Effect of Milk on Bone Metabolism in Growing Male and Female Rats

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To investigate the effect of milk on bone metabolism in growing rats, the bone mineral density (BMD), bone strength and intestinal calcium (Ca) absorption were examined. It has recently become possible to accurately and precisely measure BMD in growing animals with low bone mass by a bone densitometer. In this study, the change in BMD in growing young rats was therefore evaluated by this method.

Male and female Sprague-Dawley (SD) rats at 3 weeks of age were divided into male and female control diet groups (0.3% Ca from CaCO3) and milk diet groups (0.3% Ca from milk). The experimental period lasted for 4 weeks. The change in BMD and the intestinal Ca absorption were determined in experiment A (Exp. A), and BMD of the extracted bone, mechanical bone strength, and weight and mineral contents of the bone were measured in experiment B (Exp. B).

In Exp. A, BMD in the lumbar spine and tibia of the milk diet groups increased significantly compared to those of the control values. The intestinal Ca absorption of the rats in the milk diet groups was also significantly greater than that of the control rats. In addition, in Exp. B, BMD values, mechanical bone strength, bone weight, and Ca and P contents in the bone of the rats in the milk diet groups were also significantly greater than the control values.

These findings suggest that milk promoted bone metabolism during the growing period and confirm the effectiveness of measuring BMD of low bone mass by a bone densitometer to evaluated metabolism in vivo.

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Keywords: milk, growing rat, bone mineral density (BMD), bone strength, intestinal Ca absorption.

INTRODUCTION

Milk is one of the most nutritious sources of calcium (Ca) for bone metabolism. It is well known that milk Ca is highly absorptive in the intestine due to its casein-phosphopeptide (CPP) and lactose contents. Ezawa has already reported milk to be an effective Ca source for preventing femoral fracture in ovariectomized rats. A higher bone mineral density (BMD) in post-menopausal women with the habit of drinking milk has also been reported.

The prevention of osteoporosis, not only in elderly people but also in young people, while bones are at the actively growing stage is important; therefore, to prevent future risks of osteoporosis, sufficient Ca should be taken to acquire a higher bone mass during the growing stage. While the intake of most nutrients has become sufficient in Japan, the intake of Ca in particular has never been sufficient. Although Ca intake has nearly reached the recommended minimum daily allowance, it still remains inadequate, probably due to the relatively low quantity of milk consumed in Japan.

It has recently become possible to determine the low bone mass of small animals accurately and precisely in vivo by utilizing a bone densitometer (DCS-600R: Aloka). However, there have been few reports concerning low bone mass during the growing stage.

We have already reported that the BMD values for the lumbar spine and tibia and that the mechanical bone strength of the femur increased significantly after consuming diets with different Ca sources. Based on our previous work, we now examine the effect of milk on the bone metabolism of growing rats by using a bone densitometer (DCS-600R: Aloka). The effects of several other Ca sources on bone mass...
were also investigated by the same technique (using bone densitometer).

**MATERIALS AND METHODS**

**Experimental design**

Twenty-nine female Sprague-Dawley (SD) rats and 29 male SD rats at 3 weeks of age (just after weaning; Japan SLC Co., Hamamatsu), were used in these experiments. The protocol for the present study is shown in Fig. 1. All the rats were acclimatized for 5 days, before being randomly divided into three groups for each sex based on the body weight: the male base-line group (MB, n=5), female base-line group (FB, n=5), male control-diet group (MC, n=12), female control-diet group (FC, n=12), male milk-diet group (MM, n=12), female milk-diet group (FM, n=12). All the rats were kept separately in a cage during the experiment. The rats in the control-diet groups (MC and FC) and the base-line groups (MB and FB) were fed on a control diet containing 0.3% Ca and 0.3% phosphorus (P) (Table 1). The Ca source for the control diet was solely CaCO3. The rats in the milk-diet groups (MM and FM) were fed on a milk diet containing 0.3% Ca and 0.3% P (Table 1). The Ca source of the milk diet was solely skimmed-milk. The concentrations of the main nutrients such as protein and lipids were identical in each diet. Throughout the experiment, all the rats were allowed free access to food and ion-exchanged distilled water, the temperature was kept at 23 ± 1°C, and the humidity was maintained at 50 ± 5%. A 12-h light-dark cycle was maintained for all groups, with lights on from 7:00 a.m. to 7:00 p.m.

1. **Experiment A (Exp. A)**

Seven rats in each group (MC, MM, FC and FM), based on their body weight, were used to determine the change in BMD by in vivo measurements and the Ca absorption. In Experiment A (Exp. A), the rats were dissected at three different times and the breaking strength, bone weight, mineral contents, and bone mass by in vitro measurements were determined at each dissection.

2. **Experiment B (Exp. B)**

The rats in the MB and FB groups were sacrificed at the start of the experimental period for base-line control. The other four groups (MC, FC, MM and FM) were fed with the experimental diets. Two weeks after the start of the experimental period, 5 rats in each group were dissected. The remaining rats (7 rats for each group) were kept for 2 more weeks, before which they were finally sacrificed. The mechanical bone strength, bone weight, Ca and P contents, and bone mass in vitro (the extracted bone) were determined at each dissection.

Fig. 1. Experimental protocol for Exp. A (in vivo study) and Exp. B (in vitro study) ( ) n shows the number of dissected rats in Exp. B. Experiment A (Exp. A) determined the change in BMD by in vivo measurements and the Ca absorption. In Experiment B (Exp. B), the rats were dissected at three different times and the breaking strength, bone weight, mineral contents, and bone mass by in vitro measurements were determined at each dissection.
The appropriated rats were weighed before dissection at the start of the experiment, 2 weeks later, and at the end of experiment. Then, after overnight deprivation of food, the rats were anesthetized with ether, and blood samples were taken from the abdominal aorta. All the serum samples were stored at −35°C. The bone samples, including the lumbar spine, tibiae and femora, were isolated after killing by exsanguination. After the adhering connective tissues had been trimmed off, the lumbar spine and tibia samples were fixed with 70% ethanol.

**Bone mineral density (BMD) and content (BMC) measurements**

1. **Exp. A (in vivo)**

BMD values for the L1-L6 lumbar spine and left tibia were determined by Dual-energy X-ray absorptiometry (DXA; Aloka DCS-600R instrument) three times in this experiment. For each rat, BMD measurements were performed under general anesthesia before starting the experimental period (0 time), 2 weeks after starting the experimental period (2 weeks) and at the end of the experiment (4 weeks). The radiation beam was aimed antero-posteriorly for the lumbar spine and laterally for the tibia. BMD values were obtained for the lumbar spine and the total tibia, including the epimetafisyeal region.

2. **Exp. B (in vitro)**

After each dissection, bone samples were taken, and BMC and BMD values were measured by directly applying the radiation beam. BMC and BMD values of the extracted bones were obtained for the lumbar spine, tibia and femur.
spine (L1-L6), total tibia, proximal one-fifth of the tibia, including the epimetaphyseal region representing the trabecula sites, and middle one-fifth of the tibia representing the cortical diaphyseal region.

**Balance study (in vivo Exp. A)**

The Ca balance was evaluated at four intervals during this study to determine the intestinal Ca absorption and Ca accumulation. For each evaluation, feces and urine were collected over a 24-h period. Urine was collected under acidic conditions by using 1 ml of 6 N hydrochloric acid to prevent Ca precipitation and putrefaction. The first evaluation was carried out for two days just before starting the experimental diet period (0 time). After starting the experimental diet period, the Ca balance was evaluated three times; phase I was during the first two days, phase II during the 13th and 14th days, and phase III during the last two days before end of the experiment. All the collected urine was centrifuged immediately after its collection at 2,500 rpm for 15 min to extract the supernatant. For the fecal determination, all daily feces were burnt to ash at 550-600°C for approximately 18 h, and the resulting ash was dissolved in 1 N nitric acid. Fecal and urinary Ca excretion was measured by atomic absorption spectrophotometry (Shimadzu AA-640-12, Kyoto, Japan). Intestinal Ca absorption and Ca accumulation were calculated by using the amount of Ca intake, fecal Ca excretion and urinary Ca excretion. The dietary Ca concentration was 0.30% for the control diet and 0.32% for the milk diet.

**Measurement of the mechanical strength, weight and Ca & P contents of the femur (in vitro Exp. B)**

At each dissection, femur samples were isolated after killing by exsanguination. After the adhering connective tissues had been trimmed off, the wet weight of the femora was measured as soon as possible. The bone strength of the middle diaphysis of the femur was then tested by measuring the mechanical strength, breaking force and energy with an Iio DYN-1255 instrument as previously reported. The force and energy necessary to produce a break at the center of the femur were measured under the following conditions: the sample space was 1.0 cm, the plunger speed was 100.0 mm/min, the load range was 10.0 kg at 0 time test and 50.0 kg after 2 weeks and 4 weeks, and the chart speed was 120.0 cm/min. After testing the bone strength, all femora were dried at 98°C for 24 h, before the dry weight of the femora of each rat was measured. Thereafter, the ash weight of the femora for each rat was measured after burning to ash at 550-600°C for approximately 24 h. The samples were then dissolved in 1 N nitric acid, and the Ca and P contents were determined. Ca was measured by the method described previously, and P by the method of Fiske-SubbaRow.

**Serum Ca, P, and total protein levels (in vitro Exp. B)**

The serum Ca level was determined by atomic absorption spectrophotometry (Shimadzu AA-640-12, Kyoto, Japan), and the serum P level was measured by the method of Fiske-SubbaRow. The total protein level was determined by the biuret method.

**Statistics**

Each data value is expressed as the means ± SEM. Student’s t-test was used to analyze the differences between groups after a F-test. The data values for the milk groups were compared with the values for the control groups (MC vs. MM and FC vs. FM). In addition, an ANOVA test (with the Scheffe test) was used to analyze the differences among the four groups. A p-value of less than 0.05 is considered statistically significant.

**RESULTS**

**Experiment A (in vivo)**

1. **Body weight gain and food intake**

The body weight, body weight gain and food intake during the experiment are shown in Table 2 (Exp. A: MC-4 weeks, MM-4 weeks, FC-4 weeks, and FM-4 weeks). There was no difference in the initial body weight between the MC and MM, and FC and FM groups. The body weight at 2 weeks and 4 weeks of the MM group was slightly higher than that of MC group, but the difference was not significant. Additionally, there was no significant difference between the FC and FM body weights. The body weight gain of the MM and FM groups was not significantly different from that of the MC group, but the difference was not significant. Additionally, there was no significant difference between the FC and FM body weights. The body weight gain of the MM and FM groups was not significantly different from that of the MC and FC groups (MC vs. MM and FC vs. FM). There was no significant difference in food intake (g/day) between the MM and MM, and FC and FM groups. There was also no difference in the food efficiency (body weight gain (g/day)/food intake (g/day)) between the MM and MM, and FC and FM groups.

2. **BMD values for the lumbar spine and tibia**

The changes in BMD values from the in vivo measurements of the lumbar spine are shown in Fig. 2. The lumbar spine BMD for each rat in 4 groups (MC, MM, FC and FM) increased according to growth during the experiment. BMD of the lumbar spine for the milk groups at the 0 time was no
Effect of Milk on Bone Metabolism

Table 2. Body weight and food intake during Exp. A (in vivo study) and Exp. B (in vitro study)

<table>
<thead>
<tr>
<th></th>
<th>Body weight (g)</th>
<th>Body weight gain (g/day)</th>
<th>Experimental food intake (g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Initial</td>
<td>2 weeks</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MB</td>
<td>5</td>
<td>48.8±2.2</td>
<td>—</td>
</tr>
<tr>
<td>MC-2wks</td>
<td>5</td>
<td>48.8±2.7</td>
<td>165.0±7.1</td>
</tr>
<tr>
<td>MM-2wks</td>
<td>5</td>
<td>49.2±2.3</td>
<td>175.6±6.5</td>
</tr>
<tr>
<td>MC-4wks</td>
<td>7</td>
<td>48.8±1.6</td>
<td>166.6±4.6</td>
</tr>
<tr>
<td>MM-4wks</td>
<td>7</td>
<td>48.6±1.5</td>
<td>176.8±4.3</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FB</td>
<td>5</td>
<td>46.4±2.6</td>
<td>—</td>
</tr>
<tr>
<td>FC-2wks</td>
<td>5</td>
<td>45.8±2.3</td>
<td>138.4±4.9</td>
</tr>
<tr>
<td>FM-2wks</td>
<td>5</td>
<td>46.4±2.6</td>
<td>138.6±3.4</td>
</tr>
<tr>
<td>FC-4wks</td>
<td>7</td>
<td>46.4±1.5</td>
<td>140.1±3.0</td>
</tr>
<tr>
<td>FM-4wks</td>
<td>7</td>
<td>46.5±1.7</td>
<td>139.0±3.4</td>
</tr>
</tbody>
</table>

Mean±SE. MB, male base-line group; FB, female base-line group; MC, male control group; MM, male milk group; FC, female control group; FM, female milk group.

different from the control groups (MC vs. MM and FC vs. FM), but increased significantly at 2 weeks (p < 0.001) and 4 weeks (p<0.01) compared to the control groups.

The changes in BMD values for the total tibia (in vivo) are shown in Fig. 3. These data are similar to those for the lumbar spine. BMD for the milk groups at 2 and 4 weeks was significantly greater than for the control groups (MC vs. MM and FC vs. FM).

3. Ca Balance

There was no significant difference during the experiment in the food intake and Ca intake between the control and milk groups.

The rate of intestinal Ca absorption is shown in Fig. 4. There was no difference in the rate of Ca absorption at the 0 time between the control and the milk groups (MC vs. MM and FC vs. FM). However, during the experimental diet period (phases I, II and III) the values for the rate of Ca absorption of the milk groups were significantly higher than those of the control groups (MC vs. MM and FC vs. FM). The data for intestinal Ca absorption of the milk groups during the experimental diet period were also significantly greater than those of the control groups (MC vs. MM and FC vs. FM; data not shown).

Fig. 2. Change in the bone mineral density of the lumbar spine (Exp. A, in vivo study)

* p<0.05 (MC vs. MM), *** p<0.001 (MC vs. MM), * p<0.05 (FC vs. FM), ** p<0.01 (FC vs. FM). In vivo measurements of the bone mineral density (BMD) of the lumbar spine for each rat were taken at three different times by dual-energy X-ray absorptiometry (ALOKA DCS-600R): before starting the experimental diet (0 time), 2 weeks after starting the experimental diet (2 weeks), and at the end of the experiment (4 weeks).
In addition, the Ca accumulation and rate of Ca accumulation during the experimental diet period were also significantly higher in the milk groups (data not shown, but these results were similar to the intestinal Ca absorption and rate of Ca absorption).

**Experiment B (in vitro)**

1. Body weight gain and food intake
   The body weight, body weight gain and food intake during the experiment are shown in Table 2, the values being similar to those in Exp. A. There was no significant difference in the body weight, body weight gain, and experimental food intake between the milk diet and the control-diet groups at any time (MC vs. MM and FC vs. FM).

2. Serum calcium, phosphorus, and total protein levels
   At each dissection (Exp. B: 0 time, at 2 weeks, and at 4 weeks), the serum Ca, P and total protein values were within the normal levels in all groups. There was also no significant difference in each parameter between the control and the milk groups (MC vs. MM and FC vs. FM; data not shown.)

3. Bone strength of the femur
   The values for the strength, breaking strength and breaking energy at the center of the femur, increased

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Footnotes:

**p < 0.01 (MC vs. MM), *p < 0.05 (MC vs. MM), ***p < 0.001 (MC vs. MM), **p < 0.01 (MC vs. MM), *p < 0.05 (FC vs. FM), **p < 0.01 (FC vs. FM), ***p < 0.001 (FC vs. FM). The mechanical bone strength of the femur of each rat was measured by a breaking property (fracture) test at each dissection: at the start of the experimental diet (0 time), 2 weeks after starting the experimental diet (2 weeks), and at the end of the experiment (4 weeks).**
Effect of Milk on Bone Metabolism

Table 3. Weight and mineral contents of the femur in Exp. B (in vitro study)

<table>
<thead>
<tr>
<th></th>
<th>Bone weight (g)</th>
<th>Mineral contents (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wet</td>
<td>Dry</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MB</td>
<td>5 0.395±0.031</td>
<td>0.123±0.010</td>
</tr>
<tr>
<td>MC-2wks</td>
<td>5 1.138±0.053</td>
<td>0.439±0.016</td>
</tr>
<tr>
<td>MM-2wks</td>
<td>5 1.182±0.038</td>
<td>0.491±0.019**</td>
</tr>
<tr>
<td>MC-4wks</td>
<td>7 1.484±0.034</td>
<td>0.684±0.014</td>
</tr>
<tr>
<td>MM-4wks</td>
<td>7 1.577±0.062</td>
<td>0.758±0.024***</td>
</tr>
</tbody>
</table>

| Female |                 |                       |                      |          |          |
| FB     | 5 0.354±0.030   | 0.115±0.010           | 0.044±0.006          | 14.5±1.6 | 7.6±0.6  |
| FC-2wks| 5 0.934±0.040***| 0.393±0.016**         | 0.191±0.007          | 65.6±2.9 | 33.7±1.3 |
| FM-2wks| 5 0.991±0.034   | 0.413±0.005           | 0.204±0.007          | 70.5±2.6 | 36.3±1.4 |
| FC-4wks| 7 1.185±0.028***| 0.584±0.011***        | 0.307±0.005          | 106.±2.0 | 53.7±1.7 |
| FM-4wks| 7 1.193±0.041   | 0.620±0.022**         | 0.335±0.015          | 116.4±4.9| 59.4±2.6 |

Mean±SE. MB, male base-line group; FB, female base-line group; MC, male control group; MM, male milk group; FC, female control group; FM, female milk group. **p<0.01: MC vs. FC by Student's t-test, ***p<0.001: MC vs. FC by Student's t-test, *p<0.05: MC vs. MM by Student's t-test, **p<0.01: MC vs. MM by Student's t-test, ***p<0.001: MC vs. MM by Student's t-test, *p<0.05: FC vs. FM by Student's t-test. The statistical results by ANOVA are similar to the results of Student's t-test.

in all groups according to growth during the experiment, as shown in Fig. 5. Compared with the control groups, the breaking force and the breaking energy in the milk groups were both significantly higher at 2 and 4 weeks (MC vs. MM and FC vs. FM).

4. Bone weight and mineral contents in the femur (Table 3)

The femoral weights (wet, dry and ash) increased according to growth during the experiment. The femoral dry weight of the milk groups was significantly higher than that of the control groups (MC vs. MM and FC vs. FM). The wet and ash weights, and the Ca and P contents of the femur in the milk groups were also significantly higher than those of the control groups.

5. BMD and BMC values for the lumbar spine, tibial proximal metaphysis and tibial diaphysis

BMD values for the extracted lumbar spine and tibia are shown in Figs. 6 and 7, those of the milk groups being markedly greater than those of the control groups (MC vs. MM and FC vs. FM). The BMD values for the trabecula sites (the proximal metaphysis) and cortical sites (diaphysis) of the milk groups were also greater than those of the control groups (MC vs. MM and FC vs. FM), as shown in Table 4. The BMC values for each region were relatively similar to the BMD values (data not shown).

DISCUSSION

The results of this study clearly show that BMD from the in vivo (Exp. A) and in vitro (Exp. B) measurements, mechanical bone strength, and intestinal Ca absorption of those rats fed with the milk diet were significantly higher than those of the control groups.

BMD for in the lumbar spine and tibia of the milk-fed groups (MM and FM) was significantly higher than that of the control groups (MC and FC) in in vivo Exp. A. Compared with the control groups (MC and FC) BMD values for the lumbar spine and tibia of the milk diet groups (MM and FM) at dissection in each period were also significantly greater in in vitro Exp. B. Since the dietary contents of such main nutrients as Ca, P and protein was identical in this study, the results show that milk was an effective source of Ca for increasing BMD. Furthermore, the BMD values for the milk groups at 2 weeks were already significantly higher than those for the control groups (in vivo Exp. A and in vitro Exp. B); thus milk could be effective for enhancing the bone mass increase.

The BMD change in vivo Exp. A and BMD value of the extracted bone (in vitro Exp. B) were measured. In in vivo Exp. A, the change in BMD for the same rats was determined longitudinally. In Exp. B (in vitro
measurement of the extracted bone), the BMD values for different rats were measured at each dissection. The in vivo and in vitro values for BMD in Exp. A and Exp. B are similar and, in addition, the weight and mineral contents of the femur in the rats fed on the milk diet were significantly greater than those of the control group in the in vitro Exp. B. There was a significant positive correlation between the results of BMC by dual-energy X-ray absorptiometry (MCA; Hologic) and the ash weight, and the DCS-600 BMC value and that of the other densitometer.17) The region used in this study for measuring BMC and BMD was different from that for measuring the bone weight and mineral contents. However, there was a significant positive correlation between the values for BMD and BMC (r=0.869, p<0.001), bone weight and Ca and P contents (r=0.977, p<0.001), and BMD and Ca and P contents (r=0.387, p=0.042). These correlations and the findings reported in our previous paper indicate that measurement of bone mass by the DCS-600R instrument is suitable for assessing bone metabolism in vivo. Moreover, the longitudinal measurement of BMD (in vivo) could be valuable to estimate changes in BMD.

In addition, the mechanical bone strength of the rats fed on the milk diet was significantly greater than that of the control animals. Based on the results of the bone mass, bone weight, Ca and P contents in the bone, and mechanical bone strength in in vitro Exp. B,
we suggest that milk might be an effective and valuable Ca source for bone metabolism.

The intestinal Ca absorption and rate of Ca absorption by the rats fed on the milk diet were also significantly higher than those of the control animals during in vivo Exp. A. Although the mechanism for the Ca absorption increase in the milk diet was not investigated in this study, CPP, lactose, or other unknown components in milk could have affected intestinal Ca absorption.

The results of this study confirm the effectiveness of milk as a Ca source for bone metabolism in the growing period, and indicate lower bone mass measurements by the DCS-600R instrument to be a suitable method. To prevent osteoporosis at the later stage of life, acquiring a higher bone mass during the bone-growing period is important, and the intake of milk, as an outstanding Ca source, should be promoted.

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成長期雌雄ラットの骨代謝に対するミルクの効果

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本研究では、成長期雌雄ラットの骨に対するミルクの効果を検討した。骨成長測定装置による低骨成長測定は、機械の精度上、以前は正確かつ精密に評価することは極めて困難であった。しかし、近年、低骨成長でも正確な測定が可能となった。そこで、本研究では成長期の骨に対するミルクの効果を骨成長測定を中心に行った。

離乳直後の3週齢SD系雌雄ラットを使用し、雌雄いずれも、ミルクのみをカルシウム（Ca）源とするミルク食（Ca 0.3%，リン（P）0.3%）を摂取するミルク群・ミルク群と、炭酸Caのみをカルシウム源とするコントロール食（Ca 0.3%，P 0.3%）を摂取するコントロール群・コントロール群に分けた。なお、実験飼育期間は4週間とした。実験Aでは、生体における骨成長変化、およびCa出納を検討した。すなわち、実験開始時、実験食2週目、実験終了時（実験食4週目）の計4回の骨成長・骨成長測定、および実験飼育開始直前に含む計4回のCa出納試験を実施した。実験Bでは、実験飼育開始直前、実験食2週目、実験終了時（実験食4週目）の計3回解剖を行い、大腿骨、腰椎、脛骨を採取し、骨成長度、骨重量および骨中Ca・P含量、摘出骨成長を測定した。

その結果、実験Aにおいて、ミルク群の股関節・股骨成長度（生体測定）および腸管からのカルシウム吸収がコントロール群に比べ有意な高値を示した。また、実験Bでは、ミルク群の摘出股関節・摘出股関節成長度、および大腿骨成長度、骨重量、骨中Ca・P含量が、コントロール群に比べ有意な低値を示した。以上のことから、ミルクが成長期の骨代謝に極めて効果的であることが示唆された。さらに、DXA法を用いた生体における微量骨成長変化の測定の有用性が示された。

キーワード：ミルク、成長期ラット、骨成長、骨成長度、腸管カルシウム吸収。