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Calcium Does Not Inhibit the Absorption of 5 Milligrams of Nonheme or Heme Iron at Doses Less Than 800 Milligrams in Nonpregnant Women[1,2]

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Abstract

Calcium is the only known component in the diet that may affect absorption of both nonheme and heme iron. However, the evidence for a calcium effect on iron absorption mainly comes from studies that did not isolate the effect of calcium from that of other dietary components, because it was detected in single-meal studies. Our objective was to establish potential effects of calcium on absorption of nonheme and heme iron and the dose response for this effect in the absence of a meal. Fifty-four healthy, nonpregnant women were selected to participate in 4 iron absorption studies using iron radioactive tracers. We evaluated the effects of calcium doses between 200 and 1500 mg on absorption of 5 mg nonheme iron (as ferrous sulfate). We also evaluated the effects of calcium doses between 200 and 800 mg on absorption of 5 mg heme iron [as concentrated RBC (CRBC)]. Calcium was administered as calcium chloride in all studies and minerals were ingested on an empty stomach. Calcium doses ≥1000 mg diminished nonheme iron absorption by an average of 49.6%. A calcium dose of 800 mg diminished absorption of 5 mg heme iron by 37.7%. In conclusion, we demonstrated an isolated effect of calcium (as chloride) on absorption of 5 mg of iron provided as nonheme (as sulfate) and heme (as CRBC) iron. This effect was observed at doses higher than previously reported from single-meal studies, starting at ~800 mg of calcium. J. Nutr. 141: 1652–1656, 2011.

Introduction

Iron is an essential mineral; it plays a key role in hemoglobin synthesis and also oxidoreduction reactions by donating and accepting electrons. Humans obtain iron from the diet associated to hemoglobin or myoglobin (heme iron) or not associated to these proteins (nonheme iron) (1). Iron deficiency is the most common single nutrient deficiency in the world, affecting even developed countries. This is due to low iron intake and the fact that iron absorption is diminished by other dietary components, such as phytic acid, polyphenols, and calcium (2–5), especially in populations consuming diets low in meat (6).

Calcium is the only known component in the diet that might affect absorption of both heme and nonheme iron. Hallberg et al. (7) reported that 40–300 mg of calcium (as chloride) has a dose-dependent inhibitory effect on the absorption of 5 mg nonheme iron (as sulfate) but no further inhibition greater than these amounts. Furthermore, this group reported that 165 mg of calcium (as chloride) diminished absorption of 5 mg heme iron (as rabbit hemoglobin) (7,8); a dose response curve for the calcium effect on absorption of heme iron was not established. The evidence for a calcium effect on iron absorption mainly comes from the studies by Hallberg et al. (7–9), who did not isolate the effect of calcium from that of other dietary components, because they were detected in single-meal studies. In contrast, the only study that evaluated the effect of calcium (as carbonate) on absorption of nonheme iron (as sulfate) taken on an empty stomach does not support the hypothesis of an inhibition (10). In this study, 600 mg calcium and 37 mg nonheme iron, or 300 mg calcium and 18 mg nonheme iron, were ingested together. This study also did not establish a dose response curve for the effect of calcium on the absorption of either nonheme or heme iron.

Currently, a large percentage of the population in most countries does not consume the recommended amount of calcium and is encouraged to increase their intake (11,12). On the other hand, women from regions with a high prevalence of anemia should be supplemented with iron (13). Combined calcium and iron supplements would be an interesting strategy to achieve both goals. However, it is important to clarify whether calcium affects the

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absorption of iron. Our objective was to establish potential effects of calcium on the absorption of nonheme iron and to establish a dose response curve for this effect when both minerals are ingested on an empty stomach. We also explored the effect of calcium on heme iron absorption as these forms of iron are known to be absorbed by different mechanisms (14,15).

Methods

Fifty-four women between 34 and 46 y of age were selected to participate in 1 of 4 iron absorption studies (15 in studies A and C, 13 in study B, and 11 in study D). None of the women were pregnant or lactating and all had to be using intrauterine devices as their method of contraception at the time of the study. Exclusion criteria were obesity (BMI >30) and any known acute or chronic disease, as evaluated by a physician. Informed consent was obtained from all the volunteers before the study began. The protocol was approved by the Ethics Committee of the Institute of Nutrition and Food Technology, and the doses of radioactive isotopes used were approved by the Chilean Commission of Nuclear Energy.

Study design. Studies were performed to characterize the dose response curves to graded levels of calcium (as calcium chloride) on absorption of nonheme iron (as ferrous sulfate) and heme iron (as CRBC[5]). **Table 1** summarizes the absorption studies conducted. Study A was conducted to evaluate the effect of 0-, 200-, 400-, and 800-mg calcium doses on the absorption of nonheme iron. Based on the results, we designed study B to evaluate the effects of 0-, 1000-, 1250-, and 1500-mg calcium doses on the absorption of nonheme iron. Study C was conducted to evaluate the effect of 0-, 200-, 400-, and 800-mg calcium doses on the absorption of heme iron. Based on these results, we designed study D to evaluate the effects of 0-, 500-, 600-, and 800-mg calcium doses on the absorption of heme iron. In all studies, a labeled 5-mg iron dose was administered on d 1, 2, 14, and 15, with 37 kBq ^{59}Fe given on d 1 and 14 and 111 kBq ^{55}Fe given on d 2 and 15. Increasing calcium doses were administered with iron during d 2, 14, and 15. Doses were administered after a nocturnal fast, with volunteers not being allowed to eat again until 4 h after ingestion of the doses. Iron isotopes of high specific activity were used as tracers (NEN, Life Science Products). Doses of nonheme iron labeled with the iron isotopes were given to the participants in 50 mL distilled deionized water. Doses of labeled CRBC and calcium were given in gelatin capsules (number 0; Reutter).

Labeling of CRBC. The labeled CRBC was prepared by using RBC from rabbits based on the method described by Asenjo et al. (16). Briefly, New Zealand rabbits, ~3 kg in weight, received an i.v. injection of 74 MBq of ^{55}Fe or 37 MBq of ^{59}Fe as ferric citrate (NEN, Life Science Products) diluted in 10 mL of 0.16 mol NaCl/L. Fifteen days later, the rabbits were exsanguinated through cardiac puncture. The radioactive RBC were centrifuged (1000 × *g* for 15 min at 22°C) and washed with saline, hemolyzed by freezing, and finally dehydrated by lyophilization. Labeled freeze-dried CRBC with a specific activity of 475 kBq of ^{59}Fe and 2.46 MBq of ^{55}Fe/mg of heme iron was obtained. This was mixed in dry form with unlabeled bovine red cells, resulting in a dose of 37 kBq ^{59}Fe or 111 kBq ^{55}Fe/5 mg of elemental iron. We added unlabeled bovine CRBC to the labeled rabbit CRBC to increase the volume of CRBC given that the amount obtained from rabbit exsanguination was too small to conduct the heme iron absorption studies. Elemental iron was determined by atomic absorption spectrometry (Perkin-Elmer Model SIMAA 6100; Perkin Elmer). The compounds were packaged in gelatin capsules.

Blood samples. Venous blood samples were obtained on d 14 and 28 to measure circulating radioactivity and determine the iron status of the volunteers. Hb and MCV were determined in a CELL-DYN 1700 instrument (ABBOTT Diagnostics). FEP was determined in a hematofluorometer (ZP-M206D, AVIV Biomedical). SF was determined by ELISA (17) and Sat was calculated as previously reported (18). Anemia was defined as Hb <120 g/L and iron deficiency without anemia as Hb >120 g/L but at least 2 other parameters outside the normal range (MCV <80 fL, and/or FEP >700 μg/L RBC, Sat <15%, and/or SF <12 μg/L) (19).

Radioisotope analysis. For the calculation of total radioactivity ingested, aliquots of the compounds were counted in sextuplets as standards. Measurement of blood radioactivity was performed in duplicate venous samples according to the Eakins and Brown technique (20). Samples were counted within a sufficient time period to obtain a counting error of ~3% in a liquid-scintillation counter (Beckman LS 5000 TD; Beckman Instruments). The percentages of absorption were calculated on the basis of blood volume estimated for height and weight (21) and assuming 80% incorporation of the radioisotope into erythrocytes in all volunteers, independent of iron status (22). This method is reproducible in our laboratory with a CV of 5%.

Statistical analysis. Because the percentages of iron absorption and SF concentrations had a skewed distribution, these values were log-transformed before calculating means and SD or performing statistical analyses. Results were retransformed to recover original units and are expressed as geometric means (−1 SD, +1 SD). Iron nutritional status, iron absorption on d 1 between participants of studies A and B and between participants of studies C and D were compared by an independent samples *t* test. Iron absorption differences in each study were determined by repeated-measurements ANOVA and Dunnett's post hoc test and the absorption of iron on d 1 was considered as control. $P < 0.05$ was considered significant. The dose response curves for the calcium effect on absorption of nonheme and heme iron were obtained by using the ratios between the absorption of iron ingested with calcium (d 2, 14, and 15) divided by the absorption of iron ingested without calcium (d 1). The data were then analyzed by polynomial regression. A sample of 15 participants/group was estimated to detect a 5% difference in iron absorption within the same participant, with $\alpha = 0.05$, 80% power, and allowing for an estimated 25% loss to follow-up. All analyses were performed using the statistical software SPSS (SPSS for Windows, version 11.0, SPSS).

Results

Three volunteers presented with iron deficiency anemia (1 in study C and 2 in study D). Ten volunteers were iron deficient without anemia (2 in study A and 4 in studies B and C). Hb and MCV differed between volunteers in studies A and B. There were no other differences in BMI and iron nutritional status between volunteers in studies A compared to B or C compared to D (**Table 2**). The absorption of iron on d 1 was similar in volunteers who participated in the nonheme iron absorption studies (17.9% for A and 21.3% for B; $P = 0.47$). Similarly, the absorption of iron on d 1 was similar in volunteers who participated in the heme iron absorption studies (13.9% for C and 11.1% for D; $P = 0.29$) (Table 2).

In study A, calcium doses between 0 and 800 mg did not affect the absorption of 5 mg nonheme iron ($P = 0.09$) (**Table 3**). In study B, calcium doses ≥1000 mg diminished nonheme iron absorption by 49.6% ($P < 0.05$) (Table 3). **Figure 1***A* shows the adjusted curve for the effect of calcium on nonheme iron absorption.

In study C, the 800 mg calcium dose diminished absorption of 5 mg heme iron ingested as CRBC by 37.7% ($P < 0.05$) (Table 3). In study D, calcium doses of 500–700 mg did not affect the absorption of 5 mg heme iron ($P = 0.37$) (Table 3). Figure 1*B* shows the adjusted curve for the effect of calcium on heme iron absorption.

Discussion

The evidence suggesting an inhibitory effect of calcium on absorption of iron was obtained in volunteers who ingested iron

[5] Abbreviations used: CRBC, concentrated RBC; FEP, free erythrocyte protoporphyrin; Hb, hemoglobin; MCV, mean corpuscular volume; SF, serum ferritin.

TABLE 1 Iron absorption studies conducted in nonpregnant women[1]

Study (*n*)	Day 1	Day 2	Day 14	Day 15
Nonheme iron absorption studies[1]				
A (15)	Iron dose+ ^{59}Fe + 0 mg Ca	Iron dose + ^{55}Fe + 200 mg Ca	Iron dose + ^{59}Fe + 400 mg Ca	Iron dose + ^{55}Fe + 800 mg Ca
B (13)	Iron dose + ^{59}Fe + 0 mg Ca	Iron dose + ^{55}Fe + 1000 mg Ca	Iron dose + ^{59}Fe + 1250 mg Ca	Iron dose + ^{55}Fe + 1500 mg Ca
Heme iron absorption studies[2]				
C (15)	Iron dose + ^{59}Fe + 0 mg Ca	Iron dose + ^{55}Fe + 200 mg Ca	Iron dose + ^{59}Fe + 400 mg Ca	Iron dose + ^{55}Fe + 800 mg Ca
D (11)	Iron dose + ^{59}Fe + 0 mg Ca	Iron dose + ^{55}Fe + 500 mg Ca	Iron dose + ^{59}Fe + 600 mg Ca	Iron dose + ^{55}Fe + 700 mg Ca

[1] Five-mg iron doses were provided as sulfate (A and B) or concentrated RBC (C and D). Calcium was provided as chloride.

and calcium together in a meal (7–9). Cook et al. (10) published the only study that isolated the effect of calcium on absorption of iron from other dietary components. They did not find any effect of calcium when they administered 300 mg calcium (as carbonate) and 37 mg nonheme iron (as sulfate) doses to healthy volunteers, which corresponds to a Ca:Fe molar ratio of 11:1. They also evaluated a higher Ca:Fe molar ratio (46:1) and did not find any effect. Our studies were designed to clarify the effect of increasing calcium doses (as chloride) on 5 mg of nonheme (as sulfate). We decided to evaluate the absorption of 5 mg iron based in doses previously administered by Hallberg et al. (7) who published the most relevant data supporting the hypothesis of an inhibitory effect of calcium on the absorption of iron; this dose represents the typical content of iron in a meal. We found that calcium doses <800 mg did not affect the absorption of 5 mg nonheme iron (Ca:Fe molar ratio ≤223:1); however, calcium doses ≥1000 mg diminished nonheme iron absorption by 49.6%. Thus, at a Ca:Fe molar ratio of ~280:1 and above an inhibitory effect was observed.

In study A, the 800-mg calcium dose diminished the absorption of nonheme iron by 37.8%; however, this difference was not significant. This is likely explained by the large inter-individual differences in iron absorption. It is possible that a larger sample size was needed to observe a significant effect. Thus, we conclude that the inhibitory effect of calcium on absorption of 5 mg nonheme iron starts at a level of ~800 mg of calcium. Thus, the dose of calcium that we report as inhibitory of iron absorption is higher than the dose reported by Hallberg et al. (7). The difference may be explained by an interaction among calcium, iron, and other dietary components in the former study. Actually, Hallberg et al. (3,7) concluded that one part of the inhibition of iron absorption was caused by an inhibitory effect of calcium on the enzymatic degradation of phytate, leading to an increased content of phytate, which is a known inhibitor of nonheme iron absorption. Cook et al. (10) found that the addition of calcium diminished the absorption of nonheme iron when the minerals were ingested in a meal but did not have any effect when ingested on an empty stomach. Our results and those of previous reports lead us to think that the calcium effect on absorption of nonheme iron, at the doses these minerals are ingested in a normal diet, is explained by an interaction between calcium and the food matrix in the intestinal lumen rather than by a direct effect on the enterocyte. However, we have obtained data in Caco-2 cells that suggest that a level of calcium, higher than those ingested in the normal diet, may affect iron absorption by a direct effect on the enterocyte (D. Gaitán, S. Flores, F. Pizarro, M. Olivares, M. Suazo, and M. Arredondo, unpublished data).

Surprisingly, in the absence of calcium, we obtained a mean heme iron absorption of 13.9 and 11.1% in studies C and D, respectively. These percentages are considerably lower than those previously reported (~20%) in volunteers who ingested iron in a meal (4). However, we observed that the absorption of heme iron was ~10% when it was ingested as Hb or as heme moiety on an empty stomach (F. Pizarro, M. Olivares, S. Flores, V. Weinborn, D. Gaitán, and A. Brito, unpublished data). The mechanism that explains a low absorption of heme when it is ingested in the absence of proteins or another dietary component is not understood. Further research is needed to clarify it. On the other hand, the CRBC contained mostly a low amount of lipids from the erythrocyte membranes. Thus, the influence of any other component on the absorption of iron may be negligible. The effect of calcium on absorption of heme iron has been poorly studied, but an inhibitory effect has been reported in single-meal studies (7,8). Our study showed that 800 mg of calcium diminished the absorption of heme iron by 37.7%, whereas there was no significant effect at lower levels of calcium. In contrast, Hallberg et al. (7,8) reported that absorption of 5 mg on heme iron (as rabbit hemoglobin) was diminished, directly,

TABLE 2 Iron status of the nonpregnant women studied[1,2]

	Nonheme iron studies			Heme iron studies		
	Study A *n* = 15	Study B *n* = 13	*P*	Study C *n* = 15	Study D *n* = 11	*P*
BMI, *kg/m²*	25.1 ± 3	25.1 ± 3	0.98	26.9 ± 3	25.7 ± 2	0.24
Hb, *g/L*	133 ± 8	127 ± 6	<0.04	131 ± 8	132 ± 14	0.82
MCV, *fL*	87 ± 3	84 ± 2	<0.01	87 ± 6	89 ± 8	0.47
FEP, *mg/L RBC*	0.61 ± 0.09	0.63 ± 0.14	0.61	0.62 ± 0.13	0.67 ± 0.13	0.30
Transferrin saturation, %	22.6 ± 7.4	16.3 ± 10.1	0.08	19.5 ± 8.5	25.1 ± 11.0	0.15
SF, *μg/L*	20 (8–48)	18 (9–36)	0.71	17 (8–38)	17 (5–60)	0.85
Basal iron absorption, %	17.9 (7.0–45.6)	21.3 (10.1–45.0)	0.47	13.9 (8.7–22.1)	11.1 (6.2–19.8)	0.29

[1] Values are mean ± SD or geometric mean (−1 SD, +1 SD).
[2] FEP, free erythrocyte protoporphyrin; Hb, hemoglobin; MCV, mean corpuscular volume; SF, serum ferritin.

TABLE 3 Effects of various doses of calcium on women's absorption of 5 mg of iron given as nonheme or heme iron

Study	*n*	d 1	d 2	d 14	d 15	*P*
		Absorption, %				
Nonheme iron						
A	15	17.9 (7.0–45.6)	15.9 (7.6–33.6)	15.3 (5.8–40.2)	11.9 (4.7–30.5)	0.09
B	13	21.3 (10.1–40.5)	10.7* (5.3–21.8)	12.7* (6.2–26.0)	13.3* (6.6–26.7)	<0.05
Heme iron						
C	15	13.9 (8.7–22.1)	11.5 (6.9–19.1)	11.6 (6.8–19.9)	8.6* (4.2–17.4)	<0.05
D	11	11.1 (6.2–19.8)	9.0 (4.7–17.0)	9.6 (5.3–17.5)	10.1 (5.8–17.5)	0.37

[1] Values are geometric mean (−1 SD, +1 SD). *Different from d 1 (no calcium), $P < 0.05$.

by the addition of 165 mg calcium (as chloride). However, these studies did not isolate the minerals from other dietary components. Therefore, it is not possible to conclude that this dose of calcium directly affects the absorption of heme iron; it may be an indirect effect related to the interaction between calcium and other dietary components.

The mechanism by which calcium affects absorption of iron is still debated. Our results agree with Hallberg et al. (7), who postulated that calcium affects absorption of iron by an interaction between these 2 minerals at a point common for nonheme and heme iron absorption. The uptake of nonheme and heme iron at the apical membrane is mediated by 2 proteins. Nonheme iron uptake occurs via divalent metal transporter 1 (DMT1) (14) and heme is taken up by Heme Carrier Protein 1 (15). However, after heme reaches the cytoplasm, it is included into endosomes, where it is degraded by heme-oxygenase which releases iron from the heme group. Iron is then released to the cytoplasm by a DMT1 variant located on the endosome membrane (23). Thus, both types of dietary iron form a common pool in the enterocyte cytoplasm. This pool of iron is stored as ferritin or transported to enteric blood vessels by ferroportin, which is located at the basolateral membrane (24). Hallberg et al. suggested that calcium may affect ferroportin activity (7). We suggest that calcium may modulate DMT1 located in both endosomes and the apical membrane, which may explain the effect of calcium on absorption of both nonheme and heme iron. Actually, Thompson et al. (25) reported that increasing calcium doses decrease DMT1 expression at the apical membrane and suggested that it may explain the effect of calcium on the absorption of nonheme iron. Because they were not able to differentiate between the two DMT1 variants, it is not possible to draw any conclusion about the effect of calcium on the absorption of heme iron. On the other hand, calcium may modulate any process involved in the trafficking of iron in the cytoplasm; however this process is poorly understood. The effect of calcium on these mechanisms needs to be studied further.

It is important to emphasize that we evaluated the effect of calcium given as chloride on iron absorption. Unlike some other calcium salts, chloride is highly dissociated in the gastrointestinal tract. Thus, the maximal interaction between calcium and iron in the gastrointestinal tract in the absence of any other dietary component would be expected. Some reports suggest that the effect of calcium on the absorption of iron depends on the calcium salt administered. Cook et al. (10) showed that 600 mg calcium, as citrate or phosphate, which are also well dissociated, diminished absorption of 18 mg iron (as sulfate) by 50% when they were ingested on an empty stomach, but this effect was not observed when the same amount of calcium was ingested as calcium carbonate. Further, Monsen and Cook (26) observed that absorption of 4.3 mg iron (present in a meal) was diminished 50% by addition of 178 mg calcium as phosphate and an additional amount of phosphate salt. However, Roughead et al. (27) did not find any effect of 450 mg of calcium (as citrate) on the absorption of dietary calcium. Due to these differences between calcium salt effects on iron absorption, our data should not be extrapolated to other salts.

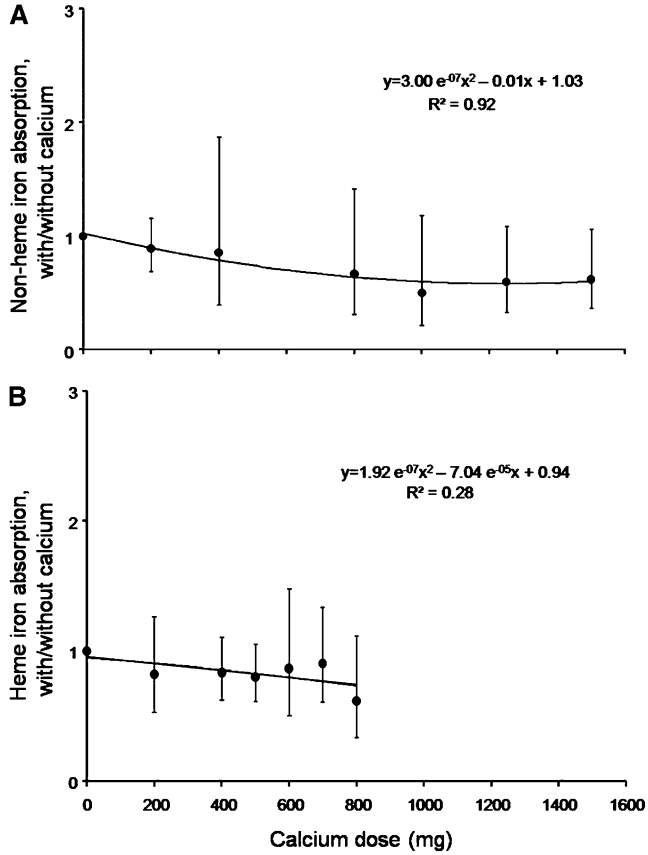

FIGURE 1 The effect of various doses of calcium (as chloride) on absorption of nonheme iron (*A*) and heme iron absorption (*B*) in nonpregnant women. The ratios were calculated by dividing the absorption of iron in the presence of calcium (d 2, 14, and 15) by the absorption of iron in the absence of calcium (d 1) during studies A (n = 15) and B (n = 13) (*A*), and C (n = 15) and D (n = 11) (*B*). Data points are geometric mean; bars are −1 SD, +1 SD. Data were analyzed by polynomial regression.

Due to the inhibitory effect on iron absorption that has been attributed to calcium (7–9), some studies have evaluated the impact of an increased calcium intake by calcium supplementation on iron status. Kalkwarf and Harrast (28) did not find any effect of 500-mg calcium supplements, taken twice per day for 6 mo, on iron status of lactating women, when they were ingested with the main meals. A similar result was obtained by Molgaard et al. (29) in 12- to 14-y-old girls who ingested 500 mg calcium with their evening meal during 1 y. One proposed mechanism to explain the difference between acute and expected chronic effects of calcium on iron absorption is adaptive responses by the intestinal mucosal cell (30).

Women from regions with a high prevalence of anemia should be supplemented with iron (13). On the other hand, the provision of calcium as supplements is one strategy to increase intake in those populations who do not consume the recommended amounts of calcium (11,12). Based on our results, it would be possible to provide supplements with combined therapeutical doses of iron and calcium, if their Ca:Fe molar ratio is lower than 220:1. Further studies are needed to confirm this.

In summary, we described the isolated effect of calcium (as chloride) on absorption of 5 mg iron doses, as nonheme (as sulfate) and heme (as CRBC) iron. This effect is present at doses near to 800 mg of calcium for both nonheme and heme iron, which are higher than previously reported. It may be possible to design supplements that improve both calcium and iron nutritional status in populations at risk.

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