Calcium from LactoCalcium™ Milk Mineral after Digestion with Pepsin Stimulates Mineralized Bone Nodule Formation in Human Osteoblast-Like SaOS-2 Cells in Vitro and May Be Rendered Bioavailable in Vivo

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Many individuals cannot obtain the optimum calcium requirement from food for a variety of reasons. Therefore, calcium supplements are important sources of dietary calcium. One of the calcium sources commercially available is LactoCalcium™ (milk minerals) that has 28% calcium, and a 2:1 ratio of calcium to phosphorus. The objectives of this study were (a) to examine whether calcium can be released from LactoCalcium™ by using digestive enzymes and (b) to determine its biological activity by examining its ability to stimulate bone formation. LactoCalcium™ was treated in vitro by using simulated gastric and intestinal fluids or porcine gastric, pancreatic and intestinal extracts. Our results indicate the role of enzymes or bile extract in the digestion of the product. We show that, by increasing the concentration of pepsin at a fixed concentration of LactoCalcium™ (substrate), the percentage of released calcium increased in a dose-dependent manner, showing that, at the right enzyme concentration, as much as 100% of the calcium present in LactoCalcium™ can be made available. The biological activity of the digested calcium was demonstrated by the stimulation of mineralized bone nodules in SaOS-2 cells in a dose-dependent manner. Thus, 1 mM and 3 mM calcium released from LactoCalcium™ increased the nodule area by 23.17 mm² (p < 0.0001) and 77.78 mm² (p < 0.0001), respectively, as compared to a value of 0.99 mm² at 0.5 mM calcium from LactoCalcium™. These results demonstrate the in vitro bioavailability and bioactivity of calcium from LactoCalcium™ and serve as a basis for carrying out in vivo analyses to determine the suitability of using LactoCalcium™ as a source of calcium for individuals at risk of developing osteoporosis.

Key words: calcium bioavailability; LactoCalcium™; milk mineral; osteoblast; SaOS-2 cells

Calcium is the principal mineral present in the human body. Ninety-nine percent, (nearly 2.5 kg) of all calcium in the body is found in bones, making it the most important mineral in maintaining bone health.1) An adequate dietary intake of calcium is essential to achieve the genetically programmed peak of skeletal mass during growth and development, and to maintain bone mass in the elderly, particularly in osteoporotic individuals.2) Osteoporosis is a multifactorial disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture.3) One of the risk factors for osteoporosis is lifestyle, and an inadequate dietary intake of calcium falls under this category.4) Although 70% of dietary calcium is generally obtained from milk and dairy products, calcium supplements also serve as important sources of calcium for individuals unable to obtain their optimum calcium requirement from dairy sources.5)

Various calcium supplements are commercially available, including calcium carbonate, calcium citrate, coral calcium and the calcium preparation from milk whey, the milk mineral. In order to have the desired physiological effect, it is essential that the calcium consumed from these supplements is biologically available. The bioavailability of the various calcium preparations has been tested both in vivo6–8) and in vitro.9,10) Bioavailability studies have also been carried out on human subjects, but inter-individual variability, cost and time constraints, and ethical concerns can make this a rather complex, and, at times, misleading undertaking.11) Rat models have also been used to study calcium bioavailability.12) However, the differences in metabolism between man and rodents could lead to inaccurate data, as has been seen in a study showing that rats cannot be used to assess the quantitative dietary factors in human iron nutrition.13)

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We present here our study of determining the bioavailability of calcium from LactoCalcium™, a milk mineral preparation containing 28% calcium, 14% phosphorus and 1.5% protein. Our method consists of treating the milk mineral with simulated gastric fluid or by enzymatic digestion and then determining the biological activity of the digested calcium on bone formation in mineralizing cultures of human SaOS-2 cells.

Materials and Methods

Materials. The calcium supplement milk mineral used in this study, LactoCalcium™, kindly presented by Cyvex Nutrition, Inc (Irvine, CA, USA). SaOS-2 cells were obtained from ATTC (Manassas, VA) and maintained in our laboratory by weekly passaging as we have described previously. Fetal calf serum, trypsin, and an antibiotic-antimycotic solution were purchased from Gibco (Burlington, Ontario, Canada). All other chemicals were obtained from Sigma (St. Louis, MO, USA).

Treatment of LactoCalcium™ with simulated gastric fluid. To release calcium, LactoCalcium™ was incubated with simulated gastric fluid by using a modification of the method of Maharaj. LactoCalcium™ and CaCl$_2$·H$_2$O as the positive control weighing 0.6 g, were suspended in 5 ml of simulated gastric fluid consisting of a solution of 0.1 M HCl and 0.2% NaCl at pH 1.2. The suspension, with a starting pH of 5, was incubated for three hours in a 37°C water bath while constantly shaking and with the acidity adjusted to pH 2 by dropwise addition of 6 M HCl. The pH value was monitored at half-hour intervals. After three hours of incubation, the acidity was gradually decreased by raising the pH to 5 with dropwise addition of 10 N NaOH. The pH value was adjusted to 7.5 with 0.04 M NaOH. The mixture was incubated for an additional three hours at 37°C while constantly shaking, and the alkalinity monitored and maintained at pH 7.5 by dropwise addition of 10 N NaOH every half-hour. At the end of the incubation period, the mixture was centrifuged at 250 g for five minutes. The supernatant containing the digested calcium was collected, divided into aliquots and stored at −20°C until needed for determining the calcium concentration and for mineralization studies.

Enzymatic digestion of LactoCalcium™. The digestion was carried out by using a modification of the method of Ekmeckcioglu. Varying weights of the milk mineral and CaCl$_2$·H$_2$O were suspended in 5 ml distilled water. The acidity was adjusted to pH 2 with dropwise addition of 6 M HCl. A 100-µl pepsin solution (0.2 g of pepsin powder dissolved in 5 ml of simulated gastric fluid) was added, the pH value was measured every thirty minutes and adjusted to pH 2 with 6 M HCl, and the mixture incubated for a total of three hours. Subsequently, 10 N NaOH was used to raise the pH value to 5, and 500 µl of a pancreatin-bile extract (0.3 g of pancreatin from porcine pancreas, 1.5 g of porcine bile extract suspended in 125 ml of 0.1 M NaHCO$_3$) was added to the mixture. The alkalinity was then adjusted to pH 7.0, and the mixture incubated for another three hours. The digested mixture was centrifuged at 250 g for five minutes, and the supernatant containing the released calcium was transferred to another test tube. To stop the reaction, 1% v/v fetal calf serum (FCS) and 10 mM HEPES at pH 7.4 were added to the supernatant to inhibit the proteases and stabilize the pH, respectively.

Measurement of the calcium concentration in the supernatant of the digested mixture. The calcium concentration in the supernatant obtained after the simulated gastric fluid treatment and enzyme digestion of LactoCalcium™ and CaCl$_2$·H$_2$O were measured by using a calcium assay kit (Sigma). The calcium binding reagent and calcium buffer were mixed in a 1:1 ratio according to the manufacturer’s instructions. A 10-µl amount of the supernatant containing the released calcium was added to 1 ml of this mixture, and the absorbance of the sample (A$_{\text{sample}}$) was measured at 575 nm with a Milton Roy spectrophotometer against a blank of deionized distilled water (A$_{\text{blank}}$). The absorbance from a solution with a known calcium concentration was used as the standard (A$_{\text{standard}}$). The concentration of calcium in the supernatant was calculated by subtracting A$_{\text{blank}}$ from the sample and the standard absorbance to get delta A$_{\text{Sample}}$ and delta A$_{\text{Standard}}$, respectively. The following formula was used for calculations:

$$\text{Calcium concentration of the sample} = \frac{\text{delta A}_{\text{Sample}} \times \text{Concentration of Standard}}{\text{delta A}_{\text{Standard}}}$$

Cell culture. SaOS-2 cells were maintained by passaging weekly in 75-cm$^2$ flasks containing Ham’s F-12 medium supplemented with 10% FCS, a HEPES buffer at pH 7.35, 10% antibiotic-antimycotic and 1.1 mM CaCl$_2$. The medium was changed every two to three days as we have previously described.

Determination of mineralized bone nodule formation in the SaOS-2 cell culture. To study the effect on bone formation of released calcium from LactoCalcium™, we used our previously described method. In brief, the cells were passaged with trypsin, plated at a density of 10$^5$ cells/ml in 12-well plastic dishes and cultured in a medium supplemented with 10 nM dexamethasone (Dex) and 50 µg/ml of ascorbic acid phosphate. A 10 mM concentration of β-glycerophosphate was added at day eight and at every medium change until the end of culture. To study the effect of calcium, the medium was removed at day eight and replaced with a fresh medium containing varying concentrations of calcium obtained...
from LactoCalcium™ or CaCl₂·2H₂O. Calcium was added every three days at each medium change until day 14 or 15.

Von Kossa staining of mineralized bone nodules. At the end of the culture period, the cells were fixed with 4% paraformaldehyde and stained in situ with the von Kossa reagent as we have described previously. The stained cells were covered with glycerol and the mineralized bone nodules were quantified by using a Leco image analyzer.

Statistical analysis. All data are presented as Mean ± Standard Deviation. For comparison between two groups, independent Unpaired Student’s T tests. One way Analysis of Variance (ANOVA) tests were performed for comparison between multiple groups. A calculated p-value < 0.05 was accepted as significant.

Results

Calcium released after simulated gastric fluid treatment and enzymatic digestion of LactoCalcium™

The calcium released from LactoCalcium™ was determined as aqueous suspension of LactoCalcium™ after incubating with simulated gastric fluid or after enzymatic digestion with pepsin and pancreatin. The amount of soluble calcium present in the supernatant after centrifuging the suspension of LactoCalcium™ prior to the treatment or digestion (control) was found to be 0.012–0.014 mg/g of LactoCalcium™. However, after incubating with the simulated gastric fluid, the amount of calcium released was found to be 7 mg/g of LactoCalcium™, or 580-fold more than the control value (p < 0.001), and after enzymatic digestion with pepsin and pancreatin, this value was 17.8 mg/g of LactoCalcium™, or 1270-fold more than the control (p < 0.001). The calcium released by enzymatic digestion was 2.6-fold (p < 0.001) that of Simulated Gastric Fluid, indicating that the enzymes are needed to further increase the calcium released from LactoCalcium™ (Table 1). No statistically significant differences were observed in the mass of calcium released when the positive control, CaCl₂·2H₂O, was digested with the simulated gastric fluid in comparison with using enzymes (data not shown). Since the enzymatic digestion resulted in the release of a significantly greater amount of calcium than with digestion using the simulated gastric fluid (p < 0.001), only the method using enzymatic digestion was pursued in subsequent experiments.

Effect of increasing weight of LactoCalcium™ on the calcium released after enzymatic digestion

To determine whether the amount of calcium released would be dependent on the weight of LactoCalcium™, samples weighing from 75 mg to 600 mg were digested by using the enzymatic protocol already outlined. The amount of calcium released increased in a dose-dependent manner with increasing weight of LactoCalcium™ (ANOVA, p < 0.01, Fig. 1). At the highest weight of LactoCalcium™ this increase in calcium released, although statistically significant, amounted to only 17% more than that obtained from the lowest weight (75 mg), despite the 800% (from 75 mg to 600 mg) increase in the initial weight of LactoCalcium™, digested. The possibility that the small amount of calcium released could have been caused by a limiting concentration of pepsin was tested below.

Effect of increasing pepsin concentrations on the calcium released from LactoCalcium™

In order to determine whether increasing the concentration of pepsin would result in a corresponding increase in the calcium released from LactoCalcium™, 75 mg and 150 mg of LactoCalcium™ were each digested by using an increasing concentration of pepsin from 0.8 mg/ml to 2.4 mg/ml. Although no statistically significant difference was apparent in the calcium

<table>
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<tr>
<th>Digestion method</th>
<th>Mass of calcium/weight of LactoCalcium™ (mg/g)</th>
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<tr>
<td>Before digestion</td>
<td>After digestion</td>
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<tr>
<td>1. Simulated digestion</td>
<td>0.012 ± 0.003</td>
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<tr>
<td>2. Enzymatic digestion</td>
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LactoCalcium™ (0.6 g) was suspended in water and subjected to simulated or enzyme digestion as described in the text. The concentration of the released calcium was measured before and after digestion as described in the text. Significant difference between mass of calcium released before and after simulated digestion (p < 0.001); Significant difference between mass of calcium released before and after enzymatic digestion (p < 0.001); Significant difference between mass of calcium released after simulated vs. after enzymatic digestion (p < 0.001). Each point represents Mean ± SEM of 9 determinations from three separate experiments carried out in triplicate.

Fig. 1. Enzymatic Digestion of Increasing Weights of LactoCalcium™

Varying weights of LactoCalcium™ were digested by using the same concentration of enzymes in digestive mixtures. The concentration of released calcium in the supernatant was analyzed as described in the text. Each point represents Mean ± SEM of 6 determinations from two separate experiments carried out in triplicate. The amount of calcium released increased in a dose dependent manner with increasing weight of LactoCalcium™ (ANOVA, p < 0.01).
released by CaCl$_2$·2H$_2$O when subjected to the increasing concentration of pepsin (data not shown), the amount of calcium released from LactoCalcium™ (milk mineral) was found to increase with increasing concentration of pepsin (ANOVA test, statistically significant, $p < 0.01$, Fig. 2). The amount of calcium released from 150 mg of LactoCalcium™ by using 0.8 mg/ml of pepsin was 10.4 mg compared to 18.2 mg calcium released when 2.4 mg/ml pepsin was used. A similar increase in the calcium released was observed when 75 mg of LactoCalcium™ was digested with an increasing concentration of pepsin (Fig. 2) (ANOVA test, statistically significant, $p < 0.01$). The maximum amount of pepsin required to release the maximum amount of calcium was not attained in this experiment.

Effect of the calcium released from LactoCalcium™ on the mineralized bone nodule formation in an SaOS-2 cell cultures as a measure of bioactivity

The formation of mineralized bone nodules following the addition of calcium released from the digested milk mineral is shown in Fig. 3. As can be seen in the photomicrograph, no mineralization was apparent when calcium was added at the lowest concentration of 0.5 mM (Fig. 3A). The mineralized bone nodules were observed when 1 mM of released calcium was added to the cultures (Fig. 3B and C). To quantify the area of the mineralized bone nodules, the mineral formed was stained by the von Kossa technique and quantified by using a Leco image analyser. It can be seen from Fig. 4 that the area of mineralized bone nodules increased with increasing concentration of calcium released from LactoCalcium™ added from day eight until days 14 and 15 of the culture (Fig. 4) (ANOVA test, statistically significant with $p < 0.01$). The maximum area of mineralized bone nodules (80 mm$^2$) obtained when 3 mM of released calcium from LactoCalcium™ was added to SaOS-2 cells was 90-fold higher than that obtained at 0.5 mM. These results were not statistically different from the area of mineralized bone nodules measured for the positive control when the same concentration of calcium from the enzyme-digested CaCl$_2$ was added to the cell culture. These results therefore indicate that calcium from the digested LactoCalcium™ was specific since it has the same
effect as the positive control CaCl$_2$ and bioactive at the level of bone formation.

**Discussion**

The major findings from this study are that calcium in LactoCalcium™, a milk mineral preparation intended to be used as a calcium supplement, is readily released by enzymatic digestion and is bioactive in stimulating bone formation in a human osteoblast in culture. These findings are novel and suggest that LactoCalcium™ is bioavailable and bioactive; it becomes available for absorption after digestion by gastric enzymes and it can be incorporated into bone as shown by its ability to stimulate the formation of mineralized bone nodules in osteoblast cultures.

Our results show that the amount of calcium released after digesting LactoCalcium™ with the enzymatic mixtures was greater in comparison to that released after treating with the simulated gastric fluid. Since the simulated gastric fluid did not contain either the digestive enzymes, pepsin and pancreatin, or a bile extract, these results indicate that these enzymes and components may have a role in increasing the bioavailability of calcium. The results of our experiment in which the calcium released increased with increasing pepsin, while maintaining the concentrations of the other components constant, indicate pepsin to be the active component.

However, the contributions from the other components need to be evaluated in future studies. We postulate that pepsin acted on the proteins that bind calcium. It has been shown that proteins in milk may have specific or non-specific binding sites for minerals. Thus, they can then act as chelators or carriers of various milk minerals and may enhance or inhibit bioavailability. The milk mineral, LactoCalcium™ used in the present study is a milk whey isolate which contains the proteins, β-lactoglobulin, α-lactalbumin and lactoferrin. β-lactoglobulin and α-lactalbumin, have the ability to bind calcium and can be digested by the digestive enzymes, pepsin and chymotrypsin. Therefore, our results, showing a 120-fold increase in the amount of calcium released after pepsin digestion, may be explained by the ability of pepsin to digest the 1.5% protein present in LactoCalcium™. The role of pepsin in increasing the calcium released from LactoCalcium™ is further strengthened by the finding that the amount of calcium released was enhanced in a dose-dependent manner by increasing the concentration of pepsin. Our results indicate that, although the calcium component of LactoCalcium™ is insoluble, this calcium may be made available after enzymatic digestion in vivo as we have demonstrated in vitro.

To determine the physiological action of the calcium released from LactoCalcium™, its effect on bone formation was tested and compared with that of CaCl$_2$ by using cultures of SaOS-2 cells under mineralizing conditions. We have shown in the present study that the area of mineralized bone nodules increased with increasing concentration of calcium from digested LactoCalcium™ indicating that it was biologically active. The results of another recent study, showing that rats fed with calcium from milk whey, from which the milk mineral is produced, had a higher femur calcium level and a higher breaking force of an excised femur than the control group, support our proposal that calcium supplements derived from milk whey, although insoluble in water, can be digested, absorbed and used to form bone, both in vitro and in vivo.

We do not know at the present time how the calcium released from LactoCalcium™ is able to increase the formation of mineralized bone nodules in SaOS-2 cells. However, in a previous study, we found a similar stimulatory effect of CaCl$_2$ when added in an increasing concentration to SaOS-2 cells being cultured, and the stimulatory effect of estrogen on mineralized bone nodule formation in SaOS-2 cells was also found to increase with increasing concentration of extracellular calcium. It is possible that the calcium released from LactoCalcium™ acted through the extracellular calcium-sensing receptor (CasR) that has been shown to be expressed in a number of osteoblast cell lines including.
SaOS-2 cells. The important role played by CasR in mineralization has been shown by the defective mineralization of cartilage and bone in CasR knockout mice (CasR(−/−)). The mechanisms involved in the effect of calcium released from LactoCalcium™ remains to be elucidated.

Although the role of calcium supplements in preventing osteoporosis has generated some controversy in the past, evidence linking an inadequate calcium intake to osteoporosis is accumulating. A calcium supplement of 500–1500 milligrams per day has been shown to increase bone formation in adolescents, young and old men, and postmenopausal women. A calcium supplement given to rats resulted in increased bone mineral density (BMD). In addition, a clinical study has demonstrated a significant increase in BMD at the femoral neck of patients taking a calcium supplement. Other studies have shown the beneficial effects of calcium supplementation alone or of calcium and vitamin D supplementation. More recent studies have shown the protective effects of calcium supplementation from bone loss in men and women. Although the effect of a calcium supplement on reducing bone loss was less than that of an estrogen treatment, clinical studies have indicated that it was more effective than 1α-hydroxyvitamin D₃ in preventing loss of BMD among elderly women over a four-year period. Current research has revealed that hormone replacement therapy (HRT), accompanied by a calcium supplement and monofluorophosphates or 1α-hydroxyvitamin D₃, can help increase BMD among postmenopausal women. A supplement of calcium or of calcium and vitamin D is generally prescribed to osteoporotic patients undergoing treatment with alendronate, clodronate, iripilavone, calcitriol, and disodium palmidronate. The results of these studies give credence to the view that a calcium supplement, along with other treatments, can play an important role in the treatment of osteoporosis. This information, coupled with the fact that more than half of the Caucasian women in the United States over 80 years of age had bone a density value low enough to warrant treatment, suggests that these women may benefit from using a calcium supplement with other treatments for osteoporosis.

In conclusion, we have shown that releasing calcium by enzyme digestion from the milk mineral, LactoCalcium™, and analyzing the effect of the released calcium on mineralization in a long term culture of human osteoblast-like SaOS-2 cells provided an excellent method for determining the bioavailability of calcium from a milk mineral. This model was used to provide evidence that the calcium in LactoCalcium™ is bound to the proteins present in a milk mineral and can be released and made bioavailable by enzyme digestion in vitro. Thus, our study lays the groundwork for carrying out in vivo studies to evaluate the bioactivity and bioavailability of calcium from LactoCalcium™. Such studies will then help us to determine the practicality of of using LactoCalcium™ alone or in combination with vitamin D or other approved drugs that inhibit bone resorption, for the prevention and possible treatment of osteoporosis.

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