

# Influence of ascorbic acid on iron absorption from an iron-fortified, chocolate-flavored milk drink in Jamaican children<sup>1-3</sup>

Lena Davidsson, Thomas Walczyk, Audrey Morris, and Richard F Hurrell

**ABSTRACT** The influence of ascorbic acid on iron absorption from an iron-fortified, chocolate-flavored milk drink (6.3 mg total Fe per serving) was evaluated with a stable-isotope technique in 20 6–7-y-old Jamaican children. Each child received two test meals labeled with 5.6 mg <sup>57</sup>Fe and 3.0 mg <sup>58</sup>Fe as ferrous sulfate on 2 consecutive days. Three different doses of ascorbic acid (0, 25, and 50 mg per 25-g serving) were evaluated in two separate studies by using a crossover design. Iron isotope ratios were measured by negative thermal ionization mass spectrometry. In the first study, iron absorption was significantly greater ( $P < 0.0001$ ) after the addition of 25 mg ascorbic acid: geometric mean iron absorption was 1.6% (range: 0.9–4.2%) and 5.1% (2.2–17.3%) for the test meals containing 0 and 25 mg ascorbic acid, respectively. In the second study, a significant difference ( $P < 0.05$ ) in iron absorption was observed when the ascorbic acid content was increased from 25 to 50 mg: geometric mean iron absorption was 5.4% (range: 2.7–10.8%) compared with 7.7% (range: 4.7–16.5%), respectively. The chocolate drink contained relatively high amounts of polyphenolic compounds, phytic acid, and calcium, all well-known inhibitors of iron absorption. The low iron absorption without added ascorbic acid shows that chocolate milk is a poor vehicle for iron fortification unless sufficient amounts of an iron-absorption enhancer are added. Regular consumption of iron-fortified chocolate milk drinks containing added ascorbic acid could have a positive effect on iron nutrition in population groups vulnerable to iron deficiency. *Am J Clin Nutr* 1998;67:873–7.

**KEY WORDS** Iron, ascorbic acid, bioavailability, children, iron fortification, stable isotopes, anemia, Jamaica, chocolate milk drinks

## INTRODUCTION

Anemia has been reported to be a major public health problem in all countries of the English-speaking Caribbean, including Jamaica (1–3). As in many other developing countries, the most vulnerable population groups are pregnant and lactating women and preschool and school-age children. School-age children are highly vulnerable to iron deficiency because their iron requirements for growth often exceed the dietary iron supply (4). Several strategies have been proposed to overcome this problem, including the use of iron supplements. This approach can be effective but its usefulness is often limited by low compliance. Food fortification with iron is generally considered the most effective way to

increase iron intake and can be achieved by fortifying a dietary staple such as cereal flour or by fortifying widely consumed foodstuffs such as sugar and salt. However, this strategy provides iron to all segments of the population, including adult men and postmenopausal women, who do not require additional iron. The preferred approach to increase the iron intake of children would be targeted fortification of a specialty food item that is used selectively in this age group (5). One possibility would be to fortify a chocolate-flavored milk drink with iron.

Chocolate drinks, however, contain relatively high amounts of inhibitors of iron absorption, such as polymerized polyphenols of the catechin type. These compounds are similar to the polyphenols present in red wine, which have been shown to inhibit iron absorption in human subjects (6). In addition, milk contains the iron absorption inhibitors calcium (7) and bovine casein (8). Some commercial chocolate drink powders also include malt, which contains phytic acid, another potent inhibitor of iron absorption (9, 10). Therefore, chocolate-flavored milk drinks would not be expected to be a good vehicle for iron fortification unless the iron used for fortification was in some way protected from the absorption inhibitors. The classic way to counteract inhibitors of iron absorption in industrially produced foods is to add ascorbic acid (11, 12). The aim of this study was to measure iron absorption from an iron-fortified, malted, chocolate-flavored milk drink in 6–7-y-old Jamaican children by using a stable-isotope technique and to evaluate the addition of ascorbic acid as an absorption enhancer.

## SUBJECTS AND METHODS

### Subjects

Twenty apparently healthy schoolchildren aged 6–7 y (10 boys and 10 girls) were recruited for the studies from a primary

<sup>1</sup>From the Laboratory for Human Nutrition, Institute of Food Science, Swiss Federal Institute of Technology, Rueschlikon, Switzerland, and the Caribbean Food and Nutrition Institute, Kingston, Jamaica.

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<sup>3</sup>Reprints not available. Address correspondence to L Davidsson, Laboratory for Human Nutrition, Institute of Food Science, Swiss Federal Institute of Technology, PO Box 474, CH-8803 Rueschlikon, Switzerland. E-mail: davidsson@ilw.agrl.ethz.ch.

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school in the greater Kingston, Jamaica, area. The protocol was approved by the Ethical Committees at the Ministry of Health, Kingston, Jamaica, and the Nestlé Research Center, Lausanne, Switzerland. Parents were fully informed about the aims and procedures of the study and informed consent was obtained from one of each child's parent or guardian.

### Test meals

Chocolate-flavored milk drink powder without any added iron or ascorbic acid was prepared at the Nestlé Research Center Orbe (Orbe, Switzerland). The formulation was equivalent to a commercial product (MILO; Nestlé Jamaica, Kingston, Jamaica), except for the omission of added iron. The product contained cocoa, malt, skim milk powder, sugar, butter, oil, mineral salts, and vitamins and had been vacuum-dried before being milled to produce a powder. All labeled test meals were prepared immediately before administration and consisted of 25 g chocolate drink powder, 220 g deionized water, 30 g full-fat sweetened condensed milk (Nestlé Jamaica), stable isotope solutions of  $^{58}\text{Fe}$  or  $^{57}\text{Fe}$ , and food-grade ascorbic acid (Merck, Darmstadt, Germany).

### Stable-isotope labels

Elemental iron isotopically enriched with  $^{57}\text{Fe}$  (95.28%  $^{57}\text{Fe}$ ) and  $^{58}\text{Fe}$  (91.66%  $^{58}\text{Fe}$ ) was purchased from Isotec (St Quentin, France) and dissolved in 0.1 mol  $\text{H}_2\text{SO}_4/\text{L}$  to obtain isotopically labeled  $\text{FeSO}_4$  solutions. The isotopic composition of the iron in solution was determined by negative thermal ionization mass spectrometry (NTIMS) using  $\text{FeF}_4^-$  molecular ions (13) and a magnetic sector field instrument (MAT 262; Finnigan MAT, Bremen, Germany). Iron concentrations of the labeled solutions were determined against a diluted, commercially available iron standard (Titrisol; Merck) by isotope dilution-mass spectrometry.

The isotope doses to be administered were calculated by using the estimated total amount of circulating iron in the children and the expected range of fractional iron absorption from the test meals. All calculations were based on the significantly lower precision of isotope ratio determinations attainable by the commonly used positive thermal ionization technique using  $\text{Fe}^+$  atomic ions (14, 15). The individual  $^{57}\text{Fe}$  isotope doses (5.6 mg  $^{57}\text{Fe}$ ) and  $^{58}\text{Fe}$  isotope doses (3.0 mg  $^{58}\text{Fe}$ ) were prepared by weighing the corresponding amount of isotopically labeled solution into polytetrafluoroethylene containers. The total iron content of the  $^{58}\text{Fe}$  isotope doses was equilibrated to that of the  $^{57}\text{Fe}$  isotope doses by adding iron of natural isotopic composition as food-grade  $\text{FeSO}_4$  (Merck). The filled containers were flushed with argon to keep the iron in the 2+ oxidation state.

### Study 1

The stable isotopes were administered with the test meals as follows. For meal A,  $^{58}\text{Fe}$  was added as  $^{58}\text{FeSO}_4$  plus ferrous sulfate of normal isotopic composition (total 5.9 mg Fe) with 25 mg ascorbic acid; for meal B,  $^{57}\text{Fe}$  was added as  $^{57}\text{FeSO}_4$  (total 5.9 mg Fe) with no ascorbic acid.

### Study 2

The stable isotopes were administered with the test meals as follows. For meal A,  $^{58}\text{Fe}$  was added as  $^{58}\text{FeSO}_4$  plus ferrous sulfate of normal isotopic composition (total 5.9 mg Fe) with 25 mg ascorbic acid; for meal B,  $^{57}\text{Fe}$  was added as  $^{57}\text{FeSO}_4$  (total 5.9 mg Fe) with 50 mg ascorbic acid.

### Study protocol

Labeled test meals were administered in the morning after an overnight fast. Ten children participated in each of the two studies. The two different test meals within each study were administered in the order AB ( $n = 5$ ) or BA ( $n = 5$ ). Administration of labeled test meals was done under close supervision by the investigators.

A baseline venous blood sample was drawn for determination of iron-status indexes and iron isotopic composition on the first day of the study. Height and weight were measured followed by the serving of the first test meal (A or B). The children were supervised closely during the 3 h after intake of the labeled test meals to ensure that no food or fluids were consumed. On day 2 of the study, the second labeled test meal was administered under identical conditions. The second blood sample was drawn 14 d after intake of the labeled test meals.

### Blood analyses

EDTA-treated whole blood was used to determine hemoglobin (HemoCue, Helsingborg, Sweden) and to analyze the isotopic composition. Plasma was separated from a sample of whole blood and analyzed for ferritin (enzyme-linked immunosorbent assay kit; Ramco, Houston) and C-reactive protein with an agglutination technique (Rapitex CRP; Behringwerke AG, Marburg, Germany). Quality control materials (Dade Immunoassay Controls Comprehensive Tri-levels; Baxter Diagnostics, Deerfield, IL) were analyzed together with the plasma ferritin samples.

### Analysis of isotopic composition of blood samples

Sample handling was done under clean laboratory conditions to reduce the risk of sample contamination during analysis. All containers were acid-washed and commercial chemicals and reagents were purified before being used.

Each isotopically enriched blood sample was analyzed in duplicate for its iron isotopic composition under chemical blank monitoring. Whole-blood samples were mineralized with a mixture of nitric acid and hydrogen peroxide and microwave digestion (MLS 1200; MLS, Leutkirch, Switzerland). Sample iron was separated from the matrix by anion-exchange chromatography after a solvent-solvent extraction step into diethyl ether (15, 16). All isotopic analyses were performed with NTIMS using a magnetic sector field mass spectrometer (MAT 262) equipped with a multicollector system for simultaneous ion-beam detection. The recently developed NTI technique for iron (13) was used to measure the ratio of iron isotopes. The iron separated from the sample was loaded on barium fluoride-coated rhenium filaments of a double-filament ion source together with silver fluoride to promote the formation of negatively charged  $\text{FeF}_4^-$  ions.

### Calculation of iron absorption

The amounts of  $^{57}\text{Fe}$  label and  $^{58}\text{Fe}$  label in the blood of the subjects 14 d after administration were calculated based on the shift of the iron isotope ratios in the blood samples and the amount of iron circulating in the body. Calculations followed the principles of isotope dilution and considered that the iron labels used were not monoisotopic (17). Circulating iron was calculated based on blood volume and hemoglobin concentration. Blood volume calculations were based on height and weight following empirically derived formulas (18). For calculation of fractional absorption, 90% incorporation of the absorbed iron into red blood cells was assumed. Because of the high enrich-

ment of the isotopically enriched labels and the low amounts of label incorporated into the red blood cells, the ratio of  $^{54}\text{Fe}$  to  $^{56}\text{Fe}$  ( $^{54}\text{Fe}:$  $^{56}\text{Fe}$ ) in the blood remained unchanged within the reproducibility of the isotopic analysis after incorporation of the labels. Therefore, it was possible to normalize the data for the natural isotope ratio,  $^{54}\text{Fe}:$  $^{56}\text{Fe} = 0.06370$  (19), to correct for mass-dependent isotopic fractionation effects. The normalized iron isotope ratios of blood samples taken before test meal administration were identical to the natural iron isotope ratio.

### Food analysis

The chocolate-flavored milk drink powder used in the study was analyzed for iron and calcium contents by electrothermal flame-atomic absorption spectroscopy (SpectrAA 400; Varian, Mulgrave, Australia) after mineralization by microwave digestion (MLS 1200) with a mixture of nitric acid and hydrogen peroxide. Analyses were carried out according to standard addition principles to minimize matrix effects. The phytic acid content of the milk drink powder was determined colorimetrically (MRX Microplate Reader; Dynatech, Embrach, Switzerland) as inorganic phosphorus according to the method of Makower (20), which was modified in that cerium sulfate was used instead of ferric chloride for a more selective phytic acid precipitation. The total polyphenol content of the test meals was ascertained by UV-VIS (ultraviolet visible) spectrophotometry (Uvikon 940; Kontron, Zürich, Switzerland) with Folin-Ciocalteu reagent as described by Celeste et al (21). Ascorbic acid was extracted with an aqueous solution of *meta*-phosphoric acid and quantified by HPLC (Merck-Hitachi System; Merck) with a reversed-phase column and photometric detection according to methods described previously (22, 23). Before being analyzed, dehydroascorbic acid was reduced to ascorbic acid by adding dithiothreitol (Fluka, Buchs, Switzerland). Analytic results were verified by triplicate analysis of the milk drink powder.

### Statistics

A paired *t* test was used to evaluate data from studies 1 and 2. Values for iron absorption were logarithmically transformed before statistical analysis. Results are presented as geometric means  $+1$  SD and  $-1$  SD.

## RESULTS

### Food analysis

The non-iron-fortified chocolate drink powder contained 0.44 mg Fe, 156 mg Ca, 158 mg polyphenols (gallic acid equivalents), and 58 mg phytic acid per 25-g serving. No ascorbic acid could be detected in the sample. The condensed milk contained 84 mg Ca per serving (30 g wet wt).

### Subject characteristics

Individual hemoglobin concentrations ranged from 94 to 127 g/L. All children, with one exception, were anemic (hemoglobin  $< 120$  g/L). However, the plasma ferritin analysis indicated that only three children were iron deficient (ferritin  $< 12$   $\mu\text{g/L}$ ). All plasma samples tested negative for C-reactive protein.

### Iron absorption

In study 1, geometric mean iron absorption was significantly greater when 25 mg ascorbic acid was added to the test meal than

when 0 mg ascorbic acid was added to an identical test meal (Table 1). Geometric mean iron absorption was 5.1% (range: 2.2–17.3%) and 1.6% (range: 0.9–4.2%) for the two test meals, respectively. The geometric mean iron absorption ratio (25 mg ascorbic acid:0 mg ascorbic acid) was 3.2 (range: 1.4–6.0). In study 2, a significant difference in iron absorption was observed between the two test meals containing 25 and 50 mg ascorbic acid: geometric mean iron absorption was 5.4% (range: 2.7–10.8%) compared with 7.7% (range: 4.7–16.5%), respectively. The geometric mean iron absorption ratio (25 mg ascorbic acid:50 mg ascorbic acid) was 1.4 (range: 0.71–3.2).

## DISCUSSION

As expected, iron absorption by the children from the iron-fortified, chocolate-flavored milk was low when no ascorbic acid was added, presumably because of the relatively high content of phenolic compounds (150 mg per serving) and phytic acid (58 mg per serving) in the test meals. A similar content of polyphenols (230 mg) in a glass of red wine reduced iron absorption from an iron-fortified bread roll in adults by  $\approx 70\%$  (6) whereas addition of 34 mg phytate to a bread roll decreased iron absorption in adults by  $\approx 60\%$  (9). The relatively high content of calcium in the test meal (240 mg) would also be expected to inhibit iron absorption from the chocolate drinks. A dose-effect relation between calcium content and nonheme-iron absorption was shown from small test meals consisting of wheat bread, with the most marked inhibition occurring when the meals contained between 40 and 165 mg Ca (7).

The enhancing effect of added ascorbic acid on iron absorption from the chocolate-flavored milk drink was shown clearly in the present study. Iron absorption from the chocolate drink containing the standard commercial amount of ascorbic acid (25 mg/25 g dry powder) was significantly greater than that from the chocolate drink with no added ascorbic acid. In study 2, a dose-response relation between ascorbic acid and iron absorption was shown. The geometric mean iron absorption increased from 5.4% to 7.7% when the ascorbic acid content was doubled, from 25 to 50 mg ( $P < 0.05$ ). The positive effect of ascorbic acid on iron absorption from a variety of different test meals was shown many times previously in both adults (9, 24–26) and infants (11, 27, 28).

The enhancing effect of ascorbic acid on iron absorption is believed to be due to its ability to reduce ferric iron to ferrous iron, which binds less strongly with polyphenols and phytic acid to form insoluble complexes (29). Siegenberg et al (26) showed that the addition of 30 or 50 mg ascorbic acid doubled or tripled iron absorption from a test meal containing 58 mg phytate-P ( $\approx 200$  mg phytic acid) whereas increasing the dose of ascorbic acid to 150 mg caused only a modest further increase. In the study by Hallberg et al (9), it was estimated that  $\approx 80$  mg ascorbic acid would be needed to fully counteract the inhibition of 25 mg phytate-P ( $\approx 85$  mg phytic acid). The positive effect of the addition of ascorbic acid in overcoming the inhibiting effect of polyphenols was also shown by Siegenberg et al (26) and Tuntawiroon et al (30). In these studies,  $\approx 50$  mg ascorbic acid was necessary to counteract the negative effect of  $> 100$  mg added tannic acid (26) whereas 100 mg ascorbic acid was needed to overcome the inhibiting effect of 146 mg tannic acid equivalents in a Thai vegetable meal (30).

The results from the present study showed that the addition of either 25 or 50 mg ascorbic acid per serving of chocolate-flavored

**TABLE 1**

Geometric mean values for iron-status indexes and iron absorption from an iron-fortified, chocolate-flavored milk drink containing 0, 25, or 50 mg ascorbic acid (AA) per serving

	Hemoglobin	Plasma ferritin	Iron absorption		
			0 mg AA	25 mg AA	50 mg AA
	<i>g/L</i>	$\mu\text{g/L}$		%	
Study 1					
$\bar{x}$	110	25	1.6 <sup>1</sup>	5.1	—
+ 1SD	117	47	2.7	9.3	—
-1 SD	103	13	0.9	2.7	—
Study 2					
$\bar{x}$	111	22	—	5.4	7.7 <sup>2</sup>
+ 1SD	117	43	—	8.6	11.7
-1 SD	105	12	—	3.4	5.1


<sup>1,2</sup>Significantly different from 25 mg AA: <sup>1</sup>*P* < 0.0001, <sup>2</sup>*P* < 0.05.

milk drink, containing 5.9 mg Fe as ferrous sulfate (6.3 mg total Fe), would provide a nutritionally significant amount of bioavailable iron. Even at the lower concentration of ascorbic acid (25 mg), geometric mean iron absorption was 5% and thus provided the children with 315  $\mu\text{g}$  absorbed Fe per serving, which is equivalent to  $\approx 31\%$  of the daily requirement of absorbed iron for young children (31). The energy provided per serving was  $\approx 1110$  kJ, equivalent to  $\approx 14\%$  of the recommended energy intake for this age group (31). Thus, relative to the energy content, the chocolate drink would provide about twice the required daily amount of absorbable iron and would therefore be expected to have a positive effect on iron nutrition in young children.

The proposal that ascorbic acid-fortified chocolate drinks can be a good vehicle for iron fortification depends to a large extent on the choice of the iron-fortification compound used. One problem with fortifying chocolate-flavored milk drinks is the unacceptable color changes that occur when highly soluble compounds such as ferrous sulfate are added. In this study, ferrous sulfate changed the color of the drink from red-brown to gray. Although this color change was still acceptable under experimental conditions, it would clearly be unacceptable in a commercial product. Iron absorption from commercial chocolate-flavored drink powders fortified with insoluble iron compounds such as ferric pyrophosphate was shown in adults to be 20–75% of that from drink powders fortified with ferrous sulfate (32). Although the enhancing effect of ascorbic acid on iron absorption from two commonly used insoluble iron compounds, electrolytic iron and ferric orthophosphate, has been shown in humans (33), foods and beverages fortified with these compounds would still be less useful in providing iron to the growing child. Alternative iron compounds such as ferrous fumarate (32) and ferrous succinate (34), which are absorbed similarly to or better than ferrous sulfate and cause less organoleptic problems, have been suggested for use in fortifying food products targeted for infants and children. Although it would have been ideal to have used ferrous fumarate or ferrous succinate in the present study, the cost of labeling these compounds to achieve characteristics similar to those of commercially available iron compounds was prohibitive.

There are no reports on the influence of ascorbic acid on iron absorption from ferrous succinate. However, in an earlier study with the same chocolate-flavored drink powder as used in the present study, we reported that iron absorption from ferrous fumarate added before processing was not enhanced by the addition of ascorbic acid (32). The data from this previous study are difficult to

interpret because heat processing per se increased iron absorption from ferrous fumarate to about twice that from ferrous sulfate (32) and concern was raised over the possibility that the transformed ferrous fumarate did not enter the common iron pool under these experimental conditions. Thus, the enhancing effect of ascorbic acid on iron absorption from the chocolate-flavored drink fortified with ferrous sulfate in the present study cannot be extrapolated to alternative iron compounds without being investigated further.

In conclusion, iron absorption by 6–7-y-old children from a malted, chocolate-flavored milk drink fortified with ferrous sulfate was low because of the presence of iron absorption inhibitors. The negative effect of phytic acid and polyphenols in the test meal was overcome by adding ascorbic acid, which resulted in a threefold increase in fractional iron absorption. Chocolate drinks are difficult to fortify with iron but, if iron compounds that readily enter the common iron pool can be added together with sufficient amounts of ascorbic acid, then chocolate drinks would be useful vehicles for providing nutritionally significant amounts of absorbable iron. 

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