Absorbability of Calcium from a New Calcium Supplement Prepared from Bovine Marrow-Free Bone in Rats

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(Received March 10, 1998)

Summary Powdered bovine marrow-free bone was completely solubilized with lactic and citric acids under reduced pressure. The resulting solution was lyophilized to obtain a stable powder form (total bone extract, TBE), and the calcium (Ca) absorbability of TBE from intestine was investigated in normal rats. Each animal perorally received 10 mg of Ca in 1 mL of distilled water as extrinsic 45Ca-labeled TBE, intrinsic 45Ca-labeled Ca lactate, or intrinsic 45Ca-labeled Ca carbonate. The amount of radioactivity in plasma was measured periodically up to 34 h after dosing, and pharmacokinetic parameters were calculated from the radioactivity in plasma. The time taken to reach the maximal 45Ca level (Tmax) did not differ among the three groups. The area under the plasma 45Ca level/time curve (AUC∞) and the radioactivity at Tmax (Cmax) values for the TBE group were significantly higher than those of the Ca carbonate group. Similar results were observed between the Ca lactate and the Ca carbonate groups. No significant difference was observed in the AUC∞ and the Cmax values between the TBE and the Ca lactate groups. Radioactivity in a femur 34 h after dosing was highest in the Ca lactate group and lowest in the Ca carbonate group among the three groups. Both the TBE and the Ca lactate groups showed significant higher whole-body 45Ca retention than the Ca carbonate group did, although no significant difference was found between the TBE and the Ca lactate groups. These findings indicate that the Ca absorbability of TBE is almost comparable with that of Ca lactate and higher than that of Ca carbonate. Therefore TBE would be useful as a Ca supplement with relatively high absorbability from intestine.

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Abbreviation: TBE, total bone extract.
It is generally believed that bovine bone, which consists mainly of hydroxyapatite, a crystal form of Ca phosphate, is a relatively inferior calcium source compared with other Ca sources, such as Ca carbonate, Ca lactate, Ca citrate, Ca gluconate, milk, and other dairy products. This belief results from the hypothesis that the absorbability of various Ca salts is greatly dependent on their solubility in aqueous (1) solution because Ca is believed to be absorbed as its free or ionized form from intestine (2). Therefore Ca salts must be dissolved in water before absorption. Hydroxyapatite is well known to be insoluble in water. However, Heaney et al, using a double-isotope method with $^{45}$Ca and $^{47}$Ca, reported that the absorbability of food Ca was not clearly related to the solubility of the dominant chemical form in the food concerned (3). They also suggested that even under controlled, chemically defined conditions, the solubility of a Ca source has very little influence on its absorbability and that the absorbability of Ca from food sources is mainly determined by other food components. Therefore the absorbability of Ca from bovine bone may not markedly be inferior to that from other Ca salts, regardless of poor solubility. However, only a few studies have been carried out on the in vivo absorption of Ca from bovine bone in humans and animals (3, 4). Ohtani et al recently developed a new Ca supplement prepared from bovine bone, namely, total bone extract (TBE) (5). TBE is prepared by solubilizing powdered bovine marrow-free bone with lactic and citric acids in water under reduced pressure. The resulting solution is lyophilized to obtain a stable powder form, TBE. Thus TBE mainly consists of hydroxyapatite, lactic acid, and citric acid and contains some minerals and proteins originally present in bone. In this study we compared the Ca absorbability of TBE with that of Ca carbonate and of Ca lactate, the solubility of which in water is respectively very low and relatively high (6). TBE was extrinsically labeled with $^{45}$CaCl$_2$, and Ca carbonate and Ca lactate were intrinsically labeled with the same radioisotope. Isotopic tracer procedures for measuring the absorption of Ca from Ca supplements are based on the assumption that the tracer is absorbed with the same efficiency as the intrinsic nonradioactive Ca in a supplement (7, 8).

**Key Words**  bovine marrow-free bone, solubilization with organic acids, Ca carbonate, Ca lactate, absorbability from intestine

**MATERIALS AND METHODS**

*Preparation of total bone extract (TBE).*  TBE was prepared from fresh bovine marrow-free cortical bone. The bone was frozen in liquid nitrogen and crushed into a fine powder. The resulting bone powder was mixed with lactic acid in distilled water (DW) at a weight ratio of bone powder : lactic acid : DW of 1 : 1.2 : 7.8 for 1 h under reduced pressure. The residue was subsequently mixed with citric acid at a weight ratio of bone powder (initial weight) : citric acid of 1 : 0.028 under the
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above condition. The resulting clear solution was lyophilized to obtain a stable powder form, TBE.

**Preparation of radiolabeled calcium sources.** TBE was extrinsically labeled with radioactive Ca ($^{45}$Ca) using $^{45}$Ca chloride ($^{45}$CaCl$_2$) (9, 10). Both Ca carbonate and Ca lactate were intrinsically labeled with the same radioisotope according to the method of Kanerva et al (9, 10).

- $^{45}$Ca carbonate: A $^{45}$CaCl$_2$ (Amersham, Japan) solution was combined with a sodium carbonate solution at a molar excess of carbonate, and the resulting suspension was stirred at room temperature for 2 h. The precipitate was collected by centrifugation, rinsed with distilled water, and dried in an oven at 60°C overnight.

- $^{45}$Ca lactate: A lactic acid solution was combined with a molar excess of concurrently synthesized $^{45}$Ca carbonate, and the resulting suspension was stirred at room temperature for 2 h. The precipitate was collected by centrifugation, rinsed with distilled water, and dried in an oven at 60°C overnight.

Radioactive TBE ($^{45}$CaTBE): TBE was extrinsically labeled with $^{45}$Ca by suspending TBE in distilled water, adding the $^{45}$CaCl$_2$, and stirring the mixture overnight. All other reagents were of analytical grade and were from Wako Pure Chemicals (Tokyo, Japan). The radioactivity of $^{45}$CaCO$_3$, $^{45}$Ca lactate, and $^{45}$CaTBE was 35.9, 34.8, and 36.3 kBq/10 mg Ca, respectively.

**Animals.** All animal experiments were approved by the Animal Committee of Kobe Pharmaceutical University, and the animals were maintained in accordance with the guidelines for the care and use of laboratory animals issued by Kobe Pharmaceutical University. Fifteen Wistar-strain male rats (Japan SLC, Hamamatsu, Japan) weighing 150 g each were individually housed, with free access to laboratory chow (CE2; Clea Japan, Tokyo, Japan) and distilled water, in wire stainless-steel cages in an air-conditioned room (temperature 23 ± 1°C; relative humidity 55 ± 10%) with a 12 h light-dark cycle. The animals were divided into three groups of five rats each. The rats were deprived of food for 17 h and of water for 2 h before receiving a radiolabeled Ca dose. Each rat was perorally administered 10.0 mg of Ca radiolabeled with 1.0 μCi $^{45}$Ca as TBE, Ca carbonate, or Ca lactate in a 0.5 mL distilled water dose by using a stainless-steel intubation needle and a 1.0 mL plastic syringe. Residual solution in the syringe after the first administration was combined with another 0.5 mL of distilled water, and the solution was administered to the same animal. Each rat was placed in a Bollman’s cage (Natsume, Tokyo, Japan) immediately after receiving the radioisotope. The supplies of food and distilled water were resumed 3 h after dosing. One pooled sample of urine and one pooled sample of feces from each rat were collected over a 34 h period. Twenty microliters of plasma were collected from rat tail veins at 0, 1, 3, 6, 10, 24, and 34 h after administration, and the amount of radioactivity in plasma was measured by liquid scintillation counting (2300 TRI-carb Pakard). All rats were sacrificed by overdosing with pentobarbital sodium (Nembutal) 34 h after administration of the radioisotope. A femur and kidney were removed from each rat. These samples were prepared for measurement by liquid scintillation counting of the amount of
Radioactivity they contained. The preparation involved ashing in a muffle furnace at 600°C for 4 h and subsequent dissolution of the ash in 0.3 N HCl.

Pharmacokinetics of plasma $^{45}$Ca and whole-body $^{45}$Ca retention. The area under the plasma $^{45}$Ca radioactivity level/time curve (AUC: dpm·h/mL) was calculated by using the trapezoidal rule. Whole-body $^{45}$Ca retention was calculated from the $^{45}$Ca radioactivity of feces and urine collected over a 34 h period. The whole-body retention efficiencies of $^{45}$Ca were calculated from the following formula:

$$\text{Retention efficiency (\%)} = \frac{\text{(ingested }^{45}\text{Ca} - \text{fecal }^{45}\text{Ca} - \text{urinary }^{45}\text{Ca})}{\text{(initial }^{45}\text{Ca})} \times 100$$

**Statistical analyses.** Values are expressed as the means ± SE for five animals. A statistical analysis was performed by two-way ANOVA coupled with Duncan's multiple range test, and differences were considered significant at $p < 0.05$.

RESULTS

Time-course changes in plasma $^{45}$Ca radioactivity from the rats perorally administered TBE, Ca carbonate, or Ca lactate radiolabeled with $^{45}$Ca are shown in Fig. 1. The plasma radioactivity for the three groups began to increase 1 h after administration and reached the maximal value at 3 h, followed by a gradual decline that had not reached a baseline level by 34 h. The plasma radioactivity for the Ca lactate and TBE groups was significantly higher than for the Ca carbonate group at 1, 3, 6, and 10 h after administration ($^* p < 0.05$ vs. Ca carbonate, $^{**} p < 0.01$ vs. Ca carbonate).
Table 1. Pharmacokinetics of plasma $^{45}$Ca radioactivity from TBE, Ca carbonate, and Ca lactate in rats (34 h).

| Ca compounds | Tmax (h) | Cmax (dpm/mL) | MRT (h) | AUC$\infty$ (dpm·h/mL) |
|--------------|----------|---------------|---------|------------------------|
| TBE          | $3.0 \pm 0.0^a$ | $3,140 \pm 230^{b**}$ | $12.4 \pm 0.7^d$ | $36,800 \pm 3,900^{**}$ |
| Ca carbonate | $2.6 \pm 0.4^a$ | $1,660 \pm 160^c$  | $12.7 \pm 2.0^d$ | $20,000 \pm 3,800^f$  |
| Ca lactate   | $3.0 \pm 0.0^a$ | $3,300 \pm 160^{b***}$ | $12.8 \pm 0.3^d$ | $42,800 \pm 4,400^{***}$ |

Data not sharing the same subscript are significantly different (*p < 0.05, **p < 0.01, ***p < 0.001).

Fig. 2. $^{45}$Ca radioactivity in the femur of rats perorally administered TBE, Ca carbonate, or Ca lactate radiolabeled with $^{45}$Ca. Each column represents the means ± SE of five rats. The $^{45}$Ca radioactivity of the TBE and the Ca lactate groups was significantly higher than that of the Ca carbonate group ($p < 0.001$). The $^{45}$Ca radioactivity of the Ca lactate group was significantly higher than that of the TBE groups ($p < 0.05$).

Ca absorbability of TBE with those of Ca carbonate and Ca lactate, the AUC$\infty$ was calculated from the plasma $^{45}$Ca radioactivity. Table 1 summarizes the AUC$\infty$ (dpm·h/mL), the time taken to reach the maximum $^{45}$Ca level (Tmax, in h), and the amount of radioactivity at Tmax (Cmax, in dpm) for the three groups. The AUC$\infty$ and the Cmax values for the TBE group were both significantly higher than those for the Ca carbonate group. Similar results were observed for the Ca lactate and the Ca carbonate groups. Analyses of variances revealed no significant differences in the AUC$\infty$ and the Cmax values between the TBE and the Ca lactate.
Fig. 3. $^{45}$Ca radioactivity in the kidney of rats perorally administered TBE, Ca carbonate, or Ca lactate radiolabeled with $^{45}$Ca. Each column represents the means ± SE of five rats. The $^{45}$Ca radioactivity in kidney did not differ significantly among the three groups.

Fig. 4. Whole-body $^{45}$Ca retention in rats perorally administered TBE, Ca carbonate, or Ca lactate radiolabeled with $^{45}$Ca. Each column represents the means ± SE of five rats. The whole-body $^{45}$Ca retention of the TBE and the Ca lactate groups was significantly higher than that of the Ca carbonate group ($p<0.001$). The whole-body $^{45}$Ca retention did not differ significantly between the TBE and the Ca lactate groups.

groups. Figure 2 shows the amount of radioactivity in the femur of rats 34h after the administration of $^{45}$Ca. The radioactivity level was highest in the Ca lactate group and lowest in the Ca carbonate group among the three groups. Figure 3 shows the amount of radioactivity in kidney. No significant difference was observed among the three groups. The percent of whole-body $^{45}$Ca retention was calculated as the difference between the amount of $^{45}$Ca ingested and the amount of $^{45}$Ca excreted in the feces and urine over a 34h period. The TBE and the Ca lactate groups both showed significantly higher whole-body $^{45}$Ca retention than the Ca
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carbonate group did, though no significant difference was noted between the TBE and the Ca lactate groups (Fig. 4).

DISCUSSION

The results reported here provide evidence that Ca absorption from TBE, which was prepared by solubilizing powdered bovine marrow-free bone with lactic acid and citric acid in distilled water, is comparable to and higher than that from Ca lactate and Ca carbonate, respectively. Furthermore, Ca from TBE is incorporated into bone more effectively than that from Ca carbonate without inducing high Ca accumulation in kidney. It is generally believed that Ca is largely absorbed in its free or ionized form in intestine, and therefore it is likely that differences in solubility correlate directly with differences in the availability of Ca for absorption. Thus it is widely believed that Ca from bovine and fish bone may not be absorbed easily because these bones consist mainly of hydroxyapatite, a water-insoluble form of Ca phosphate. The results obtained here, however, demonstrate clearly that the absorbability of Ca from TBE was almost comparable to that of Ca from Ca lactate. However, TBE does not yield a clear solution in water; thus it is difficult to accurately measure the solubility of TBE in water. Several possibilities may explain the relatively high absorbability of Ca from TBE. First, TBE may be more soluble in the intestine than Ca carbonate and Ca phosphate are. To confirm this, the solubility of Ca in TBE in a luminal fluid must be determined. Second, the inhibitory effect of phosphate on Ca absorption may not occur for TBE. Ca balance studies in humans have demonstrated that phosphate has little effect on Ca absorption unless the Ca and phosphate intakes are both high (12). In this study, the amount of Ca present in the TBE ingested was 10.0 mg. In our preliminary experiment using Ca carbonate, it was confirmed that the dose-dependent absorption of Ca takes place in the range from 5.0 to 20.0 mg Ca under the same conditions. Therefore it is likely that the absorption of 10.0 mg of Ca from the intestine is nutritionally sufficient and that the phosphate present in TBE does not affect Ca absorption. Third, Ca chelators, such as lactic acid and citric acid, may improve Ca absorption either by keeping absorbable Ca in solution as the pH rises or by favoring Ca absorption as a Ca-chelate complex (13–15). The latter possibility is supported by the fact that Ca in the form of a Ca-phosphopeptide complex (CPP) isolated from milk is highly absorbable (16). Furthermore, it has been reported that calcium-citrate-malate (CCM), a mixture of Ca carbonate, citric acid, and malic acid, exhibits approximately six times the solubility of either Ca citrate or Ca malate, both of which are much more soluble in water than Ca carbonate is (17). It is also well known that Ca from CCM is better absorbed in humans and animals than Ca from Ca carbonate is (18–21). Similar to CCM, TBE is a composite salt of Ca phosphate derived from bovine bone, citric acid, and malic acid. Therefore it can be assumed that a Ca phosphate–citric acid–malic acid complex is more soluble and absorbable than Ca from Ca carbonate and bovine...
bone. At present we cannot confirm this, but investigations are under way.

In this study we used a single isotope extrinsic labeling method for TBE. Marshall and Nordin (11) compared several methods for estimating Ca absorption from various Ca salts and concluded that a dual isotope labeling method was not more effective than a single isotope labeling method. Miller et al (18) reported that Ca absorption from CaCl₂, milk, or other dairy products was similar whether an intrinsic or extrinsic radiolabeling method was used. Further experiments should be designed to elucidate the mechanism for the rapid and significant increased absorption of Ca from TBE. These studies should include the analysis of a chemical form of Ca from TBE and the contributions of organic acids, such as lactic acid and citric acid. Further studies are needed to determine the effects of TBE on skeletal development in animals.

This study indicates that the bioavailability of Ca from TBE is almost comparable to that from Ca lactate and much higher than that from Ca carbonate. We conclude that TBE would be a useful Ca supplement with relatively high absorbability from intestine.

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