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Bioavailability of calcium from bovine-bone-marrow calcium (BBMCa) and calcium carbonate in vitamin D-deficient rats

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Bovine-bone-marrow calcium (BBMCa) has been widely used for the enrichment of Ca in foods. However, its biological availability in humans and animals has not yet been fully clarified. We have therefore investigated the bioavailability of Ca from unsterilized BBMCa (UBBMCa), sterilized BBMCa (SBBMCa) (both of which are commercially available), and Ca carbonate as a control compound. Vitamin D-deficient rats were used to examine the Ca bioavailability of the BBMCa, these being unaffected by the vitamin D nutritional status in the animals. Vitamin-D-deficient rats were fed *ad libitum* a vitamin D-free 1·20% Ca diet prepared by using UBBMCa, SBBMCa, or Ca carbonate as Ca sources for 28 days. Body weight plasma Ca level, and alkaline-phosphatase activity (Alp) of the rats were measured periodically. The femoral-bone-mineral density and biomechanical strength of the rats were also measured after the feeding period. The UBBMCa and SBBMCa groups showed a rapid increase in plasma-Ca level to the normal range and an increase in bone-ash weight, bone-mineral density, and biomechanical strength, the values of which were significantly higher than those in the Ca carbonate group. The bioavailability of Ca from UBBMCa was substantially equal to that from SBBMCa in increasing plasma Ca level, bone-mineral density, and bone biomechanical strength. These findings suggest that the bioavailability of Ca from UBBMCa and SBBMCa is much higher than that of Ca carbonate in the vitamin D-deficient state, and they are available as Ca sources for the enrichment of Ca in food.

INTRODUCTION

Osteoporosis is a severe health problem in the elderly, producing disability through the development of hip fracture. Osteoporotic fractures increase in frequency with age and are observed more commonly among post-menopausal women, since bone strength is closely related to bone mineral density. Many cross-sectional studies and prospective studies have demonstrated the positive effect of physical activity (Martin *et al.*, 1987) and the negative effect of lifestyle factors (Wardlaw *et al.*, 1988), such a cigarette smoking and coffee consumption, on bone-mineral density. Calcium(Ca) deficiency has recently been reported to be one of the most important risk factors causing osteoporosis, and increase in dietary Ca intake may serve to prevent bone losses even in post-menopausal and elderly individuals (Lee *et al.*, 1981; Polly *et al.*, 1987). The recommended dietary allowance (RDA) for Ca in Japan is 600 mg per day for adults. However, the Japanese National Nutrition Survey reported that the average Ca dietary intake in Japanese has never reached the level of the RDA in the past decade. It has also pointed out that the dietary Ca intake in Japanese largely depends not only on milk and dairy products but also on fish, vegetables or poultry. The actual Ca intake may therefore be much lower than expected because of the low Ca bioavailability of the latter (Kanematsu, 1953). In order to increase the Ca nutritional status, newly developed Ca compounds, such as oyster shell electrolysate (Fujita *et al.*, 1988, 1990; Yoshimoto *et al.*, 1990) and Ca-citrate-malate (CCM) (Smith *et al.*, 1991) have been reported as effective Ca sources. Among these Ca compounds, BBMCa has been commercially available and widely used for the enrichment of Ca in food. However, the biological availability of Ca from BBMCa has not yet been clarified. In addition, it is still unknown as to whether the bioavailability of raw and unsterilized BBMCa (UBBMCa) and that of commercially available sterilized BBMCa(SBBMCa) are different. A number of different techniques for measuring Ca bioavailability in humans and animals have been reported. The balance

technique has been the most commonly used because of good information on intestinal absorption and the utilization of Ca from different Ca sources. However, there are many problems in the performance and interpretation of Ca-balance studies. In particular, inaccuracies in the collection of faecal Ca and in the contamination of dietary Ca cause large errors in estimates of Ca availability. Furthermore, apparent absorption, which can be calculated by the difference between Ca intake and Ca excretion into faeces and urine is not so high in small animals as that observed in humans. This technique will therefore be less satisfactory in the fed animals, especially in rats. As an alternative way of examining the bioavailability of Ca from the test materials, measurements of the factors influencing plasma Ca from metabolism and bone formation will constitute useful information on Ca bioavailability. There are numerous factors, such as hormones, vitamins, minerals, age and sex which influence both intestinal Ca absorption and mineralization. To examine the bioavailability of Ca from the BBMCa and Ca carbonate and to explore the bioavailability of the BBMCa, we used vitamin D-deficient rats. We report here the bioavailability of Ca from UBBMCa and SBBMCa in vitamin D-deficient rats. Ca carbonate was used as a control compound.

Table 1. Composition of diets

Ingredient	Composition of diet (g/100 g)			
	Diet 11	Control diet	UBBMCa diet	SBBMCa diet
Ca carbonate	1·1	3·0		
UBBMCa			14·0	
SBBMCa				14·0
Glucose monohydrate	67·5	65·6	54·6	54·5
Vitamin-free casein	18·0	18·0	18·0	18·0
Cysteine	0·2	0·2	0·2	0·2
Choline chloride	0·2	0·2	0·2	0·2
Cottonseed oil	10·0	10·0	10·0	10·0
Equimolar mixture of KH_2PO_4 and K_2HPO_4	0·9	0·9	0·9	0·9
Ca- and phosphorus-free salt mixture[a]	2·0	2·0	2·0	2·0
Water soluble vitamin mixture[b]	0·1	0·1	0·1	0·1
Concentration of Ca	0·44	1·2	1·2	1·2

[a] The Ca- and phosphorus-free salt mixture contains 57·29% of KCl, 20·9% if NaCl, 17·9% of $MgSO_4$, 3·22% of $FeSo_4.7H_2O$, 0·078 of $CuSO_4.5H_2O$, 0·113% of NaF, 0·004% $CoCl_2.6H_2O$, 0·01% of KI, 0·04% of $MnSO_4.H_2O$, 0·44% of $ZnSO_4.7H_2O$ and 0·005% of $(NH_4)\ 6Mo_7O_{24}.4H_2O$.
[b] The water-soluble vitamin mixture contains 0·5% of thiamine, 0·5% of riboflavin, 0·5% of pyridoxine, 2·8% of Ca pantothenate, 2·0% of nicotinamide, 20% of inositol, 0·02% of folic acid, 0·002% of vitamin B_{12}, 0·01% of biotin, and 73·668% of glucose monohydrate.

MATERIALS AND METHODS

Animals and diets

Eighteen male Wistar-strain rats, approximately four weeks old and averaging 40 g in weight, were purchased from Japan SLC Inc. (Shizuoka, Japan). They were housed in multiples and fed a vitamin D-free diet (Diet 11, Suda *et al.*, 1970) for six weeks and subsequently a vitamin D-free and Ca-free diet (Diet 11-Ca) for one week. During the feeding period, 0·2 ml of a fat-soluble vitamin solution containing vitamins A (25 mg), E (500/mg), and K (60 mg) in 100 ml of cottonseed oil was given orally twice a week. The vitamin D-deficient rats with a low plasma Ca level (average: 5·1 mg/100 ml) were divided into three groups of six each. One group was fed *ad libitum* a control diet containing 1·20% Ca prepared by Ca carbonate as shown in Table 1. The other two groups received *ad libitum* the BBMCa diets with demineralized water. The fat-soluble vitamin solution was given orally twice a week. The amount of diet consumed was recorded daily. All rats were kept in conventional metal-wire cages under specific pathogen-free conditions. The room temperature and humidity were kept at 24 ± 2°C and 55 ± 5%, respectively. During the feeding period, approximately 100 μl of blood samples were taken from tail veins of the overnight-fasted rats once a week, and each 20 μl of plasma were used for measuring plasma Ca concentration and alkaline phosphatase activity. Rats were fasted for 17 hours before sacrifice. After the feeding period of 28 days, blood was taken by cardiac puncture by using a heparinized syringe under light anaesthesia with diethylether. Bilateral femora were taken from each animal. Adherent soft tissue was removed and specimens were laid on an X-ray film to make a radiophotograph by using a soft X-ray radiophotographer (Softex CMB-2, SOFTEX Co., Japan).

Ca carbonate, UBBMCa and SBBMCa

Ca carbonate was purchased from Nakalai Tesque Inc. (Kyoto, Japan). UBBMCa and SBBMCa were supplied by Sumitomo Chemicals Co. (Tokyo, Japan). SBBMCa was prepared by heating UBBMCa at 80–85°C for 30 min for sterilization. The general compositions of UBBMCa and SBBMCa were similar, as is shown in Table 2. We previously reported the presence of vitamin D_3 metabolites, such as D_3, 25-hydroxyvitamin D_3(25-OH-D_3), 24,25-dihydroxyvitamin D_3(24,25-$(OH)_2$-D_3) and 1,25-dihydroxyvitamin D_3(1,25$(OH)_2D_3$) in human bone marrow at levels similar to those in plasma. BBMCa was therefore considered to contain certain amount of vitamin D_3 metabolites that may affect the bioavailability of Ca from BBMCa. We thus measured the concentration of vitamin D_3 metabolites in UBBMCa and SBBMCa by a method previously reported (Takeuchi *et al.*, 1989; Masuda *et al.*, 1992). As shown in Table 3, both UBBMCa and SBBMCa contained significant amounts of D_3, 25-OH-D_3, and 1,25$(OH)_2D_3$ but not 24,25$(OH)_2D_3$. When the total antirachitic activities were calculated by using conversion

Table. 2. General composition of UBBMCa and SBBMCa

Nutrients	Composition of BBMCa (g/100 g)	
	UBBMCa	SBBMCa
Proteins	24·4	24·3
Lipids	21·2	21·9
Carbohydrate	20·7	21·3
Fibers	0·1	0·1
Ash	30·0	28·8
Moisture	3·6	3·6
Concentrations of Ca and phosphorus in BBMCa		
Ca	8·7	8·0
Phosphorus	4·1	3·9

factors proposed by Reeve *et al.* (Reeve *et al.*, 1982), these were 40·1 and 23·1 IU/100 g, respectively. The UBBMCa and SBBMCa diets contained 5·59 and 3·41 IU/100 g of vitamin D activity, respectively. Since the rats of UBBMCa and SBBMCa diet groups consumed about 12 g and 11 g per day during the feeding period, respectively, it is concluded that they daily ingested the antirachitic activity corresponding to 1/15 and 1/27, respectively of the physiological dose (10 IU/day for rat) from their diets during the feeding period.

Plasma chemistry

Plasma Ca levels were measured by atomic-absorption spectrophotometry after dilution in 0·5% strontium solution. Plasma inorganic phosphorus (Pi) levels were measured by a colorimetric method (Chen *et al.*, 1956). Plasma alkaline phosphatase activity (Alp) was measured by a colorimetric method using *p*-nitrophenyl-phosphate as a substrate (Alakaline phosphate K-test, Wako Pure Products KK, Japan). Plasma levels of 25-OH-D_3 were measured by a method previously reported (Okano *et al.*, 1981).

Table 3. Concentrations of vitamin D_3 metabolites in UBBMCa, SBBMCa, UBBMCa diet, and SBBMCa diet

BBMCa and BBMCa diets	Vitamin D_3 metabolites (ng/100 g)[a]			
	D_3	25-OH-D_3	24,25$(OH)_2D_3$	1,25$(OH)_3D_3$
UBBMCa[b]	114	145	N.D.	16·2
SBBMCa[b]	106	50·2	N.D.	22·0
UBBMCa diet[c]	16·0	20·3	N.D.	2·24
SBBMCa diet[c]	14·9	7·04	N.D.	3·50

[a] Abbreviations of D_3, 25-OH-D_3, 24,25$(OH)_2D_3$, and 1,25$(OH)_2D_3$ stand for vitamin D_3, 25-hydroxyvitamin D_3, 24,25-dihydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3, respectively.
[b] UBBMCa and SBBMCa represent the unsterilized bovine-bone-marrow calcium and the sterilized bovine-bone-marrow calcium, respectively.
[c] UBBMCa diet and SBBMCa diet represent the diets containing 1·20% Ca prepared by either UBBMCa or SBBMCa as shown in Table 1.

Bone-mineral density

The bilateral femora of each animal was removed and cleaned of adherent soft tissue. All the specimens were X-rayed using a soft X-ray apparatus and Fuji 100 X-ray film (25·4 × 30·5 cm, Fuji Photo Co., Ltd., Tokyo, Japan) with a 1·0-mm thickness of aluminium board on the film under conditions of 30 KVP, 3 mA, 30 s and FED of 45 cm. To assess the bone status quantitatively, the cortical-thickness index (CTI) and bone density (Σ *GS/D*) were measured by scanning a soft X-ray picture of the midpoint of the right femur by a method previously reported (Inoue *et al.*, 1988). As shown in Fig. 1, CTI is a value obtained by dividing cortical width ($d_1 + d_2$) by bone width (*D*). The parameter ΣGS is an integrated pattern area, which is determined opticodensitometrically by using an aluminium slope-wedge as a standard for mineral density. The value $\Sigma GS/D$ is obtained by dividing ΣGS by *D* and correspond to an apparent bone-mineral density.

Bone biomechanical strength

After radiography, bone-breaking tests were made on a computerized bone-strength-measuring apparatus MZ-501D (Maruto Testing Machine Co., Tokyo, Japan). In the three-point bending test, proximal and distal ends

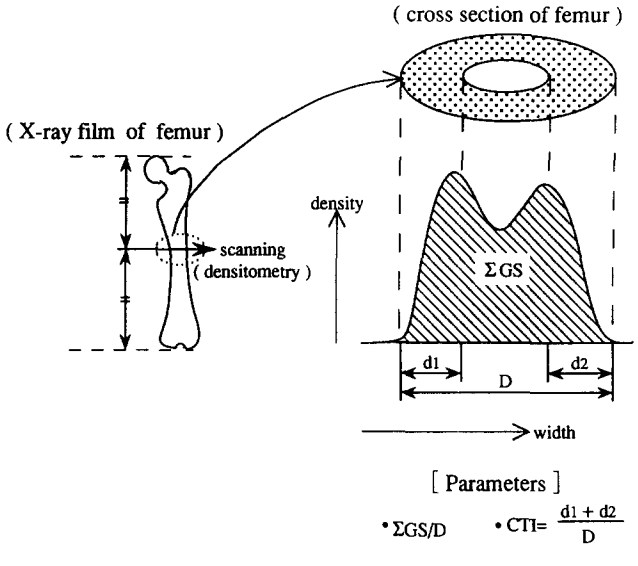

Fig. 1. Bone parameters related to mineral density. The bilateral femora of rats were X-rayed using a soft X-ray apparatus and Fuji 100 X-ray film with a 1·0-mm thickness of an aluminium board on the film under the conditions of 30 KVP, 3 mA, 30 s, and FED of 45 cm. The cortical-thickness index (CTI) and $\Sigma GS/D$ were measured by scanning a soft X-ray picture of the midpoint of the femur by densitometry. As shown in the right side of the figure CTI is a value obtained by dividing cortical width ($d_1 + d_2$) by bone width (*D*); ΣGS is an integrated pattern area measured by densitometry by using an aluminium slope-wedge as a standard for mineral density; $\Sigma GS/D$ is a value obtained by dividing ΣGS by *D* and corresponding to an apparent bone-mineral density.

of the left femur were supported by a fulcrum, and the distance between the supports (fulcra points) was 1·2 cm. The breaking force was applied perpendicularly to the long axis of the bone at midshaft with a crosshead speed of 5·0 mm/min. A specimen was broken from the anterior to the posterior plane and the load energy (kgf) and the displacement (mm) needed for bone-breaking were measured. In the torsional-loading test, the proximal and distal ends of the right femur were embedded in a 1-in × 1-in × 1-in Teflon mold fixture filled with a fast-setting dental acryl resin (GC Ostron 100 m G-C Dental Industrial Corp., Tokyo, Japan). The specimen was then mounted in a torsion apparatus. The apparatus with the fixed end of bone was equipped with a torque transducer (Maruto Testing Machine Co., Tokyo, Japan) and the rotating end was coupled to a precision angular potentiometer (Maruto Testing Machine Co., Tokyo, Japan). The rotating end was driven by a ram of a servo-hydraulic materials, and the torque and angle of deformation were recorded on an *X–Y* recorder. After loading to failure, the maximum torque (kgf/cm) and maximum angle of deformation (degree) were calculated by computer.

Bone chemistry

The left femur was placed in a mortar and heated to 600°C for 6 h in an electric furnace. The bone ash thus obtained was dissolved in 1·0 ml of hydrochloric acid, the solution being diluted to 100 ml with distilled water, and the concentrations of Ca and Pi were measured by the same procedures was described in the section above on plasma chemistry.

Statistical analysis

Statistical analysis of those parameters measured throughout the 28-day experimental period was carried out as two-way analysis of variance with variable of time and treatment. Statistical analysis of those parameters measured at the end of the experiment was carried out as a one-way analysis of variance.

RESULTS

Changes in body weights and plasma-Ca levels

As shown in Fig. 2(A), the three groups showed a progressive gain in body weight throughout the feeding period, and there were no significant differences between their body weights at the end of the feeding period. There were also no significant differences between their food intakes throughout the feeding period. As shown in Fig. 2(B), the initial hypocalcaemia of the UBBMCA group and the SBBMCa group had completely recovered to the normal range by two and three weeks, respectively, and thereafter their plasma-Ca levels were maintained within the normal range

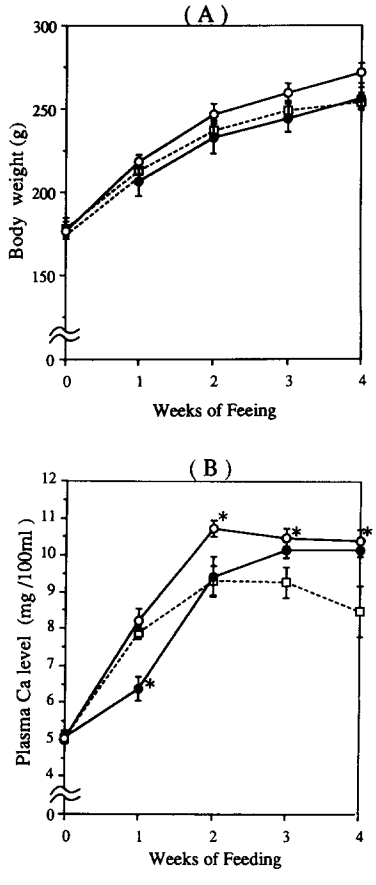

Fig. 2. Changes in body weights and plasma Ca levels of rats fed a diet containing UBBMCa, SBBMCa or Ca carbonate. Values are presented as the mean ±SEM. The value at week 0 in the figures represents that of each group measured just before the feeding of experimental diets started. (*Significantly different from control ($p < 0.05$).

(9–11 mg/100 ml) until the end of feeding, on the other hand, the control group showed similar recovery from hypocalcemia by two weeks, but then the plasma Ca level of the group fell gradually to a moderate hypocalcemia at the end of the feeding period.

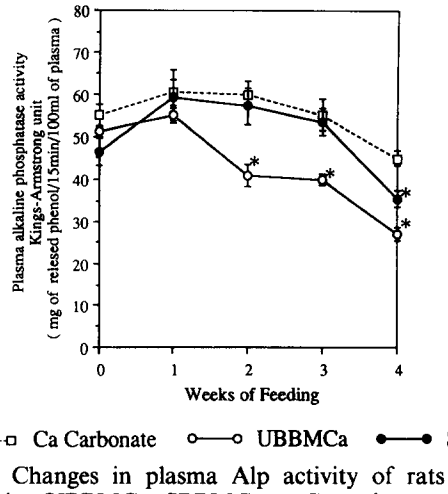

Fig. 3. Changes in plasma Alp activity of rats fed a diet containing UBBMCa, SBBMCa or Ca carbonate. Values are expressed as the mean ±SEM. The value at week 0 in the figures represents that of each group measured just before the feeding of experimental diets started. * Significantly different from control ($p < 0.05$).

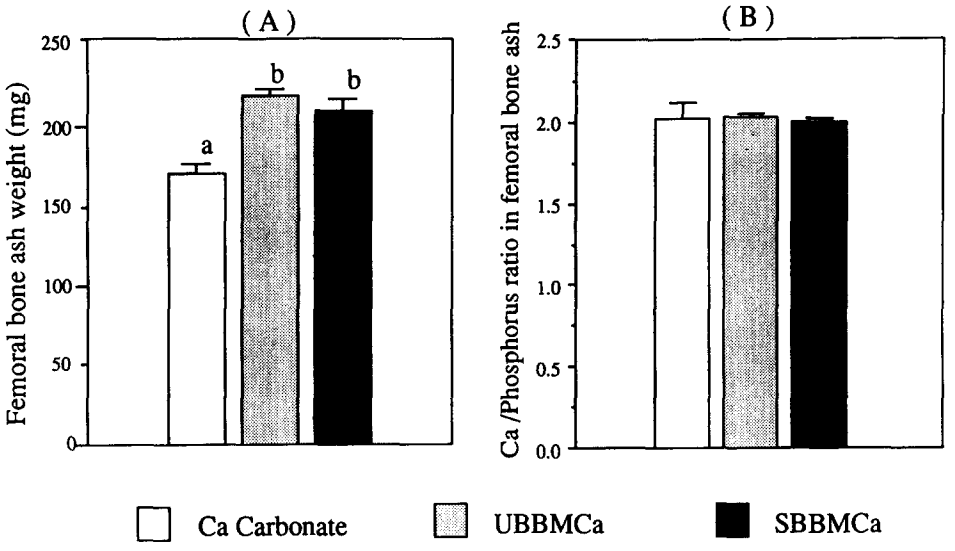

Fig. 4. Bone-ash weights and Ca/phosphorus weight ratios of rats fed a diet containing UBBMCa, SBBMCa or Ca carbonate. Values are expressed as the mean ±SEM. (a,b: Means that do not share a common letter are significantly different at $p < 0{\cdot}05$.)

Changes in plasma alkaline phosphatase(Alp) activity and plasma 25-OH-D_3 level

Figure 3 shows the plasma Alp activity of the three groups. The Alp activities of the UBBMCa group decreased continuously from week one to the end of the feeding period and were significantly lower than the levels of the other two groups. The Alp activity of the SBBMCa group was significantly lower than that in the control group at the end of the feeding period, although the Alp activities of both groups were similar to one another up to three weeks. To confirm the nutritional status of vitamin D_3 in all rats, the concentration of 25-OH-D_3 in the plasma of each group was measured at the end of the feeding period. As a result, the plasma 25-OH-D_3 level of the UBBMCa group was 1·82 ± 0·16 ng/m, while no 25-OH-D_3 was found in the other two groups.

Femoral-ash weight and Ca/Pi ratios

Figure 4(A) shows the femoral-ash weights in the three groups. The femoral-ash weight in the UBBMCa and SBBMCa groups was significantly higher than that in the control group. There was no significant difference in the femoral-ash weight between the UBBMCa group and the SBBMCa group. To examine the bone-mineral quality, we measured the concentrations of Ca and phosphorus. As shown in Fig. 4(B), there was no significant difference in the weight ratios of Ca and phosphorus in femoral ash among the three groups, which suggested that bone-mineral quality appeared to be similar among the three groups.

Bone-mineral density

Figure 5(A) and (B) shows the bone-mineral densities of the three groups. As indicated in Fig. 5(A), the

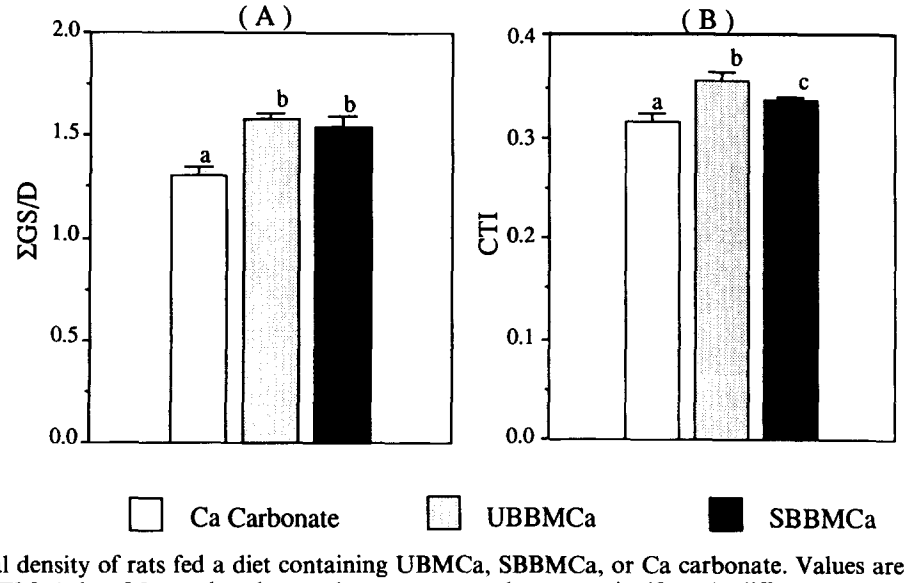

Fig. 5. Bone-mineral density of rats fed a diet containing UBMCa, SBBMCa, or Ca carbonate. Values are expressed as the mean ±SEM. (a,b,c: Means that do not share a common letter are significantly different at $p < 0{\cdot}05$.)

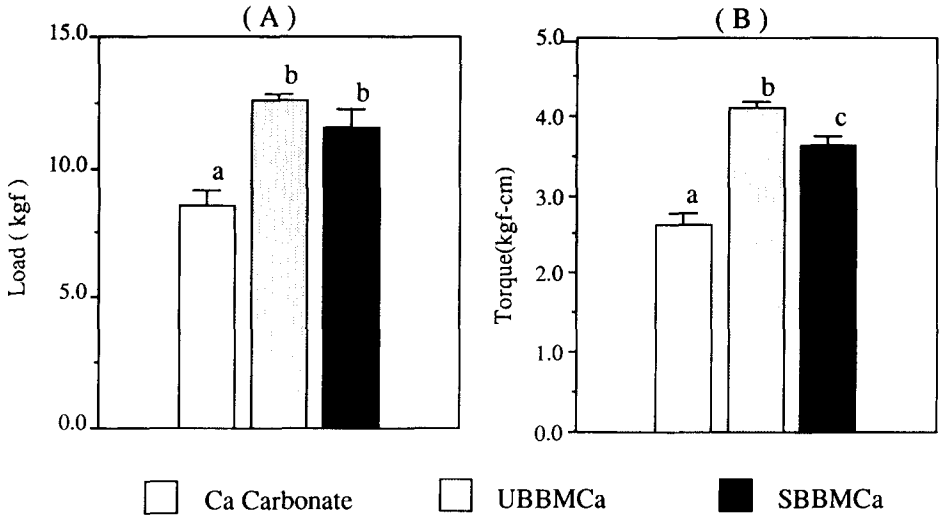

Fig. 6. Bone biomechanical strength of rats fed a diet containing UBBMCa, SBBMCa or Ca carbonate. Values are expressed as the mean ±SEM. (a,b,c: Means that do not share a common letter are significantly different at $p < 0.05$.)

values of $\Sigma GS/D$ in the UBBMCa and SBBMCa groups were significantly higher than that in the control group, and there was no significant difference in the values of $\Sigma GS/D$ between the UBBMCa group and the SBBMCa group. On the other hand, the values of CTI in the UBBMCa group and the control group were the highest and the lowest, respectively, among the three groups.

Bone biomechanical strength

Figures 6(A) and 6(B) show the bone biomechanical strength in the three groups. As indicated in Fig. 6(A), the values of load energy in the UBBMCa and SBBMCa groups were significantly higher than that in the control group, and there was no significant difference in the values of load energy between the UBBMCa group and the SBBMCa group. On the other hand, as shown in Fig. 6(B), the values of torque energy in the UBBMCa group and the control group were the highest and the lowest among the groups, respectively.

DISCUSSION

Recently, a number of studies have demonstrated that reduced Ca intake could be one of the major risk factors causing osteoporosis, hypertension, and colon cancer. These reports have prompted increased consumption of Ca in Japan, the USA, and many European countries. Nevertheless, most Japanese and more than 60–75% of American adults (Poneros & Edman, 1983) consume less than the RDAs of 600 mg or 800 mg of Ca per day. Dairy products are the major dietary source of Ca in many countries. However, these foods are not available for individuals with lactose intolerance, obesity, and restriction of food energy. Thus, it is worthwhile creating alternative food sources containing highly bioavailable Ca. Many food-Ca sources have therefore been developed in recent years. Among them, BBMCa is of particular interest because it contains not only Ca but also other nutrients, such as fat, proteins, minerals and vitamins that are required for normal bone growth. BBMCa has recently been widely used as an additive for the enrichment of Ca in food in Japan and many other countries. However, little is known about the bioavailability of Ca from BBMCa, especially SBBMCa formulated for foods. The present study is the first detailed one concerning the bioavailability of Ca from UBBMCa and SBBMCa. Vitamin D_3 is the most important factor influencing both intestinal Ca absorption and bone mineralization in mammals. The nutritional status of vitamin D_3 in animals should therefore be strictly controlled when bioavailability of Ca is evaluated. In order to make clear the role of vitamin D_3 metabolites contained in BBMCa, we used vitamin D-deficient rats as an appropriate animal model for the evaluation of Ca bioavailability. The rats were kept under vitamin D-deficient conditions during the feeding period. The bioavailabilities of UBBMCa and SBBMCa in increasing plasma Ca levels (Fig. 2) and decreasing plasma Alp activity (Fig. 3) were significantly higher than those of Ca carbonate. Furthermore, The UBBMCa and SBBMCa groups showed higher values of $\Sigma GS/D$ and CTI in bone-mineral density (Fig. 5) and higher values of load energy and torque energy in bone biomechanical strength (Fig. 6) than the respective values in the control group. In addition, the increase in bone-mineral density was strongly correlated with the increase in bone-ash weight (Fig. 4). The reason why BBMCa showed higher Ca bioavailability than Ca carbonate is unknown. However, BBMCa may contain certain amounts of vitamin D_3 metabolites (Table 3), which could be responsible for higher Ca bioavailability of BBMCa and the difference between the bioavailability of the BBMCa and that of Ca carbonate may be small or disappear in vitamin D-replete rats. Studies to identify the components responsible for the enrichment of the Ca bioavailability of BBMCa are in progress. Additional studies in rats are also needed to determine the

effects of UBBMCa and SBBMCa on bone growth when dietary Ca is lower than 1.20%. Since the prevention of bone loss is a primary concern in the elderly, feeding studies with BBMCa in older rats would be necessary. In conclusion, the findings obtained in the present study strongly suggest that the bioavailability of Ca from UBBMCa and SBBMCa in maintaining plasma Ca homeostasis and increasing bone growth is significantly higher than that of Ca carbonate in vitamin D-deficient rats and the differences in Ca bioavailability from UBBMCa and SBBMCa are due, at least in part, to the differences in vitamin D activity of the materials.

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