The effect of calcium supplement given with a mixture of calcium carbonate and calcium citrate on the mandibular alveolar bone of pubertal rats

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Abstract: To determine whether the mixture of calcium carbonate and calcium citrate, an oral calcium supplement made from sea shell that is called UNICAL, had any beneficial effect on bone debilitation, we examined the mandibular alveolar bone of pubertal Wistar male rats by bone mineral mass, Ca/P ratio microanalysis, and scanning electron microscopy. (1) Bone mineral mass of UNICAL-fed rats in the low-calcium experiment group was not significantly different than the control group, although its value was significantly higher than in pair-fed standard diet rats. However, although the bone mineral mass of UNICAL diet rats in the calcium-deficient experiment group was significantly higher than in pair-fed standard diet rats, it was significantly lower than in the control group. (2) All Ca/P ratio values in experiment groups were significantly lower than that in control group. Ca:P ratio values of UNICAL diet rats were significantly higher than those of pair-fed standard diet rats in experiment groups. Separately, Ca:P ratio was not significantly different between UNICAL diet rats of the calcium-deficient experiment group and standard diet rats of the low-calcium experiment group. (3) On scanning electron microscope observation, UNICAL diet animals were observed to have more sufficient calcareous microdepositions in bone remodeling areas, which was thought to be one of the morphological indications of bone formation, reflecting active calcium utilization in bone metabolism, than pair-fed standard diet animals. These results suggested that the mixture of calcium carbonate and calcium citrate had a positive effect on bone debilitation to a certain extent in growth-period rats.

Key words: calcium carbonate and calcium citrate mixture, bone debilitation, bone mineral mass, Ca:P ratio microanalysis, scanning electron microscopy

Introduction

Calcium is an essential nutrient for normal growth and development. Adequate dietary calcium builds the skeleton and helps to prevent skeletal disorders during childhood and adolescence [1–3]. Inadequate dietary calcium during the critical growth and building period may result in failure to reach peak bone mass [4]. A strong bone structure in young adulthood is likely to be one of the most important factors for preventing osteoporosis and associated fractures later in life [5,6]. Approximately 20%–30% of peak bone mass can be influenced by environmental factors; another 60%–80% is explained by genetic variation [7,8]. Although it is generally recognized that optimum calcium intake is best obtained from food sources, calcium supplements are destined to become an important source of dietary calcium because the lesser food intake of modern humans compared with the food intake of humans during evolution has caused difficulty in obtaining an adequate intake of calcium [9–11].

In addition, more and more dentists’ attention has been directed to the influences of systemic bone debilitation in the dental field, for instance, on bone reparation after periapical abscess or periapical granuloma [12,13], reorganization of periodontitis [14,15], or prognosis of an implant [16]. This investigation was undertaken to determine the effect of a calcium supplement, a mixture of calcium carbonate and calcium citrate made from sea urchin shell that is called UNICAL, on the mandibular alveolar bone of pubertal rats.

Materials and methods

Animals and treatments

Thirty Wistar male rats 8 weeks old and weighing about 240 g, maintained by Seiwa Experimental Animal Research Institute, were housed in small cages individu-
ally under similar conditions. All the rats’ food was made by Oriental Yeast (Tokyo, Japan). In the control group, rats were fed on a standard diet, containing 0.449% calcium, 0.608% phosphorus, and 1000IU vitamin D₃ per 100g, with tap water for 5 weeks. In the low-calcium experiment group, rats were fed a low-calcium diet (calcium component is 30% of standard diet) with tap water for the first 3 weeks; then they were allocated into two treatment groups and pair-fed on a standard diet or the UNICAL diet, which is a mixture of calcium carbonate and calcium citrate, containing 1.697% calcium, 0.612% phosphorus, and 1000IU vitamin D₃, with tap water for a further 2 weeks. In the calcium-deficient experiment group, rats were fed a no-calcium-component diet with distilled water for the first 3 weeks; they were then treated the same as the low-calcium experiment group for a further 2 weeks. These rats were killed after being fed for 5 weeks. Their mandibles were removed and divided into two parts symmetrically between the central incisors using a dental diamond disk (#62; Shfu, Japan) with spraying water, and later treated for observations of bone mineral mass, Ca/P ratio microanalysis, and scanning electron microscopy.

**Bone mineral mass**

The mandibles were kept in 10% neutral formalin until radiographs were taken. These samples were fixed with tape on a aluminum plate (length, 15.00 mm; and thickness from 1.50 to 0.01 mm overall). Soft radiographs (ESM-2; Softex, Japan) were taken at 28 kVp, 6mA, 60s, and 70-cm distance. These films were observed by a Konica Densitometer (PDS-15; Konica, Japan). The survey light passed through 1 mm in front of the molar region and vertical to the mandibular border at 0.1 mm/s (Fig. 1). The average of peak values of cortex bone mineral mass on the lingual side and the labial side was determined for the bone mineral mass of the mandibular alveolar bone and calculated according to thickness of aluminum.

**Sample preparation for Ca/P ratio microanalysis**

The samples were cut from the incisor alveolar crest to 1 mm in front of the molar area, at thickness of 3 mm, vertical to the mandibular border, by dental diamond disk (#62; Shfu with spraying water). After fixation with 10% neutral formalin, these samples were dehydrated through a graded ethanol series and treated in 2-methyl-α-propanol, dried in 1-butyl alcohol by freeze-drying (1D-2; Japanese Electric, Japan). Sections were embedded in resin, then abraded, polished, and sputtered with aurum. Later they were observed under a scanning electron microscope (JS M T-300; Japanese Electric) equipped with energy-dispersive X-ray microanalysis (JED-2000; Japanese Electric) to determine their calcium-to-phosphorus ratios.

**Sample preparation for scanning electron microscopy**

The samples were cut as in preparation for Ca/P ratio microanalysis, and fixed with 2.5% glutaraldehyde for 1 h after being cleaned with 10% sodium hypochlorite solution by supersonic vibration to eliminate adhesion. They were rinsed by buffered phosphate acid (pH 7.2) before post-fixation. Post-fixation was performed in 1% osmic acid buffer solution (pH 7.2) under 4°C for 2h. After fixation, these samples were dehydrated through a graded ethanol series, treated with 2-methyl-α-propanol, then dried in 1-butyl alcohol by freeze-drying.

**Table 1. Results of bone mineral mass (mmAl)**

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>UNICAL diet</th>
<th>Standard diet</th>
<th>UNICAL diet</th>
<th>Standard diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD</td>
<td>2.09 ± 0.09</td>
<td>1.99 ± 0.11</td>
<td>1.90 ± 0.15</td>
<td>1.85 ± 0.13</td>
<td>1.79 ± 0.17</td>
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(ID-2; Japanese Electric). Specimens were sputter-coated with gold and later observed under a scanning electron microscope (JSM T-300; Japanese Electric).

**Results**

**Bone mineral mass**

Bone mineral mass of UNICAL diet rats in the low-calcium experiment group was not significantly different than the control group, although its value was a little lower than the control group. Its value was significantly higher than pair-fed standard diet rats. However, bone mineral mass of UNICAL diet rats in the calcium-deficient experiment group was significantly higher than that of pair-fed standard diet rats, it was significantly lower than that of the control group (Tables 1, 2).

**Ca/P ratio microanalysis**

A typical spectrum of bone demonstrated strong peaks for calcium (Ca) and phosphorus (P), with a low background and no peaks from other elements interfering with calcium or phosphorus (Fig. 2). All Ca:P ratio values in experiment groups were significantly lower than that in the control group. In experiment groups, Ca:P ratio values of UNICAL diet rats were significantly higher than those of pair-fed standard diet rats. Separately, the Ca:P ratio was not significantly different between UNICAL diet rats of the calcium-deficient experiment group and standard diet rats of the low-calcium experiment group (Tables 3, 4).

![Fig. 2. The spectrum of normal bone showing strong, characteristic peaks for calcium and phosphorus with a low background, and no peaks from other elements interfering with calcium or phosphorus](image-url)
Table 3. Results of calcium-to-phosphorus molar ratio (Ca:P)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>UNICAL diet</th>
<th>standard diet</th>
<th>Calcium deficient</th>
<th>UNICAL diet</th>
<th>standard diet</th>
</tr>
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<tbody>
<tr>
<td>Mean ± SD</td>
<td>1.963 ± 0.091</td>
<td>1.818 ± 0.042</td>
<td>1.715 ± 0.023</td>
<td>1.713 ± 0.027</td>
<td>1.659 ± 0.045</td>
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Table 4. F-test results of calcium-to-phosphorus molar ratio

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
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<tbody>
<tr>
<td>A</td>
<td>*</td>
<td>**</td>
<td>**</td>
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<tr>
<td>B</td>
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<tr>
<td>E</td>
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A, control group; B, low-Ca:UNICAL diet group; C, low-Ca:standard diet group; D, Ca-deficient:UNICAL diet group; E, Ca-deficient:standard diet group

*, P < .05; **, P < .01

Fig. 3. Scanning electron microscopy (SEM) image of bone surface in control group. Osteocytic lacunae, at the wall of which bone canaliculi are opening, are emerging on the glossy bone surface. Bar 10 μm

Fig. 4a,b. SEM image of calcium-deficient:UNICAL diet animal. Large area of bone resorption shows deep concave resorption foveas and clear division with bone matrix background (a). Sufficient calcareous microdepositions appear in the bone matrix of resorption area while many slender collagen fibers are seen on the upper surface of the bone matrix (b). Bar 10 μm

Scanning electron microscopy

In comparison with the control group (Fig. 3), the experiment groups readily showed large areas of bone resorption. In calcium-deficient experiment groups, concave resorption foveas were observed deep with a clear division from the background of bone matrix. There were many lacunae, at the wall of which bone canaliculi were opening, for emergence in resorption foveas. In UNICAL diet animals were found sufficient calcareous microdepositions appearing in the bone matrix of resorption areas and many slender collagen fibers on the upper surface of bone matrix (Fig. 4). In comparison with UNICAL diet animals, pair-fed standard diet animals showed gross resorption foveas precipitating some calcareous microdepositions. The margins of concave resorption fovea were irregular; some areas did
not show a very clear division from the bone matrix background. Collagen fibers were more often observed in bone matrix, and in some places they were seen crossed into networks on the upper surface of the bone matrix (Fig. 5).

In the low-calcium experiment group, large areas of bone resorption also were seen. However, the resorption foveas were shallower and flatter and their margins were smoother and clearer than in the calcium-deficient experiment groups. In UNICAL diet animals of the low-calcium group, fewer calcium microdepositions were observed than in the calcium-deficient experiment groups (Fig. 6). Correspondingly, they were rarely seen in pair-fed standard diet animals (Fig. 7).

Discussion

Calcium supplements are becoming an important source of dietary calcium and a basic defense against osteopenia [9–11]. Recent studies have also focused on a cancer-preventive role for calcium [17–19]. Oral calcium supplement is a very popular method of fortifying inadequate dietary calcium because of its cheap cost, convenient way of intake, and the minimal side effects [9,10], although a controversy still exists as to its effect in preventing the age-related decrease of bone mass [20,21]. There are now more than a dozen commonly prescribed calcium supplements and hundreds of different formulations commercially available [22–25]. Calcium carbonate is the major constituent in sea urchin shell and hydroxyapatite calcium, and is a preferred source of calcium supplements because of its high density of elemental calcium, about 40% by weight. Calcium citrate is another very popular formulation, and there are now effervescent preparations available. At 21% by weight, the citrate preparations have a lower calcium content but are considered much more soluble.
than calcium carbonate, which is especially important for achlorhydric patients. The citrate ion may help to modulate the propensity for the development of renal stones [22].

The mixture of calcium carbonate and calcium citrate (UNICAL) made from sea urchin shell is a kind of oral calcium supplement. The results of this study suggested a positive effect on bone debilitation in animals from puberty to early adulthood. In low-calcium diet experimental animals, this process was recovered by UNICAL diet. The bone mass of UNICAL diet experimental animals reached the level of control group; although the value was a little lower than that of the control group the difference between them was not significant, while the value was significantly higher than in pair-fed standard diet animals. The latter had a significantly lower value than the control group. In calcium-deficient diet experimental animals, the bone mass of UNICAL diet animals showed improvement in that the value was significantly higher than that of pair-fed standard diet animals, but it had not yet reached the level of the control group.

Inadequate dietary calcium during the critical growth and building period may result in failure to reach peak bone mass, causing osteopenia, osteoporosis, decreased skeletal integrity, and increased risk of fracture in later life [4–6]. The importance of ample calcium intake in early life has been made evident by recent clinical studies [26–28] and experimental studies [29]. In the present study, the experimental range was from puberty to early adulthood of rats, thought to be the critical period for peak bone mass gain. We gained satisfactory results of augmented bone mineral mass with the UNICAL diet in low-calcium experimental animals, but the result was not completely satisfactory in calcium-deficient experimental animals. The results suggested that the mixture of calcium carbonate and calcium citrate had a positive effect on bone debilitation to a certain extent, but it had not been observed to help complete recovery of serious bone debilitation caused by extremely inadequate dietary calcium in growth-period rats. In addition, the appearance suggested that recovery of serious bone debilitation is not easy to achieve. A longer time of giving the calcium supplement might be required. Also, it was thought questionable that complete recovery could be attained, particularly using only oral calcium supplements.

A threshold nutrient exerts a biological effect at levels below the threshold intake. The same principle applies to calcium nutrition [9], and this has been demonstrated convincingly in animals [30]. However, it is difficult to establish an optimum threshold level of calcium intake for a human population because this value can be expected to vary both with age and with the individual. In this study, although the calcium content of the mixture of calcium carbonate and calcium citrate was nearly 3.8 fold higher than standard diet, it was because its calcium content was below the threshold level that the positive effect on bone debilitation occurred. On the other hand, its high calcium content can be thought to be an important reason why the UNICAL diet animals could recover better than standard diet animals in both the low-calcium experiment and calcium-deficient experiment groups.

Phosphate is another important mineral content of bone, thought to have a close relation with calcium metabolism. Phosphate tends to decrease urinary calcium excretion and increases fecal calcium excretion. However, the net effect of a diet high in phosphate is probably not to influence the calcium balance significantly [31]. The theories in which the calcium and phosphate moieties initially combine are many and varied, and much debate continues as to the exact composition of bone mineral. However, it is generally accepted that bone is composed of carbonate-apatite (carbonapatite) with a calcium-to-phosphate molar ratio of 1.667 in humans [32–34]. In the calcium-to-phosphorus ratio analysis in the present experiment, the typical spectrum of bone was observed: strong peaks for calcium and phosphorus with low background, and no peaks from other elements interfering with calcium and phosphorus. The same appearance was reported by Cassella et al. [35]. Although Ca:P ratios of UNICAL diet animals were significantly higher than those of pair-fed standard diet animals in experiment groups, all values of experiment dietary groups were significantly lower than that of the control group. Some authors [36,37] have reported similar results. In this study, we noted an interesting phenomenon: the Ca:P ratio of UNICAL diet animals in the calcium-deficient experiment group was improved upward to the level of standard diet animals in the low-calcium experiment group. The phenomenon helped to demonstrate that the mixture of calcium carbonate and calcium citrate had a beneficial effect on bone debilitation.

Generally, calcium supplementation is recognized as accelerating bone formation with less inhibition of bone resorption [22]. Observation by scanning electron microscopy was helpful to understand the morphological alteration on the bone surface during the bone remodeling process. Sissons and colleagues [38,39] found that bone resorption resulting from an inadequate calcium diet was not accompanied by osteocytic resorption when osteocyte lacunae were observed by scanning electron microscopy, and reported also that osteocyte lacunae in bone formed during the period of calcium deprivation were larger than those in comparable types of bone in control rats by light microscopy.

In the present study, we caution that the appearance of calcareous microdepositions in bone remodeling
areas, which was thought to be one of the morphological indications of bone formation, reflected active calcium utilization in bone metabolism. In UNICAL, diet animals, calcareous microdepositions were found more often than those in pair-fed standard diet animals in experiment groups. This result suggested that the mixture of calcium carbonate and calcium citrate had accelerated action on bone remodeling in animals with bone debilitation. Meanwhile, by comparison with the control group and low-calcium experiment group, the concave resorption foveas in the calcium-deficient experiment group were deep and rough and their margins were irregular. Many collagen fibers appeared in the upper surface of the bone matrix of resorption areas in the calcium-deficient experiment group. It can be understood nevertheless that there existed some obviously morphological discrepancies among control group and experimental groups after being reared on calcium complement diets for 2 weeks.

References


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