

Iron absorption from ferrous fumarate in adult women is influenced by ascorbic acid but not by Na₂EDTA

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Ascorbic acid and Na₂EDTA enhance Fe absorption from the water-soluble Fe compound FeSO₄ but their effect on poorly water-soluble Fe compounds such as ferrous fumarate is less well established. In the present study, the effects of ascorbic acid and Na₂EDTA on Fe absorption from ferrous fumarate were evaluated in adult women (ten women/study) from the erythrocyte incorporation of Fe stable isotopes (⁵⁷Fe or ⁵⁸Fe) 14 d after administration. Two separate studies were made with test meals of Fe-fortified infant cereal (5 mg Fe/meal). Data were evaluated by paired *t* tests and the results are presented as geometric means. In study 1a, the comparison between Fe absorption from ferrous fumarate- and FeSO₄-fortified cereal showed that adult women absorb Fe as well from ferrous fumarate as from FeSO₄ (3.0 and 3.1 % respectively, *P*=0.85). After addition of Na₂EDTA (Na₂EDTA:fortification Fe molar ratio of 1:1), Fe absorption from FeSO₄ was significantly higher than from ferrous fumarate (5.3 v. 3.3 % respectively, *P*<0.01; study 1b). In study 2, Fe absorption was compared from ferrous fumarate-fortified meals with and without ascorbic acid added at a 4:1 molar ratio (relative to fortification Fe) and the results showed that ascorbic acid increased Fe absorption from ferrous fumarate significantly (6.3 v. 10.4 %, *P*=0.02). The results of the present studies show that Fe absorption from ferrous fumarate is enhanced by ascorbic acid but not by Na₂EDTA, thus emphasising that not all findings from Fe absorption studies made with FeSO₄ can be extrapolated to Fe compounds with different solubility properties.

Iron fortification: Ferrous fumarate: Ascorbic acid: Na₂EDTA

Ferrous fumarate is a poorly water-soluble Fe compound, which is soluble in dilute acid (such as gastric acid) and is as well absorbed as ferrous sulfate in healthy Western adults (Hurrell *et al.* 1989, 1991, 2000). In infants, Fe absorption from this compound is significantly higher than from ferric pyrophosphate (Davidsson *et al.* 2000). Besides being readily bioavailable, ferrous fumarate is a useful fortification compound since it provokes fewer organoleptic changes in fortified foods during storage than freely water-soluble Fe compounds such as ferrous sulfate (Hurrell, 1999). Presently, ferrous fumarate is used to fortify industrially manufactured infant cereals in Europe, maize flour in Venezuela and has been suggested as the fortificant for chocolate-drink powders (Hurrell, 1999; Garcia-Casal & Layrisse, 2002).

Bioavailability of fortification Fe depends on several factors, including the solubility of the Fe compound in the gastric content during digestion and the composition of the meal, i.e. on the presence of enhancers and inhibitors of Fe absorption. Ascorbic acid is a potent enhancer of Fe absorption and can overcome the inhibitory effect of phytic acid in cereal-based meals on native Fe as well as on freely water-soluble Fe compounds such as ferrous sulfate

(Sayers *et al.* 1973, 1974; Björn-Rasmussen & Hallberg, 1974; Gillooly *et al.* 1984; Cook *et al.* 1997). Ascorbic acid has also been reported to enhance Fe absorption from the water-insoluble Fe fortificants ferric orthophosphate and electrolytic Fe (Forbes *et al.* 1989). Its influence on the absorption of ferrous fumarate is, however, less well established. Hurrell *et al.* (1991) reported no enhancing effect of ascorbic acid on Fe absorption from a ferrous fumarate-fortified chocolate-drink powder and only a modest enhancing effect when added to a ferrous fumarate-fortified liquid formula meal.

In a similar way, Na₂EDTA has also been shown to enhance Fe absorption from ferrous sulfate in inhibitory meals containing phytic acid (MacPhail *et al.* 1994; Hurrell *et al.* 2000; Davidsson *et al.* 2001). Its advantage over ascorbic acid is that it is stable during heat processing and storage; however, there are several recent reports that Na₂EDTA does not enhance Fe absorption from poorly water-soluble and water-insoluble Fe compounds. For example, when added to a ferric pyrophosphate-fortified infant cereal, Na₂EDTA did not increase Fe absorption (Hurrell *et al.* 2000). Further, Fe absorption was not improved by the addition of Na₂EDTA to cornflakes

Abbreviation: Hb, haemoglobin.

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fortified with hydrogen-reduced Fe (Fairweather-Tait *et al.* 2001), or when added to a typical Guatemalan meal fortified with ferrous fumarate (Davidsson *et al.* 2002).

The aim of the present studies was to compare the influence of Na₂EDTA on Fe absorption from ferrous fumarate and ferrous sulfate and to further evaluate the influence of ascorbic acid on Fe absorption from ferrous fumarate. Fe absorption was measured in healthy women from a wheat-based infant cereal by using a stable-isotope technique based on the incorporation of Fe stable isotopes into erythrocytes 14 d after administration.

Subjects and methods

Subjects

Twenty apparently healthy adult women (20–30 years; maximum body weight 60 kg) were recruited from the student and staff population at the Swiss Federal Institute of Technology Zurich and the University of Zurich. The women were randomly allocated to two separate studies (ten women/study). Exclusion criteria included pregnancy or lactation and known gastrointestinal or metabolic disorders. No medication (except oral contraceptives) or vitamin and mineral supplements were allowed during the study. All women consuming vitamin and mineral supplements regularly (*n* 6) discontinued supplementation 2 weeks before the start of the study.

The study protocol was reviewed and approved by the ethical committee at the Swiss Federal Institute of Technology Zurich, Switzerland. Subjects were informed orally and in writing about the aims and procedures of the study. Written informed consent was obtained from all study subjects.

Study design

Two studies were performed (Table 1). In study 1, each subject received four test meals and Fe absorption from ferrous fumarate- and ferrous sulfate-fortified meals without (study 1a) and with (study 1b) added Na₂EDTA was compared. In study 2, each subject received two test meals only and Fe absorption from ferrous fumarate-fortified meals with and without ascorbic acid was compared.

Fe absorption was based on the erythrocyte incorporation of Fe stable isotope labels 14 d after the intake of labelled test meals. The Fe compounds were labelled with ⁵⁷Fe or ⁵⁸Fe and added to the different test meals as described later (pp. 1082–1083). All test meals were fed between 07.00 and 09.00 hours, after an overnight fast, under standardised conditions. A crossover study design was used with each woman acting as her own control. On the day before the intake of the first test meal (day 0), a venous blood sample was drawn after an overnight fast for the determination of Fe-status parameters (haemoglobin (Hb), and plasma ferritin) and body weight and height were measured. In study 1a, labelled test meals (meals A and B) were fed on the following 2 d (days 1 and 2). A second venous blood sample was drawn 14 d after intake of the second test meal (day 16). The second pair of labelled test meals (meals C and D) was fed on days 17 and 18 (study 1b) and a final blood sample was obtained 14 d after the administration of the last test meal (day 32). In study 2, the first labelled test meal (meal E) was fed on day 1 and a second venous blood sample was drawn 14 d later (day 15). On day 16, the second labelled test meal (meal F) was fed and a final blood sample was obtained on day 30. No intake of food or fluids was allowed for 3 h after the test-meal intake.

Test meals

All test meals consisted of 50 g roller-dried wheat-based infant cereal (Nestlé PTC, Orbe, Switzerland) fed with reconstituted milk (8 g milk powder, Sano Lait; Coop Schweiz, Basel, Switzerland, and 75 ml deionised water). The infant cereal was made from 79.7% wheat flour, partially hydrolysed, 10% sucrose, 4% honey, 3% palm oil, 3% water and 0.3% calcium carbonate. Except for Ca, no minerals or vitamins were added. Each test meal contained 5 mg added Fe, 4 mg Fe as ⁵⁸FeSO₄ plus 1 mg Fe as FeSO₄ of natural isotopic composition or 5 mg Fe as [⁵⁷Fe] ferrous fumarate. Deionised water (200 g) was served as a drink. To test meals C and D in study 1, Na₂EDTA (Akzo Nobel, Herkenbosch, The Netherlands) was added at a Na₂EDTA:fortification Fe molar ratio of 1:1 (26.7 mg Na₂EDTA). To test meal F in study 2,

Table 1. Iron absorption by healthy adult women (ten women per study) from infant cereal fortified with ferrous sulfate or ferrous fumarate (5 mg iron) with and without Na₂EDTA (studies 1a and 1b) and from ferrous fumarate with and without ascorbic acid (study 2)* (Geometric means and standard deviations)

Study no.	Test meal	Plasma ferritin (µg/l)			Fe absorption (%)		
		Geometric mean	-1 SD	+1 SD	Geometric mean	-1 SD	+1 SD
Study 1a	A: Ferrous sulfate	16.9	10.5	27.1	3.1 ^a	1.3	7.4
	B: Ferrous fumarate				3.0 ^a	1.6	5.5
Study 1b†	C: Ferrous sulfate + Na ₂ EDTA	14.9	7.7	29.0	5.3 ^a	3.2	8.6
	D: Ferrous fumarate + Na ₂ EDTA				3.3 ^b	2.2	5.0
Study 2‡	E: Ferrous fumarate	14.9	7.7	29.0	6.3 ^a	3.1	13.0
	F: Ferrous fumarate + ascorbic acid				10.4 ^b	4.0	27.0

^{a,b}Mean values within studies 1a, 1b or study 2 with unlike superscript letters were significantly different (*P* < 0.05).

* For details of subjects and procedures, see pp. 1082–1083.

† Fe: Na₂EDTA molar ratio of 1:1.

‡ Ascorbic acid: Fe molar ratio of 4:1.

ascorbic acid (Merck, Darmstadt, Germany) was added at an ascorbic acid:fortification Fe molar ratio of 4:1 (63 mg ascorbic acid).

Stable isotope labels

[⁵⁸Fe]ferrous sulfate was prepared from isotopically enriched elemental Fe (Chemgas, Boulogne, France) by dissolution in sulfuric acid and dilution to the appropriate concentration. Two separate batches of [⁵⁸Fe]ferrous sulfate were prepared. [⁵⁷Fe]ferrous fumarate was prepared in collaboration with Dr Paul Lohmann Ltd, Emmerthal, Germany. It has been used in previous human studies (Davidsson *et al.* 2000, 2002).

Ascorbic acid and Na₂EDTA solutions

Aqueous solutions of food-grade ascorbic acid and food-grade Na₂EDTA were prepared freshly each morning and added to the test meals at the time of serving.

Quantification of iron isotopes in labelled iron fortificants

Isotope-dilution MS was used to determine the concentration of ⁵⁷Fe and ⁵⁸Fe stable isotopes in the ferrous fumarate and ferrous sulfate solutions. For analysis, [⁵⁷Fe]ferrous fumarate was dissolved in concentrated nitric acid. An accurately measured amount of Fe of natural isotopic composition was added to samples taken from the prepared solutions of labelled Fe fortificants. The Fe standard was prepared gravimetrically from an isotopic reference material (IRMM-014; EU Institute of Reference Materials, Geel, Belgium). Isotopic analyses were performed using negative thermal ionisation-MS (Walczyk, 1997). Fe concentrations of each labelled Fe fortificant were calculated based on the shift in Fe isotopic abundances, the determined isotopic abundances of the pure isotopic labels and the natural Fe isotopic abundances (Walczyk *et al.* 1997).

Iron status measurements

Venous blood samples (7 ml) were drawn in EDTA-treated tubes at each sampling. The samples were analysed for Fe-status indices (Hb, plasma ferritin) and for the incorporation of ⁵⁷Fe and ⁵⁸Fe into erythrocytes (days 15 to 16 and days 30 to 32). Whole-blood samples were sampled for the analysis of Hb and isotopic composition and plasma was separated, sampled and frozen for the later analysis of plasma ferritin. Hb was measured by the cyanmethaemoglobin method (Sigma kit; Sigma, St Louis, MO, USA) and plasma ferritin by ELISA (Ramco Laboratories, Houston, TX, USA). Commercial-quality control materials (DiaMed, Cressier sur Morat, Switzerland and Ramco Laboratories) were analysed together with the samples analysed for Hb and plasma ferritin, respectively.

Quantification of iron stable isotopes in blood

Each isotopically enriched blood sample was analysed in duplicate for its Fe isotopic composition as previously

described by Walczyk *et al.* (1997). The blood samples were mineralised by microwave digestion using a mixture of nitric acid and H₂O₂. Fe was separated from the matrix by anion-exchange chromatography and a solvent-solvent extraction step into diethyl ether. Isotopic analyses were performed by negative thermal ionisation-MS (Walczyk, *et al.* 1997).

Calculation of iron absorption

The amounts of ⁵⁷Fe and ⁵⁸Fe isotopic labels in blood 14 d after the test-meal administrations were calculated based on the shift in Fe isotope ratios and on the amount of Fe circulating in the body. The calculations were based on the principles of isotope dilution and took into account that the Fe isotopic labels were not monoisotopic (Walczyk *et al.* 1997). Circulating Fe was calculated based on blood volume and Hb concentration (Kastenmayer *et al.* 1994). Blood-volume calculations were based on height and weight according to Brown *et al.* (1962). For calculations of fractional Fe absorption, 80% incorporation of the absorbed Fe into erythrocytes was assumed (Hosain *et al.* 1967).

Food analysis

Cereal and milk powder were analysed for Fe and Ca by electrothermal-flame atomic absorption spectroscopy (SpectrAA 400; Varian, Mulgrave, Australia) after mineralisation by microwave digestion (MLS-Ethos plus; Mikrowellen-Labor-Systeme, Leutkirch, Switzerland) in a nitric acid-H₂O₂ mixture, using standard addition techniques to minimise matrix effects. Phytic acid in the infant cereal was determined by a modification of the Makower (1970) method in which Ce replaced Fe in the precipitation step.

Statistics

Fractional Fe absorption values are presented as geometric means and standard deviations (−1 SD, +1 SD) (geometric means are back-transformed log-scale means). Student's paired t test was used to compare the absorption data within each study (studies 1a, 1b and study 2 respectively). Absorption values were logarithmically transformed before statistical analysis (Excel 2002; Microsoft Corporation, Redmond, WA, USA). The correlation between Fe absorption and plasma ferritin concentration was evaluated by Spearman's rank correlation.

Results

None of the women were anaemic (Hb concentration < 120 g/l). However, six women had no Fe stores, indicated by low plasma ferritin values (< 12 µg/l).

The test meals contained 0.6 mg native Fe (1.1 mg Fe/100 g infant cereal, 0.15 mg Fe/100 g milk powder), 167 mg Ca (148 mg Ca/100 g infant cereal, 1159 mg Ca/100 g milk powder) and 84 mg phytic acid (168 mg phytic acid/100 g infant cereal). The ascorbic acid content was not measured as it was assumed to be negligible.

Isotopic enrichments were $95.99 \pm 0.03\%$ ^{57}Fe (^{57}Fe -labelled ferrous fumarate) and 92.99 ± 0.02 and $91.6 \pm 0.1\%$ ^{58}Fe (^{58}Fe -labelled ferrous sulfate) in studies 1a and 1b respectively.

The results of the Fe absorption studies are shown in Table 1. In study 1a, no significant difference between Fe absorption from the ferrous sulfate- and ferrous fumarate-fortified infant cereal was found (geometric mean 3.1 *v.* 3.0% respectively, $P=0.85$). When Na_2EDTA was added to the test meals, Fe absorption was statistically significantly higher from the ferrous sulfate- than from the ferrous fumarate-fortified test meals (geometric mean 5.3 *v.* 3.3% respectively, $P<0.01$; study 1b). In study 2, the addition of ascorbic acid (ascorbic acid:Fe molar ratio of 4:1) to the ferrous fumarate-fortified cereal increased Fe absorption significantly (geometric mean 6.3 *v.* 10.4%, $P=0.02$). A statistically significant correlation ($P<0.05$) between Fe absorption and plasma ferritin concentration was only found for test meal F.

Discussion

Studies investigating the influence of enhancers and inhibitors on Fe absorption from fortified foods have most often evaluated the commonly used water-soluble Fe compound ferrous sulfate. Only a few studies have been performed with poorly water-soluble Fe compounds, primarily due to the technical difficulties involved in producing experimentally labelled compounds. While the preparation of labelled ferrous sulfate is relatively easy, special care has to be taken when preparing poorly water-soluble Fe compounds, such as ferrous fumarate, so as to ensure that the labelled compound is similar to the commercial Fe fortificant. Besides the technical difficulties associated with using a down-scaled production method, the high cost involved in the preparation of such compounds is a limiting factor, especially when stable Fe isotopes are used. In the present study, the labelled ferrous fumarate was produced in collaboration with a manufacturer of commercial ferrous fumarate and had a similar solubility in dilute acid (Davidsson *et al.* 2000).

Ascorbic acid and Na_2EDTA have repeatedly been shown to enhance Fe absorption from ferrous sulfate (Sayers *et al.* 1973, 1974; Björn-Rasmussen & Hallberg, 1974; Gillooly *et al.* 1984; el Guindi *et al.* 1988; MacPhail *et al.* 1994; Cook *et al.* 1997; Hurrell *et al.* 2000; Davidsson *et al.* 2001). However, due to the results obtained by Hurrell *et al.* (1991) and Davidsson *et al.* (2002) it is unclear whether ascorbic acid and Na_2EDTA also enhance Fe absorption from ferrous fumarate and the present study was performed for clarification.

The influence of Na_2EDTA on Fe absorption from ferrous fumarate and ferrous sulfate was compared in study 1. The present results show that healthy Western women absorb Fe as well from ferrous fumarate as from ferrous sulfate. Further, the results showed that Fe absorption was significantly higher from ferrous sulfate than from ferrous fumarate in the presence of Na_2EDTA . This indicates that Na_2EDTA does not enhance Fe absorption from ferrous fumarate-fortified infant cereal and thus confirms previous evidence that Na_2EDTA has no effect

on Fe absorption from poorly water-soluble and water-insoluble Fe compounds (Hurrell *et al.* 2000; Fairweather-Tait *et al.* 2001; Davidsson *et al.* 2002).

The lack of effect on Fe absorption after the addition of Na_2EDTA to ferrous fumarate-fortified foods is probably related to the solubility of this Fe compound. Fe from ferrous sulfate can be expected to enter the common non-haeme Fe pool rapidly, while ferrous fumarate has to dissolve in the gastric juice before Fe is released and enters the Fe pool. During this time, Na_2EDTA can form complexes with other minerals and trace elements, resulting in a reduced binding capacity of Na_2EDTA with the more slowly solubilised Fe compounds. EDTA-Fe complexes, which are assumed to be responsible for the enhancing effect of Na_2EDTA , would thus not be formed.

The results from study 2 show that ascorbic acid added at a 4:1 molar ratio (relative to Fe) enhances Fe absorption from ferrous fumarate significantly. Thus, Fe does not seem to be competing with other minerals and trace elements or with other food components present in the gastric juice for the reducing and chelating properties of ascorbic acid, which are thought to be responsible for its enhancing effect on Fe absorption (Conrad & Schade, 1968).

Until now, the enhancing effect of ascorbic acid on Fe absorption from ferrous fumarate had not been demonstrated directly. Results from earlier studies had indicated that ascorbic acid enhanced Fe absorption from ferrous fumarate to the same extent as from ferrous sulfate, as there was no significant difference between Fe absorption from infant cereals fortified with these compounds in the presence (Hurrell *et al.* 1989) and in the absence (Hurrell *et al.* 2000) of ascorbic acid. However, another study had reported ascorbic acid to have no significant effect on Fe absorption from a chocolate-drink powder and a liquid formula meal fortified with ferrous fumarate (Hurrell *et al.* 1991). These findings can probably partly be explained by the relatively low ascorbic acid:Fe molar ratio (1.7:1) and by the high level of Fe-absorption inhibitors in the chocolate drink (casein and Ca from milk, phytate from malt and polyphenols from cocoa). This assumption is supported by the observation in the same study that native Fe absorption from the unfortified chocolate drink was not increased even though the ascorbic acid:Fe molar ratio was approximately 14:1. Further, although Fe absorption from a ferrous fumarate-fortified liquid formula meal did not increase significantly on the addition of ascorbic acid at a 4.4:1 ascorbic acid:Fe molar ratio, a 1.6-fold increase ($P=0.14$) was observed in this earlier study. Based on the results from the present study, showing that ascorbic acid enhances Fe absorption significantly from ferrous fumarate, it would seem that the lack of statistical evidence in the earlier study was presumably due to the small sample size (eight subjects) and the large variations within the group.

The results of the present studies confirmed that healthy adults absorb Fe as well from ferrous fumarate as from ferrous sulfate and thus indicate that, in adults, ferrous fumarate dissolves completely in the gastric juice. Further, the results showed that ascorbic acid enhances Fe absorption from ferrous fumarate, while Na_2EDTA has

no significant influence on Fe absorption from ferrous fumarate. Thus, the present results emphasise that not all findings from Fe absorption studies made with ferrous sulfate can be extrapolated to Fe compounds which have different solubility properties.

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