Milk Calcium Taken with Cheese Increases Bone Mineral Density and Bone Strength in Growing Rats

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We investigated the calcium bioavailability of milk calcium, taken with or without cheese. Twenty-four 6-week-old male rats for a meal-feeding experiment were trained to consume an AIN-76 diet within 2 h (2 times per day) for 2 weeks. The rats were then divided into three experimental groups, each fed 2 types of experimental diets: Control group, Cheese group, and Ca-Cheese group. The rats were each alternately given 2 types of experimental diets at 2-h meal-feeding for 31 days. The breaking force and energy of the femur in the Ca-Cheese group were significantly higher than in the control group. The bone mineral density (BMD) of the lumbar spine and the femur in the Ca-Cheese group was also significantly higher than in the other two groups.

These results indicate that milk calcium taken with cheese increases bone strength and BMD efficiently, results that may be useful for the prevention of osteoporosis.

Key words: cheese; milk calcium; bioavailability; bone; rats

With the prolongation of the human life span, the incidence of metabolic diseases such as osteoporosis and bone fracture increase in the elderly. Osteoporosis is a disorder characterized by bone loss and is associated with an increased risk of fractures and back or joint pain. It is more frequently observed in the elderly and in women after menopause. In particular, the bone mass of many women after menopause decreases and the risk of fracture increases rapidly, since the bone mass of women is generally lower than that of men.1,2) Femoral neck fracture is one of the most serious injuries found in elderly patients in orthopaedics. It is important for the prevention of osteoporosis to take enough dietary nutrients, especially foods with a high content of calcium. Dietary calcium, which is more absorbable and bioavailable, is essential when considering calcium requirements in the elderly, since the absorbability and bioavailability of calcium declines with age.

Milk is a safe food that can be taken over a long time, and it has been useful for human health. Milk is also an excellent source of calcium, considering the absorption of calcium and its bioavailability.3,4) Calcium in milk is more absorbable and bioavailable than calcium in fish or vegetables and as calcium carbonate.3,6,7) Milk also contains lactose, casein phosphopeptides (CPP) that formed by proteolytic digestion of casein in gut, and vitamin D, which accelerate calcium absorption.8,9)

Recently, to increase daily calcium intake, commercially available calcium-fortified foods have been placed on the market. These foods are fortified with many calcium sources such as calcium carbonate, calcium phosphate, cattle bone powder, and milk calcium. In particular, milk calcium is more absorbable than any other calcium source. Also, colloidal calcium and calcium caseinate in milk are considered to be more absorbable and bioavailable than ionic calcium, and the chemical form of milk calcium may affect calcium absorbability and bioavailability.10,11) We prepared food consisting basically of skim milk powder, fortified with milk calcium. The calcium-fortified food was a good calcium source, considering an increase of bone mineral contents and bone strength.12)

Calcium from cheddar cheese and processed cheese is as well utilized as from cow milk.13) The absorbability of calcium from cheese is high, since cheese contains CPP, which are formed by proteolytic digestion of casein in gut and increases calcium absorption.13) Ripened cheese is an especially good calcium source for lactase-deficient individuals.14)

The purpose of this study is to investigate the calcium bioavailability of milk calcium, taken with...
or without cheese, by examining bone strength and bone mineral density (BMD) in growing male rats.

Materials and Methods

Preparation of milk calcium. Milk calcium was obtained from milk whey as follows. Milk whey was prepared from fresh milk, which was defatted by centrifugation and adjusted to pH 4.2 with HCl. Milk whey was concentrated by evaporation 10 times and cooled to 4°C for 12 h, and the lactose was precipitated and separated by centrifugation at 3,000 × g. Its supernatant was adjusted to pH 6.8 with 1 N NaOH. The solution was centrifuged at 3,000 × g and the residual lactose was removed. Its precipitate was washed with deionized water and the whey protein was separated by ultrafiltration (molecular mass cut-off, 100 kDa). Its retentate was collected and spray-dried. The concentrations of calcium, phosphorus, and magnesium in the milk calcium were 163, 100, and 10 g/kg, respectively (Table 1).

Composition of cheese fortified with milk calcium. Cheese fortified with milk calcium used in this experiment was processed cheese, which was prepared from two types of natural cheeses (Gouda and cheddar) and milk calcium. The composition of cheese fortified with milk calcium is shown in Table 2. The calcium concentrations of cheese fortified with milk calcium and non-calcium fortified cheese, which was processed cheese and prepared from the same natural cheese as cheese fortified with milk calcium, were 1,200 mg/100 g and 630 mg/100 g, respectively (Table 2).

Diets. The composition of the pre-experimental diet is shown in Table 3. The diet was prepared according to the AIN-76 diet. The composition of experimental diets is shown in Table 4. During the experimental period, each group was given 2 types of experimental diets (Type I diet and Type II diet). In the control diet, soy protein isolate was used as the sole source of protein, and calcium carbonate and milk calcium were used as the sources of calcium (Type I diet: calcium carbonate 0.5%; milk calcium 1.1%. Type II diet: calcium carbonate 0.5%). In the Cheese diet, 25% soy protein isolate and 50% calcium carbonate in the control diet were replaced by non-calcium-fortified cheese, because the same amount of milk calcium and cheese as cheese fortified with milk calcium was fed separately (Type I diet: calcium carbonate 0.5%; milk calcium 1.1%. Type II diet: cheese 35%). In the Ca-Cheese diet, 25% soy protein isolate, 50% calcium carbonate, and 100% milk calcium in the control diet were replaced by cheese fortified with milk calcium, because milk calcium and cheese were fed at the same time (Type I diet: cheese fortified with milk calcium 35%. Type II diet: calcium carbonate 0.5%). The amounts of protein, sulfur-containing amino acid, calcium, phosphorus, and magnesium were equal in the three experimental diets of 2 types each.

Animals. Twenty-four 6-week-old male Sprague-Dawley rats (Charles River Inc., Kanagawa, Japan) were housed in individual stainless steel wire-mesh cages in a temperature- and humidity-controlled room (23°C and 60 ± 5% relative humidity) with a 12-h light/dark cycle. All rats were treated in accordance with the NIH “Guide for the Care and Use of Laboratory Animals”.

### Table 1. Composition of Milk Calcium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>40</td>
</tr>
<tr>
<td>Protein</td>
<td>85</td>
</tr>
<tr>
<td>Fat</td>
<td>5</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>60</td>
</tr>
<tr>
<td>Ash</td>
<td>810</td>
</tr>
</tbody>
</table>

### Table 2. Compositions of Cheese and Cheese Fortified with Milk Calcium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Cheese</th>
<th>Cheese fortified with milk calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture (g/100 g)</td>
<td>46.3</td>
<td>46.7</td>
</tr>
<tr>
<td>Fat</td>
<td>25.3</td>
<td>23.6</td>
</tr>
<tr>
<td>Protein</td>
<td>21.2</td>
<td>21.2</td>
</tr>
<tr>
<td>Ash</td>
<td>4.6</td>
<td>5.6</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>2.6</td>
<td>2.9</td>
</tr>
</tbody>
</table>

### Table 3. Composition of Pre-Experimental Diet* (%)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>20.0</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>15.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>5.0</td>
</tr>
<tr>
<td>Corn oil</td>
<td>5.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>50.2</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.3</td>
</tr>
<tr>
<td>Vitamin mixture**</td>
<td>1.0</td>
</tr>
<tr>
<td>Mineral mixture***</td>
<td>3.5</td>
</tr>
</tbody>
</table>

* This diet was that of AIN-76 (American Institute of Nutrition, 1977).
** Identical to AIN-76 vitamin mixture.
*** Identical to AIN-76 mineral mixture.
trained to consume an AIN-76 diet within 2 h (2 times per day) and given free access to deionized water for 2 weeks. After a 2-week period, they were separated into three experimental groups of 8 rats each, and each rat had a similar body weight of 248 g. All rats were alternately given the Type I diet (morning on the odd-numbered days and evening on the even-numbered days) and the Type II diet (evening on the odd-numbered days and morning on the even-numbered days) for each of the 31 times of a 2-h meal-feeding (2 times per day). They were also given free access to deionized water for 31 days. Body weight was recorded once a week, and food intake was monitored daily. After the 31-day feeding period, the rats were deprived of food overnight and anesthetized with diethyl ether. Blood was collected from the vena cava inferior and the serum prepared. The femora and the fourth lumbar spine were both excised from the rats, and the muscles and connective tissues were removed. The bone samples were kept in phosphate buffer before measurement.

Serum analysis. Serum calcium was measured by an automatic analyzer (Fuji Drychem Systems, Tokyo, Japan).

Measurement of BMD of the fourth lumbar spine and the femur. The BMD of the fourth lumbar spine and the femur were measured by dual-energy X-ray absorptiometry, with a Dichroma Scan DCS-600A (Aloka, Tokyo, Japan) adapted for measuring small animals with beam energies of 22 and 53 keV. The scanning speed was 10 mm/s, and each scanning step was 1 mm.

Measurement of bone strength of the femur. The breaking force and energy of the femoral diaphysis (the center of the femur) were measured by a three-point bending rheometer (RX-1600, Aitechno, Japan), as in the methods of Ezawa et al. and Takada et al. The measurement conditions were as follows: sample space, 1.0 cm; plunger speed, 20 mm/min; and load range, 40.0 kg.

Statistical analysis. Statistical analysis was done by one-way ANOVA. When F ratios were significant, individual means were compared by a Tukey-Kramer test. All calculations were performed with SuperANOVA software (Abacus Concepts, CA). Differences with $P<0.05$ were taken to be significant.

Results

Body weight gain, food intake and food efficiency. The amounts of daily calcium intake were equal in the three experimental groups (52.5 mg/day). The body weight gain, food intake, and food efficiency were not significantly different among the experimental groups (Table 5).
Table 5. Body Weight Gain, Food Intake, and Food Efficiency

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight gain (g/day)</th>
<th>Food intake (g/day)</th>
<th>Food efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.7 ± 0.3</td>
<td>15.9 ± 0.2</td>
<td>23.3 ± 1.7</td>
</tr>
<tr>
<td>Cheese</td>
<td>3.4 ± 0.4</td>
<td>16.0 ± 0.0</td>
<td>21.3 ± 2.5</td>
</tr>
<tr>
<td>Ca-Cheese</td>
<td>3.3 ± 0.3</td>
<td>15.9 ± 0.1</td>
<td>20.7 ± 2.1</td>
</tr>
</tbody>
</table>

Values are means ± SD, n = 8.

Table 6. Concentration of Serum Calcium (Ca) in 12-Week-old Male Rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Cheese</th>
<th>Ca-Cheese</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca (mg/100 ml)</td>
<td>8.7 ± 0.2</td>
<td>8.7 ± 0.3</td>
<td>8.8 ± 0.2</td>
</tr>
</tbody>
</table>

Values are means ± SD, n = 8.

**Fig. 1.** Effects of Experimental Diets on Bone Mineral Density (BMD) of the Fourth Lumbar Spine in 12-Week-old Male Rats. Datum points are means, and bars show 95% confidence intervals, n = 8.

* Significantly different from the control group (P < 0.05).

**Fig. 2.** Effects of Experimental Diets on Segmental Bone Mineral Density (BMD) of the Femur in 12-Week-old Male Rats. Datum points are means, and bars show 95% confidence intervals, n = 8.
a; Significantly different from the control group (P < 0.05).
b; Significantly different from the control and the Cheese group (P < 0.05).
c; Significantly different from the Cheese group (P < 0.05).

**Fig. 3.** Effects of Experimental Diets on Breaking Force and Breaking Energy of the Femur in 12-Week-old Male Rats. Datum points are means, and bars show 95% confidence intervals, n = 8.
* Significantly different from the control group in breaking force (P < 0.05).
† Significantly different from the control group in breaking energy (P < 0.05).

**Serum analysis**

The serum concentration of calcium was normal and not significantly different among the experimental groups (Table 6).

**BMD of the fourth lumbar spine and the femur**

The BMD of the lumbar spine in rats fed the Cheese diet and Ca-Cheese diet were significantly higher than in rats fed the control diet (Fig. 1). In the 9 segmental BMD of the femur, the segment 2 BMD in rats fed the Ca-Cheese diet was significantly higher than in rats fed the control diet; the segment 3 BMD in rats fed the Ca-Cheese diet was significantly higher than in rats fed the control and Cheese diets; and the segment 4 BMD in rats fed the Ca-Cheese and control diets were significantly higher than in rats fed the Cheese diet (Fig. 2).

**Breaking force and energy of the femur**

The breaking force and energy of the excised femur in rats fed the Ca-Cheese diet were significantly higher than in rats fed the control diet (Fig. 3). Significantly positive correlation was found between the BMD and the breaking force of the femur, and the BMD and the breaking energy of the femur, respectively (breaking force, \( r = 0.60 \) and \( P < 0.05 \); breaking energy, \( r = 0.50 \) and \( P < 0.05 \)).

**Discussion**

Cheese is an excellent calcium source, which has absorbable calcium. The absorbability of calcium...
from cheese is high, since cheese contains CPP, which are formed by proteolytic digestion of casein in gut and increases calcium absorption.\(^1,5\) In this study we evaluated the calcium bioavailability of milk calcium, which is well known as a good calcium source,\(^10,20,21\) taken with or without cheese.

The breaking force and energy of the femur in rats fed cheese fortified with milk calcium are significantly higher than in rats fed the control diet (Fig. 3). Milk calcium taken with cheese increased bone strength efficiently. Bone strength is increased when the absorption or retention of calcium is increased. In this study, the BMD of the lumbar spine in rats fed cheese fortified with milk calcium was higher than in rats fed the control diet (Fig. 1). And in segmental BMD of the femur in rats fed cheese fortified with milk calcium was higher than in rats fed the Cheese diet or the control diet (Fig. 2). Also, significantly positive correlation was found between the BMD and the breaking force of the femur, and the BMD and the breaking energy of the femur. These results indicate that the increase of bone strength in rats fed milk calcium taken with cheese is due to the increased bone mineral. From this study, milk calcium taken with cheese increased bone strength and BMD efficiently. Therefore the increased bone strength and BMD in rats fed cheese fortified with milk calcium is due to their taking milk calcium and cheese, which contain absorbable calcium, at the same time.

In conclusion, these results indicate that milk calcium taken with cheese is an excellent calcium source, better than milk calcium taken without cheese. Also, milk calcium taken with cheese increases bone strength and BMD efficiently, which may be useful for the prevention of osteoporosis.

References