Calcium Chloride and Vitamin D Bioavailability from Fortified Sports Drink in Wistar Rats

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Abstract

Calcium and vitamin D play a critical role in the prevention of metabolic diseases including osteoporosis, osteomalacia, and stress fractures; but average intake for most Americans is below their Recommended Dietary Allowance. The objective of this study was to test bioavailability of a water-soluble form of vitamin D and calcium chloride as fortifiers for sports drink solutions—something that could be consumed by any populations low in these nutrients. Vitamin D fortifying ingredient was prepared as a spray-dried complex with bovine beta-lactoglobulin. Flavored beverages were formulated with various ratios of calcium and vitamin D in a 4x4 factorial design. Female Wistar rats were housed under incandescent lighting for a 4-week depletion phase, then given drink formulations with low calcium, low vitamin D diet for an additional six weeks. Blood and femur bones were analyzed. The fortified drink solutions could be accurately formulated to contain calcium chloride at 0, 1, 2 and 2.5 g Ca/L with palatability to rats. Serum vitamin D was significantly greater (P<0.0001) in rats receiving the vitamin D-fortified drinks (10, 20, and 40 μg/L), but no dose-response was evident. The reduced level of vitamin D in the deficient diet was adequate to maintain serum calcium concentrations, bone strength and bone mineralization at normal rates. Water-soluble vitamin-D can successfully fortify aqueous products with this fat-soluble vitamin. Regular consumption of flavored sports drink fortified with calcium and vitamin D may significantly increase dietary calcium and vitamin D in populations that often have low intakes of these nutrients.

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Introduction

Vitamin D plays a critical role in the homeostatic control of calcium and phosphorus. Adequate intake of vitamin D increases the efficiency of intestinal calcium absorption by increasing intracellular calbindin D, which facilitates skeletal development[1]. Calcium and vitamin D prevent metabolic diseases including osteoporosis, osteomalacia, rickets, and the stress fracture component of the female athlete triad. Optimizing calcium and vitamin D intake are recommended components in treatment and prevention of the female athlete triad[2]. Epidemiological research indicates that average intakes of these nutrients are well below the Recommended Dietary Allowance (RDA), and greater intake has been correlated with a reduction infractures, prevention of osteoporosis, and increased bone mass[3]. The vitamin D and calcium RDAs were established in 2010 at levels higher than earlier Adequate Intake (AI) values[4], but that generally has not translated to more vitamin D fortification or greater intake of calcium. For example, the vitamin D RDA was set to 15 μg/day for adults under 70 years, rather than the AI of 5 μg/day for adults under 50 years. Supplementation above the current RDA yielded a decrease[5-7]. These research studies lay the framework for more fortification of various food products with calcium and vitamin D. However, fortification of low fat food products or non-fat beverages with currently available forms of vitamin D often results in problems with stability of vitamin D in the food, binding of this lipophilic nutrient to plastic containers, or poor bioavailability from emulsions and suspensions. Our research program has aimed at reduction or elimination of these problems.

We have developed a method for creating a water-soluble fortifier for vitamin D[8]. Beta-lactoglobulin (BLG) has many functional properties including a transportation mechanism of molecules such as vitamins. BLG tightly binds to retinol (vitamin A), cholesterol and vitamin D[9]. Lipophilic vitamins structurally bind to the hydrophobic core of the BLG molecule. Previous studies suggest that BLG has a higher affinity for vitamin D compared to other lipophilic vitamins[9, 10]. Spray drying is an application that can facilitate the binding of beta-lactoglobulin and lipophilic vitamins for use as a fortifier of low fat[11,12]. Because this complex can form a water-soluble form of vitamin D, it can be paired with calcium in aqueous solutions that use calcium chloride because of its highly ionized state that may promote calcium absorption. The purpose of this study was to examine the effects of the water-soluble form of vitamin D and calcium chloride as fortifiers for aqueous sports drink solutions with a rat bioavailability assay.

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Materials and Methods

β-lactoglobulin and vitamin D complex preparation

BioPure β-lactoglobulin was purchased from Davisco Foods International, Inc. (LeSueur, MN). Vitamin D₃ (MW 384.65) and vitamin D₂ (MW 396.65) were purchased from Sigma Chemical Company (St. Louis, MO) and Roche Vitamins Inc. (Nutley, NJ), respectively. Four hundred mL of 2% w/v of β-lactoglobulin (~8g) in DI water was prepared. The solution was mixed on a magnetic stirring plate at low speed (2) to prevent foaming until homogenous clear solution was obtained. All work conducted with vitamin D was performed under dim yellow light and used amber containers or glassware enclosed in aluminum foil to prevent degradation of vitamin D from direct light exposure.

The complex was spray dried on a pilot scale dryer (Anhydro, Denmark). Prior experiments determined optimum drying conditions to protect both BLG and vitamin D to be 120°C inlet air temperature and 68-70°C outlet temperature. The system was flushed with deionized water via a MasterFlex peristaltic pump (Model 7518-10, Cole-Parmer Instrument Co., Vernon Hills, IL) to stabilize the unit before the solution could be added. The protein-vitamin stock solution was pumped into the machine at a flow rate ~2 mL/min while continuously monitoring the inlet and outlet temperatures to ensure that heat denaturation would not occur. The powder was weighed, placed in an amber vial with aluminum foil surrounding the exterior, flushed with nitrogen, and stored in the freezer(-20°C) for further analyses.

HPLC analysis for vitamin D content

The percent recovery was calculated for the vitamin D in the spray-dried powder. The vitamin D₃ content of the β-lactoglobulin-vitamin D complex was determined by High Performance Liquid Chromatography (HPLC) using vitamin D₃ as internal standards as described by Liu[13]. HPLC analyses were performed on a Waters Millipore Automated Gradient Controller with UVIS linear detector and a manual loading injector (Waters Associates, Milford MA). Reversed-phase 4.6 x 250nm Vydac TP201 C18, 5 µm column with a guard column (Vydac, Hesperia, CA) was used with a mobile phase consisting of acetonitrile/ethyl acetate/chloroform (88:8:4; v/v/v) at a consistent flow rate of 1 mL/min. Methanol was used to wash and equilibrate the column before and after each sample injection. A wavelength of 264 nm was used to quantify the results and Dynamax Software Package (Waters Associates) was used to integrate the peak areas of vitamin D. Fifty μL of the extracted sample was injected into the HPLC system. Standards were injected before injecting the unknown samples. Area under the curve (AUC) was recorded for vitamin D₃ and D₂, respectively, where vitamin D₃ AUC was used as an internal standard. The retention time was also obtained and recorded for the peak values under both vitamins.

Rat bioavailability assay

The North Carolina State University Institutional Animal Care and Use Committee (IACUC) approved the protocol for this study. Female Wistar rats (Charles River Laboratory, Raleigh, NC) were individually housed under incandescent lighting with no UV-B component with a 12- hour light/dark cycle. Rats were randomly divided into 16 treatment groups and 4 control groups. Immediately after arrival five of the weanling (4-week old) rats were sacrificed for baseline data. The remaining rats were made deficient in vitamin D for 4 additional weeks, by feeding a modified AIN-93G diet with normal calcium (5.0 g Ca/kg) level and lower vitamin D (0.11g/kg), as analyzed after the study concluded. After completion of the reduction phase, an additional five rats were sacrificed and serum was collected and stored for later measurement of 25-hydroxycholecalciferol (25(OH)D₃).

A 4X4 factorial design was utilized to divide the animals into subgroups for the drinking water supplementation. The concentrations approached or bracketed our estimate of intake from normal dietary AIN 93G diet, equivalent to 2.88 g Ca/L and 14.4 μg vitamin D₃/L. These values indicate the estimated daily requirement (EDR) for calcium and vitamin D respectively, based upon previous research conducted in our laboratory that measured feed and water intake in rats of comparable ages that were fed AIN 93G diet with standard levels of vitamins and minerals. The rats received the fortified water for 6 weeks, or until approximately 14 weeks of age. The vitamin D contents of the sports drinks were formulated to be 0, 10, 20, or 40 μg/L. Calcium concentrations were 0, 1, 2, 2.5 g/L as CaCl₂(Tetra Technologies, The Woodlands, TX). The treatments were given identifying letters as shown in Table 1 with five rats in each treatment group. Fruit flavor and non-caloric sweetener was added to increase palatability. For the duration of the vitamin D repletion stage, animals’ drink and food intake were recorded and weight was obtained twice per week. Calcium intake for the 6-week repletion period was calculated for food and water (weights of offering - refusals on weekly basis) x calcium content in food and water[11]. Rats were sacrificed with a ketamine (650 mg/100 g body weight) + xylazine (140mg/100 g body weight) anesthetic to achieve unconsciousness, followed by exsanguination. Blood was removed by cardiac puncture and serum vitamin D was determined by ELISA (Octeia 25-Hydroxy Vitamin D ELISA Kit, IDS Inc., Fountain Hills, AZ) as described below.

Bone assay for calcium analysis

The left femurs were weighed and measured. The right femurs were removed, ashed and analyzed for calcium content. All work pertaining to assay of bone was conducted under the fume hood. Prior to analysis of collected bones, test tubes, glassware and crucibles were acid-washed to remove all foreign material and minerals. The left femur bones were cleaned manually byremoving accessible excess tissue. The femurs were soaked overnight in a test tube containing 100% ethanol; solvent was discarded then the bones were soaked in chloroform over night to ensure that all lipid tissue material was removed from the femur bones. Solvent was discarded and the bones air-dried for approximately five minutes under the fume hood.
Calcium and vitamin D intake from the fortified beverage and diet had no significant effect on any of the bone parameters as determined by one-way analysis of variance (overall P> 0.05).

The femurs were placed in pre-weighed, acid-washed, ceramic crucibles, weighed and ashed as follows. The bones were placed in the muffle furnace at 100°C for 8-12 h to remove excess moisture and cooled in desiccators. The dry weight was obtained and the cooled crucibles were placed back into the muffle furnace at 649°C for 24 h, and cooled 8 h. The crucibles containing the ash were re-weighed. The ashed material was transferred to a test tube and dissolved in 10 ml of 3 N HCl and diluted 1:100 using 0.5% lanthanum in 0.1 N HCl. Atomic absorption spectrophotometry (Perkin Elmer Model 3100, Norwalk, CT) was utilized to obtain calcium content from the bone ash. Ash data were expressed as percent of dry defatted bone weight.

Bone strength test

Mechanical properties of the rats’ femurs were determined with an Instron Universal Testing Instrument (Model 1122 Instron, Canton, MA). The right femurs were dissected from the body and visual soft tissue and muscle were removed from the bone. The bones were individually sealed in plastic bags and labeled and stored at -4°C until needed for strength testing. The femurs were thawed and three measurements (mid width, joint width, and length, all measured in millimeters) were ascertained with electronic calipers, and the three point bending testing. The femurs were thawed and three measurements (mid width, joint width, and length, all measured in millimeters) were ascertained with electronic calipers, and the three point bending test was performed.

Serum analysis for calcium and vitamin D

At the point of sacrifice, blood samples were collected in 4 ml Vacutainer tubes, coated with clot activator. The samples were allowed to rest for 30 minutes at room temperature and centrifuged at 760 x g for 15 minutes. The serum was collected in amber vials and stored at -20°C until analyzed. The serum samples were diluted 1:50 using 0.5% lanthanum in 0.1N HCL and calcium content was determined by atomic absorption spectrophotometry. The Octeia 25-Hydroxy Vitamin D Elisa Kit (IDS Inc., Fountain Hills, AZ) used to analyze serum 25-OH vitamin D was approved by the Food and Drug Administration (FDA) for analyzing 25-OH vitamin D status in humans. We verified reactivity in rat serum in a preliminary experiment. For this ELISA, 25 µl of each calibrator, control and sample were added to polypropylene test tubes. One mL of 25-OH D3 biotin solution was added. Each test tube was vortexed 10 seconds and 200 µL of each diluted calibrator; control and sample were added to the appropriate well of the antibody-coated plate in duplicate. The plate was the sealed and incubated at 18-25°C for 2 hours. Next, the plates were washed manually three times with 250 µL of buffer solution provided in the kit and emptied. Two hundred µL of enzyme conjugate was added to each well and incubated at 18-25°C for 30 minutes. The manual wash step was repeated, and 200 µL of tetramethylbenzidine substrate was added to each well using a multichannel pipette. The plate was again sealed and incubated at 18-25°C for 30 minutes. One hundred µL of 0.5 M HCl was added to each well with a multichannel pipette to stop the reaction. Within 30 minutes of adding the 0.5 M HCl the absorbency was measured at 450 nm using a microplate reader (Thermo Electron Corporation, Vantaa, Finland).

Statistical methods

SAS software (Cary, NC) was used for the statistical analysis of the data. Correlation analysis compared calcium and vitamin D in water, calcium and vitamin D in food to other dependent variables. Differences among groups for measurement of nutrient intake were tested with one-way Analysis of Variance using an overall significance probability of α = 0.05. Rat serum data were analyzed by 2-way ANOVA with Scheffe’s post hoc test. Relying on the F-test in the ANOVA for analysis of the 4 x 4 factorial designs minimized the use of multiple comparison post hoc tests and use of individual treatment standard errors for unplanned comparisons and resulting Type 1 errors[14].

Table 1: Body weight, food and drink intake, and bone measurement parameters by treatment group

<table>
<thead>
<tr>
<th>Group</th>
<th>[Ca] in Drink g/L</th>
<th>[Vit. D] in Drink(µg/L)</th>
<th>Final Mean Body Weight (g)</th>
<th>Ashed bone weight (g)</th>
<th>Total Drink Intake (mL)</th>
<th>Total Food Intake (g)</th>
<th>Ca in Bone Ash (%)</th>
<th>Mid width (mm)</th>
<th>Joint width (mm)</th>
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Results

Calcium and vitamin D intake

After spray drying, the vitamin D-protein complex was analyzed and quantified by HPLC analysis. The quantity of vitamin D measured in the protein vitamin complex was 102% of the theoretical value from the formulation. An average concentration of vitamin D in the BLG complex was calculated and used to design animal diets. Additionally, the vitamin D content of the diet was analyzed. The results showed that vitamin D was present in the normal diet at 0.32 mg/kg (compared to label specification of 0.25 mg/kg), and was also present in the vitamin D deficient diet at 0.11 mg/kg. The measured concentrations in the diets were used in the calculation of total vitamin D intake.

Calcium intake for the 6-week repletion period as calculated from food and water offering minus refusals are shown in Figure 1. Groups R and T received the positive control diet and tap water. Group D (2.5 g/L Ca; 0 vitamin D in drink) consumed the greatest amount of calcium via beverage and diet consumption. Furthermore, the animals in the group D were able to consume an amount of calcium via beverage and diet equivalent to the positive control (calcium intake from feed only). The calcium via food intake was consistent among all treatment groups. The highest mean calcium intake from water was seen in treatment groups B, C, and D (Figure 1).

Vitamin D intake for the 6-week repletion period was calculated for water (weights of offering - refusals on weekly basis) x vitamin D content in water. Groups A, B, C, and D had no vitamin D from fortified sports drink (Figure 2). However, as previously mentioned, vitamin D was contained in the vitamin D deficient diet in the amount of 0.11 mg/kg.

Serum calcium, serum vitamin D and serum protein

Serum calcium was analyzed by a 2-way ANOVA with calcium and vitamin D intakes as main effects. Data among all treatment groups were not significantly different from each other (Figure 3). Serum vitamin D concentration in all treatments was adequate to compensate for low Ca intake. The fact that serum Ca did not change suggests that there may be no treatment effects observed for other biological functions of calcium, such as maintaining bone mass or bone Ca content. Lower levels of vitamin D in the diet and serum might have lowered serum calcium concentration and bone Ca measurements.

Serum 25-OH D3 analysis from competitive ELISA is shown in Figure 4. After the depletion phase the animals’ serum 25-OH D3 values were reduced, but the concentration was restored to varying extents in the 6-wk repletion phase of the experiment. Groups receiving no vitamin D in water were significantly different from the other groups (P<0.05). A dose-
response among treatments was not evident among the groups consuming drink with 10, 20 or 40 µg/L of vitamin D. The ELISA assay used recognizes vitamin D<sub>3</sub> 75% as effectively as vitamin D<sub>2</sub>, however the rats in this study were not fed any vitamin D<sub>2</sub>.

Serum 25-OH D<sub>3</sub> in rats fed control diet for 4 weeks was 55.24 ±15.24 nmol/L. Serum 25-OH D<sub>3</sub> in rats fed low vitamin D<sub>3</sub> diets for 6 weeks were 91.15 ± 9.26 nmol/L.

**Bone parameters and correlations**

As illustrated in Table 1, the final mean body weights were not significantly different among all groups. Bone ash weight was derived from drying and ashing the femur bones of the rats. The average ash weights were not significantly different among the groups.Additionally, beverage intake was measured to determine the total intake of calcium and vitamin D. Water consumption was greatest among those groups whose water was composed of 0 or the lowest amount of CaCl<sub>2</sub>. The percent of Ca in the bone ash did not significantly differ among treatment groups. The total intake of Ca from fortified beverages did not correlate with percentage of Ca in bone (R = - 0.109). Furthermore, the femur mass did not correlate with the intake of either calcium or vitamin D (P> 0.05) (Figure 6).

![Figure 6: Correlation between Bone Mass and Vitamin D (top panel) and Calcium (bottom panel) Intake among all rats: Femur weights were not significantly affected by the vitamin D content in water. Femur weights were not significantly affected by the ratio of calcium intake, regardless of vitamin D content in water. Low calcium intake of bone was compensated for by vitamin D. The slope of the regression line was not significantly different from zero in either panel. Each point represents an individual rat.](image)

**Discussion**

The purpose of this study was to examine the effects of a water-soluble form of vitamin D and calcium supplementation via an aqueous solution on various bone parameters in Wistar rats fed a vitamin D deficient diet. β-lactoglobulin has been shown to bind with vitamin D<sup>[9,10]</sup>. Vitamin D activity was
maintained by using a carbohydrate carrier during spray dryings\(^{12,15}\). This complex has been shown as an effective fortifier of aqueous solutions\(^{13,16}\). Previous research\(^{16}\) also showed the calcium sensory threshold for humans to be 70 mg/L of CaCl\(_2\) (25 mg/L of Ca) in water, and about 10 fold higher in flavored water, which is less than the concentration used in all of the calcium fortified beverages in this study. The present data confirmed that proposed vitamin D concentration for flavored water or sport drink was achievable using the protein complex to make a water-soluble form of vitamin D.

The Wistar rat model system was used to determine the bioavailability of the vitamin D and calcium in such solutions. The experiment was designed to supply vitamin D only in the flavored drinking water, and reduce the calcium level in the diet to a point where vitamin-D dependent calcium absorption would be needed to maintain normal bone mass. However, the vitamin D deficient diet was analyzed and found to have contained vitamin D. Thus, the baseline vitamin D intake from diet, although only one third as high as control diet, provided more vitamin D that the water in most treatments.

Bone strength has been correlated with bone mass and bone quality\(^{17}\). Calcium and vitamin D supplementation in a vitamin D deficient diet did not result in a stimulated increase of food or beverage intake as seen in other supplementation studies\(^{19}\). Supplementation with the fortified aqueous solution containing calcium and vitamin D did not result in a significant difference in live weight\(^{19}\), contrary to other study\(^{20}\). Moreover, supplementation with the fortified beverages had no significant treatment effect on serum calcium. The aqueous vitamin D supplementation significantly increased serum 25-OH vitamin D in this study (P< 0.01). However, the lack of dose-response among the different levels of vitamin D in the drink suggest that Wistar rats may have a homeostatic mechanism controlling serum 25-OH vitamin D concentrations. In humans, “Increasing intake of vitamin D results in higher blood levels of 25-OH, although perhaps not in a linear manner”\(^{24}\).

The low calcium intake via food and beverage may have resulted in an increased serum PTH and stimulated more efficient intestinal calcium absorption\(^{18,21,22}\). The adequate levels of serum calcium may also have facilitated consistent bone strength and length among the different treatment groups. Uchikura \(^{23}\), reported that female Wistar rats’ bone mass peaked at 10-12 months. Therefore, this study’s duration may have not covered time periods when bone mass differences with rat groups had enough vitamin D to adapt to low calcium intake. None the less, the aqueous vitaminD significantly increased serum 25-OH D\(_3\), a primary indicator of vitamin D status, compared to groups that had no vitamin D in the drinking water.

References