Inhibition of Iron Absorption by Calcium Is Modest in an Iron-Fortified, Casein- and Whey-Based Drink in Indian Children and Is Easily Compensated for by Addition of Ascorbic Acid1,2

Thomas Walczyk,3,4* Sumithra Muthayya,5 Rita Wegmüller,3 Prashanth Thankachan,5 Aafje Sierksma,6 Leon G. J. Frenken,6 Tinku Thomas,5 Anura Kurpad,5 and Richard F. Hurrell3

3Laboratory for Human Nutrition, Swiss Federal Institute of Technology (ETH) Zurich, Zurich, Switzerland; 4Departments of Chemistry (Science) and Biochemistry (Medicine), National University of Singapore, Singapore; 5Division of Nutrition, St. John’s Research Institute, Bangalore, India; and 6Unilever Research and Development, Vlaardingen, The Netherlands

Abstract

Background: Calcium inhibits and ascorbic acid (AA) enhances iron absorption from iron-fortified foods. Absorption efficiency depends on iron status, although the interaction is unclear.

Objective: We investigated the ability of AA to overcome calcium-induced inhibition of iron absorption in children differing in iron status.

Methods: The effect of calcium (0, 100, and 200 mg/test meal) on iron absorption in the absence and presence of AA (0, 42.5, and 85 mg/test meal) from a casein/whey-based drink fortified with ferrous sulfate was assessed in a series of randomized crossover studies both in iron-replete (IR) Indian schoolchildren and in children with iron deficiency anemia (IDA) (6–11 y; n = 14–16/group) by using stable isotopes.

Results: In the absence of calcium and AA, iron absorption from the casein/whey-based drink was 20% lower in IR children than in children with IDA. The addition of calcium reduced mean iron absorption by 18–27%, with the effect being stronger for high added calcium (P < 0.01). AA at a 2:1 or 4:1 molar ratio enhanced iron absorption by a factor of 2–4 and greatly overcompensated for the inhibitory effect of calcium on iron absorption in a dose-dependent manner (P < 0.001). The dose-response effect tended to be stronger (P < 0.1) in the IDA group, and iron status was of far less influence on iron absorption than the enhancing effect of AA.

Conclusion: When adding AA to iron-fortified milk products, care should be taken not to provide absorbable iron in excess of needs. J. Nutr. 144: 1703–1709, 2014.

Introduction

An adequate iron supply during childhood is required for a child to fully utilize his or her intellectual and physical potential. Iron deficiency with or without anemia has been linked to retarded mental and psychological development (1), less energy conversion for daily activities (2), and a lower immune defense (3). Growing children in developing countries are at high risk of developing iron deficiency because their diet is often based on cereals and legumes with few animal-source foods, fruits, or vegetables (4). In India, anemia affects 50–90% of children (5).

It is estimated that 50–95% of the anemia is associated with iron deficiency.

Fortification of food products is widely considered to be the best approach to fill the gap between nutrient requirements and an insufficient dietary supply. When considering the development of targeted fortified foods for children in resource-poor areas in developing countries, iron, vitamin A, and possibly zinc fortification are likely to have the most public health relevance; however, there is also a strong argument to add calcium for improving bone growth. Low calcium intake could result in children not attaining their genetically predetermined peak bone mass (6), which can lead to osteoporosis and the risk of bone fractures later in life (7).

The addition of calcium to an iron-fortified food or beverage can negatively affect iron absorption. A variety of calcium salts were shown to inhibit both heme and nonheme iron absorption
(8–13), an effect that seems to depend primarily on the calcium load and not on the calcium to iron molar ratio (14). The negative effect of calcium on the quantity of iron absorbed can be overcome by the addition of ascorbic acid (AA)7 or by increasing iron fortification levels. AA enhances iron absorption in a dose-dependent manner and is thought to exert its enhancing effect by reducing ferric to ferrous iron and by binding iron in a soluble form available for absorption (15). Iron absorption is further moderated by an individual’s iron status. In a recent study, iron absorption from a rice meal was 2-fold higher in women with iron deficiency anemia (IDA) than in women who were iron replete (IR), and the addition of AA at a 4:1 molar ratio relative to iron increased iron absorption by ~3.5-fold in both IDA and IR women (16).

The aim of the present study was to quantify the inhibitory effect of calcium on iron absorption in school-aged children from a casein/whey-based drink and to assess how much AA should be added to compensate for the inhibitory effect of calcium so as to ensure adequate iron absorption. Iron absorption was measured by a stable isotope technique in Indian schoolchildren. Studies were conducted in an identical way in IR children and in children with IDA so as to investigate the influence of iron status on the effect of calcium and AA on iron absorption.

**Participants and Methods**

The study was conducted in Bangalore, India, during the dry season so as to avoid the possible confounding effect of infection on iron absorption. Iron absorption tests were conducted in identical form in IR children and in children with IDA. Children in the IR and IDA groups were divided randomly into 3 subgroups with a target sample size of 16 children per subgroup. Children in each subgroup consumed 2 different test drinks in a crossover setting in which they were randomly assigned to the order of test drink administration while controlling equal numbers per order. In subgroup 1, we tested for the influence of 100 and 200 mg added calcium on iron absorption from an iron-fortified casein/whey-based drink. In subgroup 2, we tested for the influence of AA at molar ratios of 2:1 and 4:1 relative to iron in overcoming the inhibition of 100 mg calcium. In subgroup 3, we tested the influence of AA at molar ratios of 2:1 and 4:1 relative to iron in overcoming the inhibition of 200 mg calcium.

**Participant selection.** A total of 788 children (aged 6–11 y) were recruited from different urban slum settlements around Bangalore for iron status and nutritional status screening. Written informed consent was obtained from their parents, and oral consent was obtained from each child. The screening process was designed to select IR children and children with IDA with adequate folate, vitamin B-12, and vitamin A status on the assumption that deficiencies in these micronutrients could influence blood hemoglobin concentrations.

An EDTA-treated venous blood sample was obtained from each child. One aliquot of whole fresh blood was used for determination of hemoglobin. A second aliquot was treated with AA and stored frozen (~8°C) under minimal light exposure for erythrocyte folate analysis. Plasma was obtained from a third aliquot and was stored frozen for analysis of iron status markers [plasma ferritin (PF), soluble transferrin receptor (sTfR)] and markers of vitamin status [plasma retinol and plasma vitamin B-12] and infection [plasma C-reactive protein (CRP)].

Children were ranked for decreasing iron status on the basis of analytical results for hemoglobin, PF, and sTfR. Ranking order was by decreasing hemoglobin concentration as the primary criterion, decreasing PF concentration as the secondary criterion, and increasing sTfR concentration as the tertiary criterion. Children with significantly elevated CRP (>10 mg/L) were excluded, and the upper and lower third of remaining children were selected for further assessment of vitamin status. From the remaining children who had the fewest indications of low vitamin status (folic acid, vitamin B-12, and retinol), those who were most IR (IR group; n = 48) as well as those who had the strongest evidence of iron IDA (IDA group; n = 48) were selected. Children with the lowest CRP concentrations were preferentially included to minimize a confounding effect of infection. Cutoffs for IDA were hemoglobin <115 g/L, PF <15 μg/L, and sTfR >7.6 mg/L. Cutoffs for low vitamin status were <150 µg/L for plasma vitamin B-12, <100 µg/L for erythrocite folate, and <0.7 µmol/L for plasma retinol. Children in the IR group and the IDA group were randomly divided into 3 subgroups for the different iron absorption tests (Table 1). All of the selected children were apparently healthy with no signs of disease or use of medication at the time of the absorption tests. All absorption tests were conducted. None of the children took mineral or vitamin supplements or consumed fortified foods regularly and were not permitted to do so during the study. All screened children who had clear indications of nutrient deficiencies and/or infection were immediately treated by a local pediatrician. Children with IDA were treated with iron and folate supplements at the end of the study. The protocol of the study was reviewed independently by the ethical committees at St. John’s College of Medicine, Bangalore, and ETH Zurich, Switzerland.

**Clinical measurements.** Hemoglobin concentrations were determined in whole blood by an automated hematology analyzer (ActDiff2; Beckman Coulter). Accuracy was confirmed by daily analysis of 3 levels of quality control material (Liquchek, Hematology-16 control; Bio-Rad). PF and sTfR were determined by commercial ELISA assays (Ramco Laboratories) on frozen plasma samples. Assays were calibrated by using the standards provided by the manufacturer (horse spleen ferritin and purified sTfR solutions). A WHO quality control material (Ramco Laboratories) was processed with each batch of samples for validation of the PF assay. CRP was measured in frozen plasma samples by a particle-enhanced turbidimetric immunoassay (Dimension RXL Chemistry Analyzer; Dade). Control materials (Lyphochek; Bio-Rad) were analyzed within each run. Vitamin B-12 and erythrocyte folate were analyzed by a commercial chemoluminescence system (ACS:180; Bayer Diagnostics) (17,18) by using calibrators supplied by the manufacturer. Vitamin B-12 was determined on frozen plasma samples and erythrocyte folate in frozen whole blood. Hematocrit determined in whole blood (ActDiff2; Beckman Coulter) was used to calculate folate concentrations in lyed erythrocytes. Analytical procedures followed closely those recommended by the manufacturer for the respective assay/instrument. Plasma retinol was determined by HPLC by using a method derived from that of Bui (19) with a C-18 reversed phase column and UV detection by using retinyl acetate as an internal standard. The intrabatch CV for a pooled human plasma sample with a concentration of 1.2 μmol/L of retinol was 0.48% (n = 10), and the interbatch CV over a period of 3 wk was 5.1% (n = 15).

**Test drink composition and iron isotopic labels.** The test drinks were based on casein (80%) and whey (20%) proteins and were prepared for feeding by dissolution in hot water. The instant powder consisted of casein and whey proteins, maltodextrin, sucrose, soy fat, and K2HPO4 as a buffering agent, with no fortificants and flavors added (see Table 2). Each serving provided 586 kJ, 3.1 g fat, 23.5 g carbohydrate, and 4.5 g protein. The native calcium content of the powder was 5.9 mg/serving. The test drinks differed only in the amounts of added Ca3(PO4)2 and sodium ascorbate. Differences in phosphate content between test drinks were balanced by adding a mixture of KH2PO4 and K2HPO4 for maintaining the pH of the original preparation. The instant powder for the test drinks was prepared in bulk. On the evening before test drink administration, appropriate amounts of instant powder and Ca3(PO4)2 were dissolved in hot high-purity water (18 MΩ, 50.0 g/serving), and the casein/whey-based test drink was stored in a cool room overnight. On the following morning, the drink was divided into individual portions and iron, phosphate buffer, sodium ascorbate, sugar, and artificial strawberry flavor (20 μL, product no. 52312; Firmenich SA) were added. All additional solutions, except for the isotopic labels, were prepared freshly on the day of test drink administration. They were all added gravimetrically to the drinks except for the flavor component,
which was added volumetrically. Solutions of isotopically labeled \([^{57}\text{Fe}]-\) ferrous sulfate and \([^{58}\text{Fe}]-\) ferrous sulfate were prepared in advance at ETH Zurich by dissolution of isotopically enriched elemental iron—\([^{57}\text{Fe}]-\) metal, 95.9% enriched, and \([^{58}\text{Fe}]-\) metal, 93.2% enriched (both ETH Zurich by dissolution of isotopically enriched elemental iron—with either \([^{57}\text{Fe}}\) or \([^{58}\text{Fe}}\) (see Table 2). Within each subgroup, a drink A) and 2 test drinks (1 each of drinks B and C) that were labeled

**Study design.** The study design and protocol were similar to those used by Thankachan et al. (16). Each participant consumed 2 reference drinks (drink A) and 2 test drinks (1 each of drinks B and C) that were labeled with either \([^{57}\text{Fe}}\) or \([^{58}\text{Fe}}\) (see Table 2). Within each subgroup, a randomized crossover design was used in which each child acted as his or her own control. Each child was offered drinks A and B or drinks A and C on paired test days 1 and 2 (testing interval 1) or days 15 and 16 (testing interval 2), so that each pair of meal administrations always had a reference drink. Children were randomly assigned to start with either pair A/B or A/C in each subgroup. Children were further randomly assigned to start with the reference drink or the test drink for each pair of drink administrations. One-half of the reference drinks were labeled with \([^{57}\text{Fe}]}\) and the other half with \([^{58}\text{Fe}}\), whereas test drinks were labeled with the other tracer following a complete randomized design. Iron absorption was based on erythrocyte incorporation of isotope labels 14 d after consumption of the last labeled meal.

For the absorption test, the children arrived at the research unit in the early morning of day 1 after an overnight fast. Weight and height were measured, and the breakfast was served. The breakfast (963 kJ) consisted of tomato rice, a common South Indian morning meal, and water (150 mL). The breakfast was made from polished rice, tomato purée, and onions, which were fried in vegetable oil together with selected spices (chili, turmeric, mustard seeds). The breakfast was prepared in bulk from local ingredients, divided into individual portions, and stored frozen. Portions were defrosted overnight and warmed in a conventional microwave oven for consumption. For reasons of standardization, children had to complete the breakfast and were not given extra rations.

Reference drink A or test drinks B or C were consumed 3 h after completion of breakfast. Complete intake of the drink and isotopic labels

**TABLE 2** Composition of casein/whey protein test drinks consumed by the 3 subgroups of IR and IDA Indian schoolchildren

<table>
<thead>
<tr>
<th>All subgroups:</th>
<th>Subgroup 1</th>
<th>Subgroup 2</th>
<th>Subgroup 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>reference drink A</td>
<td>Test drink B&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Test drink C&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Test drink B&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>(no added calcium or AA)</td>
<td>(+100 mg Ca&lt;sub&gt;+100 mg Ca&lt;sub&gt;AA&lt;/sub&gt; Fe = 2:1)</td>
<td>(+100 mg Ca&lt;sub&gt;AA&lt;/sub&gt; Fe = 4:1)</td>
<td>(+100 mg Ca&lt;sub&gt;AA&lt;/sub&gt; Fe = 2:1)</td>
</tr>
<tr>
<td>Instant powder, g</td>
<td>28.2</td>
<td>28.2</td>
<td>28.2</td>
</tr>
<tr>
<td>Water, g</td>
<td>70.0</td>
<td>70.0</td>
<td>70.0</td>
</tr>
<tr>
<td>Iron as FeSO&lt;sub&gt;4&lt;/sub&gt;, mg</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Iron as [Fe&lt;sup&gt;57&lt;/sup&gt;FeSO&lt;sub&gt;4&lt;/sub&gt;] mg</td>
<td>3.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Iron as [Fe&lt;sup&gt;58&lt;/sup&gt;FeSO&lt;sub&gt;4&lt;/sub&gt;] mg</td>
<td>—</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Calcium as Ca&lt;sub&gt;3&lt;/sub&gt;(PO&lt;sub&gt;4&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;, mg</td>
<td>100</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td>K&lt;sub&gt;2&lt;/sub&gt;HPO&lt;sub&gt;4&lt;/sub&gt;, mg</td>
<td>113</td>
<td>113</td>
<td>113</td>
</tr>
<tr>
<td>Sodium ascorbate, mg</td>
<td>42.5</td>
<td>42.5</td>
<td>42.5</td>
</tr>
</tbody>
</table>

<sup>1</sup> All test drinks contained 6.0 mg added iron ([3.0 mg as isotopic label \([^{57}\text{Fe}]}\) or \([^{58}\text{Fe}}\) and 3.0 mg as iron of normal isotopic composition] and varying amounts of added calcium (0, 100, and 200 mg) and sodium (Na) ascorbate (0, 42.5, and 85 mg). K<sub>2</sub>HPO<sub>4</sub> and K<sub>2</sub>HPO<sub>4</sub> were added to correct for differences in phosphate content between meals without affecting the pH of the drink. AA, ascorbic acid; IDA, iron deficiency anemia; IR, iron replete.
was ensured by consuming the drink with a straw and by rinsing the glass 3 times with 10 mL of 18 MΩ water. The washings were likewise consumed by the child. No foods and drinks were permitted between breakfast and test drink administration and for 3 h after intake of the test drink. After a light lunch was served, children were taken back to their homes with no further restrictions on diet. The second test drink (A, B, or C) was provided in an identical manner on day 2.

A venous blood sample was drawn 14 d after intake of the second test drink (day 15) for measurement of iron isotopic composition. The second pair of test meals (A and B or A and C) was provided in an identical way on days 15 and 16. A final venous blood sample was drawn 14 d later on day 29 for measurement of isotopic composition.

Iron absorption measurements. Isotopically labeled compounds and digested blood samples were analyzed for isotopic composition at ETH Zurich by multicollector negative thermal ionization MS (Finnigan MAT 262, ThermoFinnigan) as described earlier (21). On the basis of the shift of the iron isotope ratios in the blood samples and the amount of iron circulating in the body, amounts of 57Fe and 58Fe isotopic label present in the blood 14 d after test drink administration were calculated. Calculations followed isotope dilution principles under consideration that the used iron isotopic labels were not monoisotopic (22). Circulating iron was calculated on the basis of blood volume and hemoglobin concentration. Blood volume calculations were based on height and weight according to Raes et al. (23). For calculation of fractional iron absorption, 80% incorporation of the absorbed iron into RBCs was assumed for IR children and 90% incorporation for children with IDA (24,25). A higher proportion of absorbed iron is used for erythropoiesis in IDA (26,27).

Data evaluation and statistical analysis. Measured iron isotope ratios were translated into fractional iron absorption values following algorithms laid out earlier in detail (22) and logarithmically transformed for statistical analysis. To account for intra- and interindividual variations in iron absorption efficiency, iron absorption from a given test drink (drink B or C) was normalized to iron absorption from the reference drink A in each individual participant for each study interval. Absorption ratios relative to reference drink A were calculated from non-logarithmically transformed data and were logarithmically transformed for further statistical analysis. Expression of iron absorption from a test drink relative to the reference drink in an individual minimizes uncertainties in the estimated incorporation efficiency of absorbed tracer iron into erythrocytes and the effect of iron status on iron absorption within each study group. Iron absorption data are presented in the text and tables as geometric means (−SD, +SD).

To test for differences in iron absorption between the test drinks, normalized iron absorption data were subjected to a mixed linear regression analysis in which the random effect of subject was considered by using iron absorption from the reference drink as a covariate. The analysis model contained terms for the effect of calcium and AA as well as differences in these effects on iron absorption in both subject groups (IR and IDA). In addition, terms were added to the underlying model to analyze potential crossover effects. Each individual consumed 2 different drinks on 2 consecutive days (order) on 2 occasions (offer) in a randomized design (i.e., half of the subjects consumed drinks A and B first, with half of them starting with drink A during the first interval). Relative iron absorption from the reference drink and baseline PF concentration were used as covariables to adjust for interindividual differences in iron status and iron absorption efficiency. Because test drinks were given on 2 occasions separated by a 14-d interval, iron absorption from each of the 2 test drinks was adjusted separately in each child. The models for fractional iron absorption and iron absorption ratio examined main effects and interaction effects. The main effect of calcium compared the inhibitory effect of 100 mg added calcium with that of 200 mg added calcium regardless of group and added AA, with statistical significance being indicative of a dose-response effect of calcium. The main effect of AA compared iron absorption between molar ratios of 0, 2:1, and 4:1 of added AA to iron regardless of group and added calcium amount, with statistical significance being indicative of a dose-response effect of AA. The main effect of group compared the iron absorption between IR and IDA groups regardless of added amounts of calcium and AA. Interaction effects of calcium with AA and each of these with group were considered in the model. Post hoc test comparisons were performed by using Tukey’s test. All statistical analyses were conducted by using commercial statistical software (SAS version 9.2; SAS Institute) and considered significant at P < 0.05.

Results

Participant characteristics. The children selected for the study could be classified as either IR or IDA (Table 1). Mean hemoglobin and mean PF were higher and mean sTfR lower in the IR group than in the IDA group (P < 0.01). There were no significant differences between the IR and the IDA groups in plasma vitamin B-12, plasma retinol, and CRP concentrations. The erythrocyte folate concentration was in the normal range in all of the children but was higher in the IDA group (P < 0.01). Mean height and weight of children were ~10% higher in the IR group than in the IDA group (P < 0.05). Differences can be attributed to the higher mean age (by ~10 mo; P = 0.01) and the higher proportion of boys in the IR group. Groups and subgroups were not balanced for sex and age because there are currently no indications for age- or sex-related differences in iron metabolism in the studied age group.

Iron absorption from the reference drink. Fractional iron absorption from the reference test drink without added calcium and AA was assessed in 47 IR children and 45 children with IDA (see Tables 3 and 4). Absorption measurements were conducted in each child twice (i.e., within each of the 2 study intervals). In 2 children from the IR group and 1 child in the IDA group, only data for 1 absorption test were available. Fractional iron absorption (geometric mean; −SD, +SD) from the reference drink in the IR group was 11.3% (6.2, 20.8) (n = 92 observations) when assuming an erythrocyte incorporation efficiency of the iron tracer of 80%. This increased by 25% to 14.1% (8.8, 22.8) (n = 89 observations) in the IDA group (P = 0.01) when assuming a higher tracer incorporation efficiency of 90% in response to the lower iron status.

Effect of added calcium and AA on iron absorption from the reference drink. The inhibition of iron absorption from the test drink was dose dependent (P < 0.05). The addition of 100 mg calcium decreased iron absorption by 18% in the IR group and by 8% in the IDA group, whereas 200 mg calcium decreased iron absorption by 27% in the IR group and by 24% in the IDA group (subgroup 1; Table 3). A strong dose-response effect on iron absorption was also observed for AA in both groups (P < 0.001) regardless of the dose of calcium. AA enhanced iron absorption from the calcium-fortified drinks 2- to 3-fold relative to the reference drink in IR children (P < 0.01). There were no significant differences between the IR and IDA groups regardless of added amounts of calcium and AA, with statistical significance being indicative of a dose-response effect of calcium. The main effect of group compared the iron absorption between IR and IDA groups regardless of added amounts of calcium and AA. Interaction effects of calcium with AA and each of these with group were considered in the model. Post hoc test comparisons were performed by using Tukey’s test. All statistical analyses were conducted by using commercial statistical software (SAS version 9.2; SAS Institute) and considered significant at P < 0.05.
Discussion

Our studies indicate that calcium is a modest inhibitor of iron absorption from the studied iron-fortified instant drink and demonstrate that its inhibitory effect can be easily overcome by the addition of AA. Our study shows that adequate iron absorption from foods containing both calcium and iron can be ensured by adjusting the amount of AA and iron.

The modest inhibitory effect of calcium reported in our study is similar to, or perhaps somewhat less than, that in earlier observations (8–13). In the present study, 100–200 mg of added calcium decreased iron absorption from a casein/whey-based drink fortified with 6 mg iron by 10–25% (Table 4). Hallberg et al. (11) added 40, 165, 300, and 600 mg of calcium as calcium chloride with butter to low-extraction wheat rolls after baking. The rolls contained 3.5 mg of added iron. They reported iron absorption in adults to be decreased by 18%, 47%, 53%, and 58%, respectively. Differences in fortification amounts, nature of the test meals, and subject age may explain the more modest response of iron absorption to calcium in our studies. The studies by Hallberg et al. also suggest that the effect of calcium on iron absorption is dose dependent but saturable because little further decrease in iron absorption was observed when 300 or 600 mg calcium were added to the low-calcium meal compared with 165 mg calcium. Such a saturation effect may explain why no inhibition of calcium on iron absorption could be observed in some earlier studies (28,29).

The finding that relatively small amounts of AA are needed to counteract the inhibitory effect of calcium on iron absorption is consistent with earlier radio iron absorption studies in infants fed ferrous sulfate–fortified low-fat milk (30). The milk meals in these studies contained ~2 mg iron and 180 mg calcium, and iron absorption was enhanced 2- and 3-fold, respectively, by adding AA at a 2:1 or 4:1 molar ratio with respect to iron. Additional AA did not further enhance absorption, and molar ratios of 0.5 and 1 did not enhance absorption significantly. The ease with which AA overcomes the inhibitory effect of calcium on iron absorption may also explain why some previous isotopic studies reported no influence of calcium on iron absorption. Abrams et al. (31) gave 6- to 9-y-old children a breakfast cereal or a cereal bar containing either 39 or 156 mg calcium with

| Subgroup | Test drink | Fractional iron absorption
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Test drink</td>
<td>Added calcium</td>
<td>Added AA (molar ratio of AA:iron)</td>
</tr>
<tr>
<td>1 A</td>
<td>Reference drink</td>
<td>—</td>
</tr>
<tr>
<td>B</td>
<td>Reference drink +100 mg Ca</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>Reference drink +200 mg Ca</td>
<td>0</td>
</tr>
<tr>
<td>2 A</td>
<td>Reference drink</td>
<td>—</td>
</tr>
<tr>
<td>B</td>
<td>Reference drink +100 mg Ca</td>
<td>+AA (2:1)</td>
</tr>
<tr>
<td>C</td>
<td>Reference drink +100 mg Ca</td>
<td>+AA (4:1)</td>
</tr>
<tr>
<td>3 A</td>
<td>Reference drink</td>
<td>—</td>
</tr>
<tr>
<td>B</td>
<td>Reference drink +200 mg Ca</td>
<td>+AA (2:1)</td>
</tr>
<tr>
<td>C</td>
<td>Reference drink +200 mg Ca</td>
<td>+AA (4:1)</td>
</tr>
</tbody>
</table>

1 Values are geometric means (−SD, +SD); IR group, n = 47; IDA group, n = 45. AA, ascorbic acid; IDA, iron deficiency anemia; IR, iron replete.

2 The reference drink (A) was identical in all tests and did not contain added calcium or AA. Test drinks B and C were obtained by adding calcium (100 or 200 mg) or AA to the reference drink.
significant at efficiency. Main effects of added calcium and added AA were significant at reference drink as a covariant to control for interindividual variations in iron absorption values as summarized in Table 3 by using iron absorption from the across groups.

Means without a common superscript letter differ, and 

Iron absorption from the casein/whey-based drink by the 

TABLE 5 Iron absorption from the test drink with added calcium/AA without stratification of children by iron status

<table>
<thead>
<tr>
<th>Test drink</th>
<th>Added calcium2</th>
<th>Added AA (molar ratio)3</th>
<th>Fractional iron absorption4</th>
<th>Iron absorption ratio5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference drink +100 mg Ca</td>
<td>—</td>
<td>—</td>
<td>22.6 (10.0, 51.4)</td>
<td>1.88 (0.89, 3.92)</td>
</tr>
<tr>
<td>Reference drink +200 mg Ca</td>
<td>—</td>
<td>—</td>
<td>21.4 (9.0, 50.8)</td>
<td>1.62 (0.75, 3.50)</td>
</tr>
<tr>
<td>Reference drink —</td>
<td>0</td>
<td>—</td>
<td>9.0 (5.4, 15.0)</td>
<td>0.76 (0.54, 1.07)</td>
</tr>
<tr>
<td>Reference drink + AA (4:1)</td>
<td>—</td>
<td>+ AA</td>
<td>32.3 (18.4, 56.5)</td>
<td>2.30 (1.42, 3.72)</td>
</tr>
<tr>
<td>Reference drink + AA (2:1)</td>
<td>—</td>
<td>+ AA</td>
<td>38.1 (22.1, 65.5)</td>
<td>3.13 (1.89, 5.19)</td>
</tr>
</tbody>
</table>

1 Values are geometric means (95% CI); IR and IDA groups, n = 92. Means without a common superscript letter differ, P < 0.05. AA, ascorbic acid; IDA, iron deficiency anemia; IR, iron replete.
2 Overall iron absorption ratio for drinks with added calcium irrespective of added AA amount and across groups.
3 Overall iron absorption ratio for drinks containing either 100 or 200 mg added calcium across groups.
4 Data were analyzed by mixed linear regression of log-transformed fractional iron absorption values as summarized in Table 3 by using iron absorption from the reference drink as a covariant to control for interindividual variations in iron absorption efficiency. Main effects of added calcium and added AA were significant at P = 0.027 and <0.001, respectively. The main effect of group and interaction effects was not significant. Means without a common superscript letter differ, P < 0.05.
5 Data were analyzed by mixed linear regression of log-transformed iron absorption ratios as summarized in Table 4. The main effect of added calcium and added AA was significant at P = 0.013 and <0.001, respectively. The main effect of group and interaction effects was not significant.

breakfast or dinner meals in a total diet that contained ~100 mg AA/d and reported no influence of calcium on iron absorption. Similarly, Mendoza et al. (32) reported no inhibitory effect of calcium on iron absorption in women who consumed an iron-fortified cereal-legume food supplement containing 200–400 mg calcium, 10 mg iron, and 50 mg AA. Muscle tissue, the other major enhancer of iron absorption, also appears to be capable of counteracting the inhibitory nature of calcium (33). Such an effect could explain why 400 mg calcium served with each main meal of a mixed 4-d diet including muscle tissue did not affect cumulative iron absorption (34).

Our data suggest that calcium is a less potent inhibitor of dietary iron absorption than polyphenols or phytic acid, and that the calcium inhibition is easily counteracted by the addition of AA (35–37). Both polyphenols and phytic acid form insoluble complexes with iron in the gut, making it unavailable for absorption (38). The inhibitory effect of calcium on iron absorption, on the contrary, has been postulated as occurring during transport of iron through the enterocyte or at the point of transfer from the enterocyte to the serum (12). This is because calcium, unlike other inhibitors and enhancers of iron absorption, decreases both heme and nonheme iron absorption (11). It was recently suggested, however, that calcium may influence iron absorption by >1 mechanism and that although cellular effects may be important, interactions with other meal components that affect iron absorption could also play a role, as could a direct effect of calcium on the iron transporter (14,39).

Iron absorption from the casein/whey-based drink by the children in the present study was relatively high. The amount of iron absorbed from the calcium-containing drinks fortified with 6 mg iron already provided much of the child’s daily iron requirement (~0.8 mg/d), even without the addition of AA, and was 2–4 times higher than requirements for the AA-fortified drinks. When being persistently exposed to high amounts of absorbable iron, the body would be expected to downregulate iron absorption to prevent iron overcharge (40). For this reason, single-meal studies tend to overestimate the influence of enhancers and inhibitors on iron absorption (41). Such a homeostatic response was observed by Thankachan et al. (16) by using a similar study design. At a 2:1 or 4:1 molar ratio relative to iron, AA increased iron absorption by a factor of 2–3 from a rice and tomato meal similar to our study. Iron absorption without added AA, however, was 2.5-fold higher in young women with IDA than in IR women. Differences in meal composition or participant characteristics are possible reasons why iron absorption in our study was much less influenced by iron status. Iron absorption from the reference drink without added calcium and AA was only 20% lower in the IR group than in the IDA group when assuming an incorporation efficiency of the iron tracer of 80% in IR children and 90% in children with IDA, and 29% when assuming no differences in iron incorporation efficiency. In contrast, AA increased iron absorption in both groups by a factor of 2–4 (Table 4). Such high amounts of absorbed iron may potentially induce an “order effect” for test meal administration. Iron homeostasis is maintained both by control of iron uptake by the enterocyte from the diet and by regulation of iron release from the enterocyte into the circulation. If high amounts of iron have been deposited in the mucosa from the first drink to limit systemic iron influx, less iron may enter the circulation from the drink/meals served on the following day. We indeed observed such an “order effect” for test meal administration in the IR group (P < 0.001) but not in the IDA children (P > 0.10), probably because of their higher iron demands at low iron status. However, this effect was small and did not affect our main observations, which suggests that the casein/whey-based drink should be reformulated with lower amounts of AA and/or iron to avoid providing iron in excess of needs and to avoid the possibility of potential negative health effects of excess iron (42).

In conclusion, calcium is a modest inhibitor of iron absorption in children and its inhibitory effect was readily overcome by the addition of AA at 2:1 and 4:1 molar ratios to a milk product. Such amounts of AA addition are recommended for phytic acid-containing products. A similar compensatory effect can be expected in other iron-fortified products (free of phytic acid and polyphenols) that are fortified with calcium and AA. In such iron-fortified foods containing added calcium, the iron and AA amounts should be designed to provide the children with all, or a part of, the iron lacking in the daily diet. When adding AA to iron-fortified milk products, care should be taken not to provide absorbable iron vastly in excess of needs.

Acknowledgments
T.W. and S.M. designed the research with contributions from all authors; R.W., P.T., A.S., A.K., L.G.J.F., and T.W. conducted the research in India; R.W., P.T., and S.M. analyzed samples for iron and vitamin status in Bangalore, India; T.W. and R.W. were responsible for iron isotopic analysis; T.W. analyzed the data and wrote the manuscript with contributions by R.F.H.; and T.T. contributed to the statistical analysis. All authors read and approved the final manuscript.

References


