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Experimental paper

Effects of high calcium intake on bone metabolism in magnesium-deficient rats

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Abstract. We examined the effects of high calcium (Ca) intake on bone metabolism in magnesium (Mg)-deficient rats. Male Wistar rats were divided into three groups, with each group having a similar mean body weight, and fed a control diet (control group), a Mg-deficient diet (Mg-deficient group) or a Mg-deficient Ca-supplemented diet (Mg-deficient Ca-supplemented group) for 14 d. Femoral Ca content was significantly lower in the Mg-deficient Ca-supplemented group than in the control group and Mg-deficient group. Femoral Mg content was significantly lower in the Mg-deficient group and Mg-deficient Ca-supplemented group than in the control group. Furthermore, femoral Mg content was significantly lower in the Mg-deficient Ca-supplemented group than in the Mg-deficient group. Serum osteocalcin levels (a biochemical marker of bone formation) were significantly lower in the two Mg-deficient groups than in the control group. As a biochemical marker of bone resorption, urinary deoxypyridinoline excretion was significantly higher in the Mg-deficient Ca-supplemented group than in the control group and Mg-deficient group. The results in the present study suggest that high Ca intake had no preventive effect on alteration of bone metabolism in Mg-deficient rats.

Key words: high calcium intake, bone metabolism, magnesium-deficient diet, rats

Approximately half of the total magnesium (Mg) in the body exists in bone and plays an important role in bone metabolism. Mg deficiency is one of risk factor for osteoporosis. Previous studies [1-3] have reported that Mg intake was correlated with bone mineral content and/or bone mineral density. Furthermore, experimental animals fed a Mg-deficient diet showed impaired bone growth and increased skeletal fragility [4-6].

On the other hand, calcium (Ca) is an essential mineral that plays a very important role in the maintenance of bone mass and preventing fractures. Although low Ca intake induced an increase in bone resorption and a decrease in bone mineral density [7-10], high Ca intake enhanced bone mineral content and bone mineral density [11-13]. From these observations, we hypothesize that increased Ca intake may prevent bone impairment in Mg-deficient rats. However, few studies have examined the relationship between Ca intake and bone metabolism in Mg-deficient rats. Accordingly, this study examined the effects of high Ca intake on bone metabolism in Mg-deficient rats.

Materials and methods

Animals and diets

Experimental animals were 4-week-old male Wistar rats obtained from Clea Japan (Tokyo, Japan). The rats were housed in individual stainless-steel wire-mesh cages. During the experiment, cages were located in a room with controlled lighting under a 12-h light:dark cycle (light, 0800-2000h), a tempera-

Table 1. Composition of experimental diets.

	Control diet	Mg-deficient diet	Mg-deficient Ca-supplemented diet
Ingredient (g/kg of diet)			
Cornstarch	528.657	529.486	492.028
Casein	200.0	200.0	200.0
Sucrose	100.0	100.0	100.0
Soybeanoil	70.0	70.0	70.0
Cellulosepowder	50.0	50.0	50.0
Mineralmix[a]	35.0	35.0	35.0
Vitaminmix[b]	10.0	10.0	10.0
L-Cystine	3.0	3.0	3.0
Cholinebitartrate	2.5	2.5	2.5
Tert-butylhydroquinone	0.014	0.014	0.014
MgO	0.829	–	–
$CaCO_3$	–	–	37.458
Chemical analysis (%)			
Mg	0.047	0.004	0.004
Ca	0.49	0.48	1.94

[a] The mineral mix is a modification of the AIN-93G mineral mix without magnesium oxide.
[b] AIN-93 vitamin mix.

ture of 22 ± 1°C and relative humidity of 60-65%. The study protocols were approved by the Animal Use Committee at Tokyo University of Agriculture, and animals were maintained in accordance with the university's guidelines for the care and use of laboratory animals.

The compositions of the experimental diets are shown in *table 1*. Experimental diets were based on an AIN-93G diet [14]. Magnesium oxide was excluded from the AIN-93G mineral mix in the two Mg-deficient diets. Mg and Ca concentrations in the experimental diets were as follows: control diet, 0.05% Mg and 0.5% Ca; Mg-deficient diet, Mg-free and 0.5% Ca; Mg-deficient Ca-supplemented diet, Mg-free and 2.0% Ca. The Mg and Ca concentrations as measured from an analysis of the experimental diets is shown in *table 1*. All experimental diets were stored at 4°C until used.

Experimental design

Before the study period began, there was a 7-d acclimatization period during which all rats were given free access to the control diet and demineralized water. After the acclimatization period, rats were divided into three groups of 6 rats with each group having a similar mean body weight. One of the experimental diets was assigned to each group and rats were given free access to the assigned experimental diet as well as demineralized water throughout the experimental period. Body weight and food intake were recorded daily. From days 10 to 13 of the experiment, rats were housed individually in stainless-steel metabolic cages, and feces were collected from each rat. Subsequently, urine was collected for 24h from each rat. At the end of the 14-d experimental period, all rats were killed by exsanguination from the carotid artery. Blood was collected in tubes at the time of exsanguination, and was centrifuged to obtain serum. The femur was removed and cleaned of the muscles, and connective tissues were discarded. Samples were stored at -40°C until needed for analysis.

Chemical analysis

Samples of the experimental diets, feces and femur were ashed at 550 °C for 48h in a muffle furnace, and minerals were extracted in 1 mol/L of HCl for analysis. Ca and Mg were determined by atomic absorption spectrometry (Hitachi A-2000) [15]. Osteocalcin in serum was measured with an Osteocalcin rat ELISA system (Amersham Biosciences K.K., Tokyo, Japan). Deoxypyridinoline in urine was measured with a Pyrinks-D (Quidel Corp., USA). Creatinine in urine was measured with a Creatinine-Test Wako (Wako Pure Chemical Industries, Osaka, Japan). The apparent absorption of minerals was calculated as the intake–fecal excretion.

Statistical analysis

Data are expressed as mean values with SD. Data were analyzed by one-way ANOVA. Tukey's test was used to evaluate the significance of differences in multiple comparisons among groups, with differences being considered significant at $p < 0.05$. All statistical analyses were performed using the SPSS package program Ver. 11.0 J.

Results

Body weight and food intake

Final body weight and food intake were significantly lower in the two Mg-deficient groups than in the control group, and were significantly lower in the Mg-deficient Ca-supplemented group than in the Mg-deficient group *(table 2)*.

Femoral mineral content and biochemical markers of bone turnover

Femoral dry weight was significantly lower in the Mg-deficient Ca-supplemented group than in the other two groups *(table 3)*. Femoral Ca content was significantly lower in the Mg-deficient Ca-supplemented group than in the other two groups. Femoral Mg content was significantly lower in the two Mg-deficient groups than in the control group. Femoral Mg content also was significantly lower in the Mg-deficient Ca-supplemented group than in the Mg-deficient group. Serum osteocalcin levels were significantly lower in the Mg-deficient group and Mg-deficient Ca-supplemented group than in the control group. Urinary deoxypyridinoline excretion was significantly higher in the Mg-deficient Ca-supplemented group than in the control group and Mg-deficient group.

Apparent mineral absorption and serum mineral levels

Apparent Ca absorption was significantly higher in the Mg-deficient Ca-supplemented group than in the other two groups *(table 4)*. Apparent Mg absorption was significantly lower in the two Mg-deficient groups than in the control group, and that was significantly lower in the Mg-deficient Ca-supplemented group than in the Mg-deficient group. Serum Ca levels were significantly higher in the two Mg-deficient

Table 2. Body weight and food intake in the control, Mg-deficient or Mg-deficient Ca-supplemented groups[d].

	Control group	Mg-deficient group	Mg-deficient Ca-supplemented group
Initial body weight (g)	80.5 ± 3.1	80.6 ± 2.3	80.6 ± 2.3
Final body weight (g)	185.6 ± 5.7[a]	137.7 ± 3.0[b]	117.5 ± 3.3[c]
Food intake (g/d)	14.7 ± 0.5[a]	11.3 ± 0.2[b]	10.1 ± 0.5[c]

[a,b,c] Values with different superscript letters are significantly different ($p < 0.05$).
[d] Values are means ± SD, n = 6 per group.

Table 3. Femoral mineral content, serum osteocalcin levels and urinary deoxypyridinoline excretion in the control, Mg-deficient or Mg-deficient Ca-supplemented groups[d].

	Control group	Mg-deficient group	Mg-deficient Ca-supplemented group
Femur			
Dry weight (g)	0.243 ± 0.010[a]	0.246 ± 0.011[a]	0.220 ± 0.012[b]
Ca (mg/g dry weight)	192.9 ± 9.1[a]	194.3 ± 4.6[a]	178.4 ± 5.6[b]
Mg (mg/g dry weight)	3.91 ± 0.12[a]	1.23 ± 0.09[b]	1.01 ± 0.06[c]
Osteocalcin in serum (ng/mL)	142.2 ± 12.8[a]	83.8 ± 13.9[b]	71.0 ± 16.0[b]
Deoxypyridinoline in urine (μmol/mmol creatinine)	0.76 ± 0.16[a]	0.81 ± 0.15[a]	1.05 ± 0.14[b]

[a,b,c] Values with different superscript letters are significantly different ($p < 0.05$).
[d] Values are means ± SD, n = 6 per group.

Table 4. Apparent mineral absorption and serum mineral levels in the control, Mg-deficient or Mg-deficient Ca-supplemented groups[d].

	Control group	Mg-deficient group	Mg-deficient Ca-supplemented group
Apparent absorption			
Ca (mg/d)	60.2 ± 6.1[a]	47.1 ± 6.8[a]	83.0 ± 14.7[b]
Mg (mg/d)	6.17 ± 0.36[a]	0.31 ± 0.04[b]	0.14 ± 0.02[c]
Serum			
Ca (mg/dL)	11.3 ± 0.2[a]	12.0 ± 0.3[b]	12.8 ± 0.7[c]
Mg (mg/dL)	2.07 ± 0.12[a]	0.46 ± 0.03[b]	0.34 ± 0.05[c]

[a,b,c] Values with different superscript letters are significantly different ($p < 0.05$).
[d] Values are means ± SD, n = 6 per group.

groups than in the control group, and the levels were significantly higher in the Mg-deficient Ca-supplemented group than in the Mg-deficient group. Serum Mg levels were significantly lower in the two Mg-deficient groups than in the control group, and the Mg levels were significantly lower in the Mg-deficient Ca-supplemented group than in the Mg-deficient group.

Discussion

Mg deficiency reduced final body weight in the present study. This finding may relate to food intake. In the present study, the rats were given free access to the experimental diet, and consequently food intake was decreased in rats fed the Mg-deficient diet. On the other hand, it has been reported that despite the pair-feeding method used, body weight was decreased in rats fed the Mg-deficient diet [16, 17]. From the results of the present study and previous studies, we speculate that reduced body weight in rats fed the Mg-deficient diet is not attributable to low food intake only. It is also suggested that body weight in rats fed the Mg-deficient diet may be influenced by Mg consumption rather than food consumption. Furthermore, with regard to the effects of pair-feeding on bone Ca content, rats, which were treated by *ad libitum* or pair-feeding with an Mg-restricted diet for 3 weeks, showed a similar femoral Ca content between the *ad libitum* group and a pair-fed group [16]. This finding indicates that pair-feeding has no effect on femoral Ca content. Therefore, we believe that pair-feeding did not need to be done in the present study. Probably, the femoral Ca content in Mg deficiency would be unchanged, in spite of the pair-feeding method being used in the present study.

It has been reported that bone Ca content in rats fed Mg-deficient diets was unchanged, while bone Mg content was decreased by a Mg-deficient diet [18-20]. In the present study, although the Mg-deficient diet had no effects on femoral Ca content, femoral Mg content was reduced in rats fed the Mg-deficient diet. The present study also found that femoral Mg content was lower in the Mg-deficient Ca-supplemented group than in the control group and Mg-deficient group. Reduction of femoral Mg content by the Mg-deficient Ca-supplemented diet may be related to Mg absorption in the intestine. We observed that apparent Mg absorption was lower in rats fed the Mg-deficient Ca-supplemented diet than in rats fed the control diet and Mg-deficient diets. A previous study has reported that a high Ca intake induced a decrease in apparent Mg absorption [21], and indicated that high Ca intake has an inhibitory effect on Mg absorption. In other words, we suggest that a Mg-deficient Ca-supplemented diet-induced reduction of femoral Mg content is due to a decrease in apparent Mg absorption by dietary Ca supplementation.

Bone Ca content was not affected by the Mg-deficient diet [18-20], however femoral Ca content in the Mg-deficient Ca-supplemented group was decreased in the present study. It was very interesting that despite the general belief that bone Ca content is enhanced by a high Ca intake, dietary Ca supplementation reduced femoral Ca content in rats fed the Mg-deficient diet. The mechanism responsible for the decreased femoral Ca contents in Mg-deficient Ca-supplemented group, cannot be ascertained from the results of the present study. However, the present study observed that apparent absorption and serum levels of Mg in the Mg-deficient Ca-supplemented group were lower than in the Mg-deficient group, and indicated that Mg

availability was reduced by high Ca intake. We therefore suggest that reduction in Mg availability may, at least in part, account for the reduction of femur Ca contents in Mg-deficient rats by high Ca intake, since Mg plays an important role in bone growth. On the other hand, Creedon and Cashman [8] found that dietary Ca supplementation (4 times the normal level) did not enhance bone Ca content, and concluded that increasing dietary Ca intake above the recommended level had no effect on bone mineral composition. Dietary Ca concentration in their experiment was similar to the Mg-deficient Ca-supplemented diet in the present study.

With regard to the effects of Mg deficiency on bone formation and bone resorption, Mg deficiency induces a decrease in serum osteocalcin levels [4]. Rude *et al.* [20] found that the osteoblast number of Mg-depleted rats was lower than that of control rats. We observed that serum osteocalcin levels were decreased in rats fed a Mg-deficient diet. The osteoclast number was increased in Mg-depleted rats, as measured by bone histomorphometry, and indicates that Mg deficiency also enhanced the bone resorption rate [19, 20]. These findings suggest that Mg deficiency decreases bone formation rate and increases bone resorption rate, and that these effects are the major causes of impaired bone growth of Mg-deficient rats. On the other hand, the present study observed that although there was no difference in serum osteocalcin levels between the Mg-deficient group and Mg-deficient Ca-supplemented group, urinary deoxypyridinoline excretion was higher in the Mg-deficient Ca-supplemented group than in the Mg-deficient group. This finding suggests that high Ca intake stimulates the bone resorption rate of Mg-deficient rats, but has no effect on bone formation rate. The increased urinary deoxypyridinoline excretion in the present study may be related to Mg availability. Our observation of reduced Mg availability in the Mg-deficient Ca-supplemented group suggests that increased urinary deoxypyridinoline excretion in the present study is due to the reduction of Mg availability. Furthermore, we suggest that the decrease in femoral Ca content in the Mg-deficient Ca-supplemented group was due to increased bone resorption. In other words, in rats fed a Mg-deficient diet, the high Ca intake reduced *in vivo* Mg availability, thus elevating bone resorption. Subsequently, a greater decrease in femoral Ca content was observed in the Mg-deficient Ca-supplemented group than in the Mg-deficient group.

The details of mechanisms for the changes in bone formation rate and bone resorption rate by Mg deficiency are still unclear. However, parathyroid hormone (PTH) and $1,25(OH)_2$-vitamin D are important factors in bone formation, since both hormones stimulate osteoblast activity and synthesis of procollagen and osteocalcin. Serum PTH and $1,25(OH)_2$-vitamin D levels are decreased in rats fed the Mg-deficient diet, and it is suggested that the decrease in these hormones may contribute to inhibit bone formation in Mg-deficient rats [17, 20]. Mg deficiency induces increasing substance P and tumor necrosis factor-α (TNF-α) [17, 22]. Increases in osteoclast activity and bone resorption in Mg deficiency may be due to increased substance P and TNF-α [17].

Conclusion

The effects of high Ca intake on bone metabolism in Mg-deficient rats were investigated. The rats were fed a control diet (control group), a Mg-deficient diet (Mg-deficient group) or a Mg-deficient Ca-supplemented diet (Mg-deficient Ca-supplemented group) for 14 d. Femoral Ca content in the Mg-deficient group was not changed, however femoral Ca content in the Mg-deficient Ca-supplemented group was decreased. Femoral Mg content was decreased in the Mg-deficient group and Mg-deficient Ca-supplemented group. Furthermore, femoral Mg content was lower in the Mg-deficient Ca-supplemented group than in the Mg-deficient group. Serum osteocalcin levels (a biochemical marker of bone formation) were decreased in the Mg-deficient group and Mg-deficient Ca-supplemented group. Urinary deoxypyridinoline excretion (a biochemical marker of bone resorption) was increased in the Mg-deficient Ca-supplemented group. These results suggest that a high Ca intake had no preventive effect on alteration of bone metabolism in Mg-deficient rats.

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