineral content (BMC)

The BMD of the spine at 6 weeks after consuming the experimental diets was significantly higher in the calcium supplement groups, but a difference based on the solubility of the calcium supplements was not detected (Table 6). The spinal BMDs were as follows: 0.043

g/cm^2 in the control group, 0.125 g/cm^2 in the water-soluble calcium supplemented group, and 0.129 g/cm^2 in the insoluble calcium supplemented group. Similarly, the BMC in the spine of the control group was significantly low. In the case of the femur and the tibia, both the BMD and BMC were significantly higher in the insoluble calcium supplemented group, making it appear that insoluble calcium supplements could be beneficial to bone health. Since the weight gain of the insoluble calcium supplemented group was high, it was speculated that calcium supplements that were administered according to weight mediated effects on BMD and BMC. In addition weight is considered to be an important factor for BMD and BMC (22). Thus to examine actual bone health, each value was converted to a value reflecting the body weight. Per 100 g of body weight the BMD was highest in all areas, including the femur, for the water-soluble calcium supplemented group, though the BMC per 100 g of body weight was not different between calcium supplemented groups. In other words, water-soluble calcium supplements were considered to be more effective than the insoluble calcium supplements. However, the calcium efficiency for each bone area between the two calcium supplements was not significantly different (Table 7). We speculate this may be due to the relatively short period of calcium supplementation of only 6 weeks. Thus the efficiency of the two calcium supplements was comparable within the restrictions of the experimental conditions.

In conclusion, the effects of the two calcium supplements with different solubility on the markers of blood

Table 6. Bone mineral density (BMD) and bone mineral content (BMC) of rats fed different calcium supplements

Bone	Group[1]	BMD (g/cm^2)	BMD (g/cm^2)/100 g wt	BMC (g)	BMC (g)/100 g wt
Spine	F-Ca	0.043±0.006[b3]	0.012±0.001[c]	0.133±0.026[2]b	0.025±0.003[b]
	S-Ca	0.125±0.019[a]	0.042±0.005[a]	0.504±0.086[a]	0.114±0.013[a]
	I-Ca	0.129±0.015[a]	0.036±0.004[b]	0.545±0.098[a]	0.110±0.011[a]
Femur	F-Ca	0.096±0.007[c]	0.027±0.002[c]	0.175±0.014[c]	0.051±0.002[b]
	S-Ca	0.170±0.016[b]	0.058±0.001[a]	0.326±0.040[b]	0.112±0.005[a]
	I-Ca	0.192±0.013[a]	0.054±0.003[b]	0.377±0.037[a]	0.108±0.007[a]
Tibia	F-Ca	0.066±0.002[c]	0.019±0.001[c]	0.157±0.006[c]	0.045±0.002[b]
	S-Ca	0.108±0.008[b]	0.037±0.001[a]	0.264±0.030[b]	0.091±0.009[a]
	I-Ca	0.115±0.007[a]	0.032±0.002[b]	0.291±0.029[a]	0.083±0.007[a]

[1]Refer to Table 1. [2]Mean±SD. [3]Values with different superscripts within the column are significantly different at p<0.05 by Duncan's multiple range test.

Table 7. Calcium efficiency of rats fed different calcium supplements

Group[1]	Calcium efficient[2]		
	Spine	Femur	Tibia
S-Ca	0.757 ± 0.090[3)NS]	1.027 ± 0.081[NS4)]	0.652 ± 0.032[NS]
I-Ca	0.741 ± 0.062	1.083 ± 0.060	0.647 ± 0.042

[1]Refer to Table 1. [2]Calcium efficiency = BMD (g/cm^2)/average calcium intake (g/day). [3]Mean $\pm$ SD. [4]Not significantly different.

and urine calcium metabolism and calcium efficiency for each bone area were not different. Nonetheless, the water-soluble calcium supplements were found to be beneficial to BMD in terms of weight bearing. Thus, additional studies on the bioavailability of water-soluble calcium supplements should be conducted in the future.

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