Calcium Bioavailability of Calcium Carbonate Fortified Soymilk Is Equivalent to Cow's Milk in Young Women[1,2]

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ABSTRACT Calcium (Ca)-fortified soymilk has gained popularity in the United States. Tricalcium phosphate (TCP)-fortified soymilk was shown to have a lower Ca bioavailability than cow's milk in men. However, the most popular soymilk in the U.S. is fortified with Ca carbonate (CC) and has not been evaluated. Ca bioavailability from CC-fortified soymilk (CCSM) and TCP-fortified soymilk (TCPSM) was compared with cow's milk in young healthy women using the dual stable isotope technique. In a 3-way crossover design, 20 volunteers (23 ± 2 y old) consumed 250 mg Ca in cow's milk, CCSM, or TCPSM along with 10 mg ^{44}Ca after an overnight fast. Cow's milk was extrinsically labeled, whereas each fortified soymilk was intrinsically labeled with each chemical salt of ^{44}Ca at the manufacturing facility. Another stable isotope, ^{43}Ca, was injected i.v. 1 h after the complete consumption of cow's milk or soymilk. Fractional Ca absorption was determined from the ratios of ^{43}Ca:^{42}Ca and ^{44}Ca:^{42}Ca by inductively coupled plasma (ICP)-MS in the 24-h urine samples. A mixed linear model (SAS proc mixed) was used to compare the fractional Ca absorption among groups. Fractional Ca absorption in CCSM (0.211 ± 0.057) did not differ from that of cow's milk (0.217 ± 0.040), but both were higher ($P < 0.05$) than that of TCPSM (0.181 ± 0.039). Our result suggests that calcium absorption is equivalent for CCSM and cow's milk at similar calcium loads. J. Nutr. 135: 2379–2382, 2005.

KEY WORDS: • *calcium bioavailability* • *soymilk* • *cow's milk* • *calcium carbonate* • *tricalcium phosphate*

An adequate calcium intake is essential to maintain calcium balance and build strong bones. Dairy products contain large amounts of calcium and are recommended as optimal calcium sources; >70% of dietary calcium in the United States comes from dairy products (1). Other calcium-rich sources include some vegetables, fortified foods, and supplements. Soymilk has gained popularity in the United States. However, the calcium content in natural soymilk is only 200 mg/L compared with cow's milk, which contains 1200 mg/L. Thus, manufacturers have begun to fortify soymilk with calcium to provide an alternative calcium source to cow's milk. Tricalcium phosphate (TCP)[4] and calcium carbonate (CC) are the most common calcium fortificants. Assessment of calcium bioavailability from various calcium-fortified soymilks has received little attention. In vitro bioavailability of calcium using a simulated digestion system estimated from calcium-fortified soymilk at 1000 mg calcium/L was significantly higher when CC was used as the fortificant (19 ± 0.7% at 130 mg Ca load) than for TCP-fortified soymilk (15 ± 0.7% at 137 mg Ca load), nonfortified soymilk (11 ± 2% at 27 mg Ca load), or cow's milk (17 ± 0.6%) (2). These results suggest that the calcium availability of calcium-fortified soymilk was dependent on the chemical form of the calcium salts.

Calcium bioavailability of calcium-fortified soymilk is determined mainly by the fortificant because of the low abundance of calcium in soymilk. Thus, it is the fortificant of the soymilk rather than the natural calcium in soymilk that should be labeled for such a study. Furthermore, intrinsic labeling of the fortificant should be used to reflect true bioavailability of the fortificant because extrinsic labeling can overestimate bioavailability if an incomplete exchange of tracer and calcium in the product occurs. The single human study evaluating calcium absorption from fortified soymilk compared TCP-fortified soymilk with cow's milk in healthy men using a crossover design (3). Fortificants of soymilk (TCP) were labeled either intrinsically with ^{45}Ca-TCP or extrinsically with $^{45}CaCl_2$. Cow's milk was extrinsically labeled with $^{45}CaCl_2$, a previously validated approach for this food (4). The "fortificant-intrinsically labeled" soymilk resulted in significantly lower fractional calcium absorption (0.237 ± 0.0153) compared with cow's milk (0.306 ± 0.015) and extrinsically labeled soymilk (0.358 ± 0.0167). This represents ~25% lower calcium absorption from this fortified soymilk compared with cow's milk. However, the most popular soymilk on the market

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[4] Abbreviations used: BMC, bone mineral content; BMD, bone mineral density; CC, calcium carbonate; CCSM, CC-fortified soymilk; DXA, dual energy X-ray absorptiometry; FCA, fractional calcium absorption; ICP-OES, inductively coupled plasma optical emission spectrometer; na, natural abundance; TCP, tricalcium phosphate; TCPSM, TCP-fortified soymilk.

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is fortified with CC and it has not been evaluated for calcium absorption.

Therefore, the objective of our study was to compare calcium bioavailability of calcium carbonate–fortified soymilk (CCSM) and TCP-fortified soymilk (TCPSM) using "fortificant intrinsically labeled" products with extrinsically labeled cow's milk in young women.

SUBJECTS AND METHODS

Sample size calculation. Calcium absorption efficiency from milk in young women from a previous study in our laboratory (5) was 0.243 ± 0.063 when recalculated using 24-h urinary double isotopic dilution methods (6). With an α of 0.05 (*P*-value) and power of 0.9, a 2-tailed *t* test of paired comparisons, a sample size of 19 was determined to be needed to detect a minimum change of 5% in calcium absorption (i.e., from 24 to 29%). We anticipated 5% dropout, hence 20 subjects were recruited.

Subjects. Premenopausal healthy women (n = 20) were recruited from the Purdue University community. There were 13 Caucasians, 5 Asians, and 2 African Americans. Exclusion criteria included: pregnancy, lactation, concurrent illnesses, anemia, smoking, heavy alcohol use, history of disorders or medications that influence calcium metabolism (e.g., malabsorptive disorders, steroids, thiazide diuretics), body weight outside 85–120% of ideal body weight for height, lactose intolerance, or allergy to soy or cow's milk. All of the subjects gave written informed consent to the protocol, which was approved by the Institutional Review Board at Purdue University. Nineteen subjects finished all 3 treatments; 1 subject withdrew before her last treatment (cow's milk). A whole body dual energy X-ray absorptiometry (DXA, software version 4.3e, Lunar) was performed after entry to determine total body bone mineral density (BMD) and bone mineral content (BMC).

Labeling products. Cow's milk was extrinsically labeled with $^{44}CaCl_2$ converted from $^{44}CaCO_3$ (enriched to 95.9%, Trace Sciences International) by adding 10 mg ^{44}Ca to 240 mL milk at least 12 h before consumption by subjects. Nickel et al. (4) showed that calcium absorption efficiency was identical between intrinsically labeled cow's milk (cow was injected i.v. with stable isotope so that the tracer was incorporated into milk biosynthetically) and extrinsically labeled cow's milk (stable isotope was mixed with milk overnight before testing). To match protein and fat content with calcium fortified soymilk, we used 2% reduced-fat cow's milk for our study.

CCSM was labeled as follows: $^{44}CaCO_3$ was mixed with "unlabeled" $CaCO_3$ at 10 mg ^{44}Ca/300 mg Ca and added together with other components before processing 9 L of soymilk at the pilot plant (White Wave Silk Soymilk). Thus, the ^{44}Ca would exactly reflect the calcium (fortificant) in the product. The first 2 L and the last 2 L of labeled soymilk were discarded to ensure its homogeneity. The middle flow through (~5 L) of the labeled soymilk was collected for testing.

^{44}Ca-TCP was prepared by simultaneously reacting ratios of KH_2PO_4, NaOH, and ^{44}Ca labeled Ca $(NO_3)_2$ in a large volume of hot water at pH 9.0 (7). ^{44}Ca $(NO_3)_2$ was prepared by dissolving $^{44}CaCO_3$ in HNO_3. The ^{44}Ca-TCP precipitate was washed in a Buchner funnel and air-dried. The molar ratio of Ca:P in the final product was 1.64 analyzed by an inductively coupled plasma optical emission spectrometer (ICP-OES, Optima 4300DV, Perkin Elmer). Labeled TCPSM was prepared in the same manner as CCSM except ^{44}Ca-TCP at 10 mg ^{44}Ca/300 mg "unlabeled" Ca was added.

Preparation of i.v. isotopic solution. All of the procedures were conducted under the laminar flow hood to ensure sterility. The appropriate amount of $^{43}CaCO_3$ (enriched to 60.2%, Trace Sciences International) was dissolved in 12 mol/L HCl and neutralized with 2 mol/L NaOH. The pH was adjusted to between 5.5 and 7 with 0.2 mol/L NaOH. Saline was added to the desired volume and the solution was forced through a 0.2-μm sterilization filter into the sterile evacuated container. Individual doses (5 mg ^{43}Ca/dose) were transferred into evacuated sterile vials for later use. Aliquots were sent for sterility and pyrogenicity testing (MidAmerica Clinical Laboratories) before use. All of the i.v. solutions in the study were sterile and free of pyrogens.

Test protocol. All of the Ca absorption tests were performed from September 2003 to December 2003. Subjects were asked to take 10 μg vitamin D/d 1 mo before the first calcium absorption test and throughout the entire study to minimize seasonal effects on vitamin D status. To ensure consistency of soymilk labeling, we processed each labeled soymilk in 1 batch. Due to the short shelf-life of the labeled soymilk, all the subjects had to be tested on each soymilk within the same period. Thus, subjects were randomized into only 2 sequences of receiving the treatments: *1)* cow's milk→CCSM→TCPSM or *2)* CCSM→TCPSM→cow's milk. This design allowed us to account for any seasonal effect in group comparison.

Ca absorption tests were conducted in the same phase of their menstrual cycle for all treatments. For 17 subjects, this was the follicular phase, and for 3 subjects, this was the luteal phase. There was at least a 1-mo washout period between tests. On each test day, baseline urine samples were collected. Then, either soymilk or cow's milk containing 250 mg calcium and 10 mg ^{44}Ca was administered orally with a slice of Ca-free toast. After subjects consumed the beverages, the glass was washed with deionized water twice; this was consumed to ensure the complete administration of oral isotope. The other isotope (5 mg ^{43}Ca) was injected i.v. 1 h after completion of breakfast. Syringes were weighed before and after the injection to calculate the amount of solution delivered. Subjects were asked to abstain from all additional food and liquids except deionized water for 5 h after the oral dose. Complete 24-h urine samples were collected.

Sample preparations. Aliquots of labeled soymilk and cow's milk were ashed at 600°C for 2 d. The ash was then dissolved in 2% HNO_3 (wt:v) and analyzed for total calcium by ICP-OES and isotopic ratios (^{44}Ca/^{42}Ca, ^{43}Ca/^{42}Ca). Urine samples were prepared as follows: 4 drops of 25% NH_4OH (wt:v) were added to a 2-mL urine sample to adjust the pH to 10. Then 20 μL saturated ammonium oxalate solution was added to precipitate calcium overnight. The next day, samples were centrifuged at 3000 × *g* for 20 min at 20°C and the supernatant was discarded. The pellets were then dissolved in 2% HNO_3 (wt:v) and diluted to ~1 mg/L for ICP-MS analysis.

ICP-MS procedures. Isotope ratios were determined on an ELEMENT-2 high-resolution inductively coupled plasma mass spectrometer (ThermoFinnigan) using an Aridus Desolvating Sample Introduction system with a T1H nebulizer (Cetac Technologies). The ELEMENT-2 was run in low-resolution mode using the method developed by Field et al. (8). After the instrument was tuned for maximum sensitivity and peak stability, registry settings were set to fast scan and 50 prescans. The nebulizer tip was then placed in a 100 μg/L calcium solution in 2% HNO_3 (wt:v). The ^{42}Ca peak was scanned in medium resolution with a wide window. The sweep on the Aridus system was adjusted to minimize the ArH^{2+} peak. The nitrogen gas was adjusted to optimize peak stability while ensuring that the N_3^+ peak remained small.

Initially a 2% HNO_3 (wt:v) blank was run to verify that the background ion current was low in the region of interest. Next, a 1 mg/L calcium solution was run to verify the stability and precision of the instrument. An SD of <0.2% was usually obtained for a series of 5 sample runs. Meeting these instrument criteria, samples were analyzed with the method described below. The sample set was analyzed using the Aridus Autosampler. Blanks and the 1 mg/L calcium standard solution were run every 5 samples to verify instrument stability and monitor for sample carry-over throughout the analysis.

The analog detection mode was used to monitor $^{42}Ca^+$, $^{43}Ca^+$, and $^{44}Ca^+$, and the counting detection mode was used for $^{87}Sr^{++}$. The SEM detector voltage was set at 2025 V for this study. Narrow mass windows were used, making it necessary to ensure that the peaks were smooth and flat on top. Each sample run took ~4 min, with a 2-min washout between samples to prevent sample carry-over. Operating parameters are listed in **Table 1**.

Calculations. Oral doses were calculated as follows:

For labeled soymilk and cow's milk,

$$^{44}\text{Ca dose (mmol)} = \frac{\text{drink volume (mL)} \times [\text{Ca}]}{40} \times \text{na}^{42}\text{Ca} \times \text{observed } ^{44}\text{Ca}/^{42}\text{Ca} \quad (1)$$

TABLE 1

Operating parameters of ICP-MS

Tune parameters for ELEMENT-2		Acquisition parameters		Evaluation parameters	
Gas flow	L/min	Resolution	Low	Resolution	Low
Sample	0.9–1.2	Mass window	10%	Search window	100%
Auxiliary gas	1.0	Samples/Peak	200	Integration window	100%
Cooling gas	15.0	Sample time/ms	1	Integration type	Average
		Segment time/ms	20	Runs	3
				Passes	1000
		Aridus parameters			
Sample uptake	45 s	Sweep gas	3.2–3.4 L/min		
Sample wash	120 s	Nitrogen flow	14–18 mL/min		
Analysis time	252 s	Spray chamber temperature	80°C		
Scan type	E-Scan	Desolvator temperature	160°C		

Fractional calcium absorption (FCA) was calculated using the following equations (9).

$$FCA = \frac{\Delta excess^{44}Ca\ (oral)}{\Delta excess^{43}Ca\ (iv)} \times \frac{na^{44}Ca}{na^{43}Ca} \times \frac{dose^{43}Ca}{dose^{44}Ca} \quad (2)$$

where

$$\Delta excess^{44}Ca = \frac{observed^{44}Ca/^{42}Ca - baseline^{44}Ca/^{42}Ca}{baseline^{44}Ca/^{42}Ca} \quad (3)$$

$$\Delta excess^{43}Ca = \frac{observed^{43}Ca/^{42}Ca - baseline^{43}Ca/^{42}Ca}{baseline^{43}Ca/^{42}Ca} \quad (4)$$

"Observed" ratios derive from 24-h urine samples whereas "baseline" ratios derive from baseline urine samples; "na" ^{44}Ca is the natural abundance of ^{44}Ca, and "na" ^{43}Ca is the natural abundance of ^{43}Ca.

Statistics. Data were analyzed using a mixed linear model with SAS statistical program (version 8.0, SAS Institute). The "Proc mixed" model was employed with subject treated as a random factor nested under sequence; treatment and sequence were fixed factors. LSMEANS was used for comparing multiple group means with Tukey's test. Differences were considered significant when $P < 0.05$. Data are expressed as means ± SD.

RESULTS

The general characteristics of the subjects are listed in **Table 2**. Phytate content in the fortified soymilks was <0.1% (wt:v, or 0.1 g/100 g) and the total isoflavones from a serving (240 mL) of fortified soymilk was 30.58 mg. Fractional Ca absorption did not differ between cow's milk and CCSM but both were higher ($P < 0.05$) than absorption from TCPSM (**Fig. 1**). Although the treatment sequence did not affect overall calcium absorption, there was an interaction between sequence and treatment in that fractional calcium absorption from TCPSM in sequence 2 was lower than that from sequence 1 ($P < 0.005$).

TABLE 2

General characteristics of the young women[1]

Variable	
Age, *y*	23 ± 2
Height, *cm*	163.7 ± 8.4
Weight, *kg*	61.0 ± 10.8
BMI, *kg/m²*	22.6 ± 2.7
Total body BMD, *g/cm²*	1.18 ± 0.09
Total body BMC, *g*	2600 ± 484

[1] Values are means ± SD, $n = 20$.

DISCUSSION

The results of the present study clearly showed that CCSM has calcium bioavailability similar to that of cow's milk. Our results also confirmed findings of others (3) that calcium in TCPSM is less absorbable than in cow's milk. However, there was an unexplained sequence effect on calcium absorption from TCPSM despite the fact that all of the product was from a single batch. Considering that each fortified soymilk was tested within a short period (1.5 mo), this was unlikely due to a seasonal effect. This is further supported by the lack of difference in calcium absorption from cow's milk whether tested during late summer or early winter (3 mo apart) from the 2 sequence subgroups.

Some components in soymilk may affect calcium absorption including soy isoflavones and phytate. Soy isoflavone is an estrogen-like compound and might act as a selective estrogen receptor modulator. Each serving of soymilk contains ~20–70 mg soy isoflavones (10,11). It was shown that estrogen receptors exist in various segments of the intestine including the

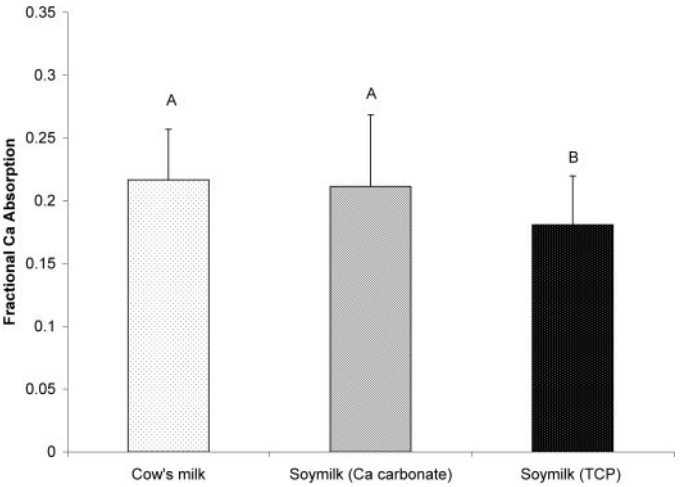

FIGURE 1 Fractional Ca absorption from cow's milk and Ca-fortified soymilks measured by the dual isotopic method in 24-h urine samples from young healthy women ($n = 20$ for soymilks, $n = 19$ for cow's milk). Values are means ± SD. Means without a common letter differ, $P < 0.05$.

colon (12–16). Intestinal calcium transport was significantly increased in rats fed an isoflavone-rich soy protein diet (17). However, in both a rat study (18) and a human study (19) conducted in our laboratory, isolated soy protein with isoflavones did not enhance calcium absorption over isolated soy protein in which the isoflavones had been removed as assessed using calcium tracer kinetics.

Phytate is a moderate inhibitor of calcium absorption and is the main form of phosphorus in soymilk, but it is in relatively low concentration at 0.12 g/100 g dry weight in soymilk (20). Heaney et al. (21) reported that the bioavailability of Ca from low-phytate soybeans (0.301 g/100 g) did not differ from that of cow's milk at a 100-mg calcium test load in humans. Given the relatively low content of phytate (<0.1 g/100 g) in the soymilk tested, phytate is unlikely to be a major inhibitor of calcium absorption in fortified soymilk.

Measurement of FCA using the dual-isotopic label method in a 24-h urine collection was validated by Yergey et al. (22). A complete collection of 24-h urine overcomes the potential problem of different calcium absorption patterns from oral and i.v. isotopes, which could confound calculation from a single serum sample.

Calcium from TCP was shown to be absorbed less than CC (23) and cow's milk (4), but due to the limited sample sizes (n = 10 and 6) in these 2 studies, differences were not significant. In the present study, the use of a powerful crossover design and sufficient sample size allowed us to detect significant differences. The lower calcium absorption from TCP may relate to the phosphate in TCP, which could precipitate calcium in the intestine after dissociation in the stomach.

In conclusion, calcium absorption efficiency is similar for CCSM and cow's milk in premenopausal women. The 3 cups (710 mL) of cow's milk daily recommended by the 2005 Dietary Guidelines would provide 855 mg total calcium and 186 mg absorbable calcium in our study population. The same amount of calcium-fortified soymilk would provide 1104 mg total calcium with 233 mg absorbable calcium if CC were the fortificant and 200 mg absorbable calcium if TCP were the fortificant (assuming the same fractional calcium absorption in divided doses as we report here). Thus, the extra calcium used to fortify soymilk makes up for the reduced bioavailability when TCP is used as the fortificant. All 3 beverages are good sources of calcium provided that the fortificant remains well dispersed and is actually consumed.

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