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Effect of Ascorbic Acid and Particle Size on Iron Absorption from Ferric Pyrophosphate in Adult Women

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Abstract: The effects of added ascorbic acid and particle size on iron absorption from ferric pyrophosphate were evaluated in adult women (9–10 women/study) based on erythrocyte incorporation of iron stable isotopes (^{57}Fe or ^{58}Fe) 14 days after administration. Three separate studies were made with test meals of iron-fortified infant cereal (5 mg iron/meal) and the results are presented as geometric means and relative bioavailability values (RBV, $FeSO_4$ = 100%). The results of study 1 showed that iron absorption was significantly lower from ferric pyrophosphate (mean particle size 8.5 µm) than from $FeSO_4$ in meals without ascorbic acid (0.9 vs. 2.6%, $p < 0.0001$, RBV 36%) and in the same meals with ascorbic acid added at a 4:1 molar ratio relative to fortification iron (2.3 vs. 9.7%, $p < 0.0001$, RBV 23%). Ascorbic acid increased iron absorption from ferric pyrophosphate slightly less (2.6-fold) than from $FeSO_4$ (3.7-fold) ($p < 0.05$). In studies 2 and 3, RBV of ferric pyrophosphate with an average particle size of 6.7 µm and 12.5 µm was not significantly different at 52 and 42% ($p > 0.05$), respectively. In conclusion, the addition of ascorbic acid increased fractional iron absorption from ferric pyrophosphate significantly, but to a lesser extent than from $FeSO_4$. Decreasing the mean particle size to 6.7 µm did not significantly increase iron absorption from ferric pyrophosphate.

Key word: Iron absorption, humans, ferric pyrophosphate, ascorbic acid, particle size, food fortification, stable isotopes

Introduction

Ferric pyrophosphate, an iron fortificant that is insoluble in water and only poorly soluble in dilute acid, is currently used by European food companies to fortify infant cereals and chocolate drink powders. Its main advantage is that it causes no adverse color and flavor changes to the food vehicle, in contrast to readily water-soluble iron fortificants such as ferrous sulfate [1]. However, due to its limited solubility, iron absorption can be expected to be low; studies in adults have reported iron absorption from ferric pyrophosphate to be in the range of 15 to 75% of that from ferrous sulfate, depending on batch and processing conditions [2–4]. In infants, iron absorption from ferric pyrophosphate-fortified foods has so far not been compared directly to that from ferrous sulfate. However, iron

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absorption from ferric pyrophosphate-fortified wheat-soy infant cereal has in 6–12 month old infants been shown to range between < 0.7 and 2.7% (geometric mean 1.3%) and was further shown to be only one-third as well absorbed as ferrous fumarate [5]. These low iron absorption values were somewhat surprising since ascorbic acid had been added at a 3:1 molar ratio relative to iron. The enhancing effect of ascorbic acid on iron absorption has been demonstrated for ferrous sulfate in several studies [6–9] but very little is known about the effect on water-insoluble iron fortificants. Ascorbic acid has been shown to enhance iron absorption from ferric orthophosphate and elemental iron [10] but the effect on iron absorption from ferric pyrophosphate has so far not been evaluated.

Furthermore, a factor which influences iron absorption from water-insoluble iron fortificants is particle size [11–14] and we have recently reported that iron absorption from micronized, dispersible ferric pyrophosphate (Sunactive Fe™) is not significantly different to that from ferrous sulfate [15]. This fortificant has an average particle size of 0.3 µm, which is over 20 times smaller than regular commercial ferric pyrophosphate (average particle size approximately 8 µm). Decreasing the particle size of hydrogen- and carbon monoxide-reduced iron powders by about 50–60%, to sizes between 7 and 10 µm, has been shown to increase iron absorption from these fortificants by approximately 50% in rats [13, 14].

The aims of the present studies were to investigate the effect of added ascorbic acid on iron absorption from ferric pyrophosphate and to evaluate the impact of ferric pyrophosphate particle sizes within the range of approximately 7–13 µm on iron absorption. Erythrocyte incorporation of stable isotopes 14 days after administration of labeled test meals was used to estimate iron absorption in healthy women. An infant cereal was used as the standardized test meal.

Subjects and Methods

Subjects

Twenty-eight apparently healthy adult women (20–30 years; max body weight 60 kg) were recruited from the student and staff population at the ETH Zurich and the University of Zurich. The subjects were randomly allocated into three separate studies (9–10 women/study). Exclusion criteria included pregnancy or lactation and known gastrointestinal or metabolic disorders. No medication (except oral contraceptives) or vitamin/mineral supplements were allowed during the study. Women regularly taking vitamin/mineral supplements discontinued the supplementation two weeks before the start of the study.

The study protocol was reviewed and approved by the Ethical Committee at the ETH Zurich, Switzerland. Subjects were informed orally and in writing about the aims and procedures of the study. Written informed consent was obtained from all women.

Study design

Three studies were made (Table I). In study 1, each subject (10 women) received four labeled test meals and iron absorption from ferric pyrophosphate- (average particle size 8.5 µm) and ferrous sulfate-fortified meals, with and without added ascorbic acid, was compared. In studies 2

Table I: Iron absorption by healthy adult women from an infant cereal fortified with ferrous sulfate or un-separated ferric pyrophosphate[1] (5 mg iron) with and without ascorbic acid[2] (study 1) and with ferric pyrophosphate of fine[3] (study 2) and coarse[4] particle size (study 3)

Study no.	n	Plasma ferritin* (µg/L)	Test meal	Iron absorption, %*	Relative bioavailability (compared to iron absorption from test meals fortified with ferrous sulfate), %*
1	10	26.6 (17.1, 41.4)	A ferrous sulfate	2.6[a] (1.3, 5.2)	
			B ferric pyrophosphate (un-separated)	0.9[b] (0.4, 2.1)	36 (24, 55)
			C ferrous sulfate + ascorbic acid[1]	9.7[c] (5.1, 18.5)	
			D ferric pyrophosphate (un-separated) + ascorbic acid[1]	2.3[a] (1.0, 4.9)	23 (16, 35)
2	9	17.8 (8.5, 37.8)	E ferrous sulfate	2.3[a] (1.2, 4.3)	
			F fine particle size ferric pyrophosphate	1.2[b] (0.7, 2.0)	52 (32, 85)
3	9	13.4 (7.4, 24.4)	G ferrous sulfate	4.9[a] (2.5, 9.5)	
			H coarse particle size ferric pyrophosphate	2.0[b] (0.8, 5.3)	42 (26, 66)

average particle sizes: [1] 8.5 µm; [2] 6.7 µm and [3] 12.5 µm

[4] molar ratio ascorbic acid:iron of 4:1; n: number of test subjects; *geometric mean (–SD, +SD)

[a, b, c] within separate studies 1, 2, and 3, mean values not sharing a common superscript letter were significantly different ($p < 0.05$)

and 3, each subject (9 women/study) received two meals only and iron absorption from fine (average particle size 6.7 μm) and coarse (average particle size 12.5 μm) ferric pyrophosphate fractions were compared to that from ferrous sulfate, respectively. Iron absorption was based on erythrocyte incorporation of iron-stable isotope labels 14 days after intake of labeled test meals. The iron fortificants were labeled with ^{57}Fe or ^{58}Fe and added to the different test meals as described below. All test meals were fed, after an overnight fast, under standardized conditions. A crossover study design was used with each woman acting as her own control. On the day before intake of the first test meal (day 0), a venous blood sample was drawn after an overnight fast for determination of iron status parameters [hemoglobin (Hb), and plasma ferritin] and body weight and height were measured. Two test meals were fed on the following days (days 1 and 2) between 0700 and 0900 hours. No intake of food or fluids was allowed for 3 hours after test meal intake. A second venous blood sample was drawn 14 days after intake of the second test meal (day 16). In study 1, a second pair of labeled test meals (meals C and D) was fed on days 17 and 18 and a final blood sample was obtained 14 days after administration of the last test meal (day 32).

Test meals

The 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 deionized water). The infant cereal was made from 79.7% partially hydrolyzed wheat flour, 10% sucrose, 4% honey, 3% palm oil, 0.3% calcium carbonate, and 3% water. Except for calcium, no minerals or vitamins were added to the infant cereal. Each test meal contained 5 mg added iron, 4 mg iron as $^{58}FeSO_4$ plus 1 mg iron as $FeSO_4$ of natural isotopic composition, or 5 mg iron as ^{57}Fe ferric pyrophosphate. In study 1, the labeled ferric pyrophosphate used was comparable to commercial ferric pyrophosphate. Fine and coarse particle size fractions of ferric pyrophosphate, as described below, were used in studies 2 and 3, respectively. To test meals C and D in study 1, food grade ascorbic acid (Merck, Darmstadt, Germany) was added at the time of serving as an aqueous solution at a molar ratio of ascorbic acid to fortification iron of 4:1 (63 mg ascorbic acid). Aqueous ascorbic acid solutions were prepared freshly each morning. Deionized water (200 g) was served as a drink.

Stable isotope labels

[^{58}Fe]Ferrous sulfate was prepared from isotopically enriched elemental iron (Chemgas, Boulogne, France) by dissolution in sulfuric acid and dilution to appropriate concentration. [^{57}Fe]Ferric pyrophosphate (hereafter referred to as un-separated ferric pyrophosphate) was prepared in collaboration with Dr. Paul Lohmann Ltd., Emmerthal, Germany. It has been used in previous human studies [5]. Part of the un-separated labeled ferric pyrophosphate was used to prepare the fine and coarse particle size fractions. The coarse fraction was collected by trapping large particles by means of filtration. For this, un-separated labeled ferric pyrophosphate was suspended in deionized water and filtered through a polyamide monofilament screening fabric (pore size 11 μm, Sefar Nitex 03–11/6, Sefar Inc., Rueschlikon, Switzerland). The filter cake representing the coarse particle size fraction was washed thoroughly with deionized water and dried on the filter at room temperature. The fine fraction was subsequently separated from the filtrate by sedimentation. Deionized water was added to the filtrate to a total volume of 2 L and this suspension was filled into a tall beaker (24 cm) and left for 20 hours at room temperature. After this time period, the particles that remained in suspension were recovered by filtering the suspension through a 0.45 μm filter (Alltech Associates, Inc., Deerfield, IL, USA). The resulting filter cake (fine particle size fraction) was dried on the filter at room temperature. Particle size distribution of the un-separated, fine, and coarse ferric pyrophosphate fractions was measured by laser light diffraction (Mastersizer X, Malvern Instruments Ltd., Malvern, UK). Average particle size, calculated as surface area moment mean (Sauter mean diameter), of the un-separated, fine, and coarse ferric pyrophosphate fractions, was 8.5 μm, 6.7 μm, and 12.5 μm, respectively (Fig. 1). The Sauter mean diameter, which is based on surface area as well as volume, was con-

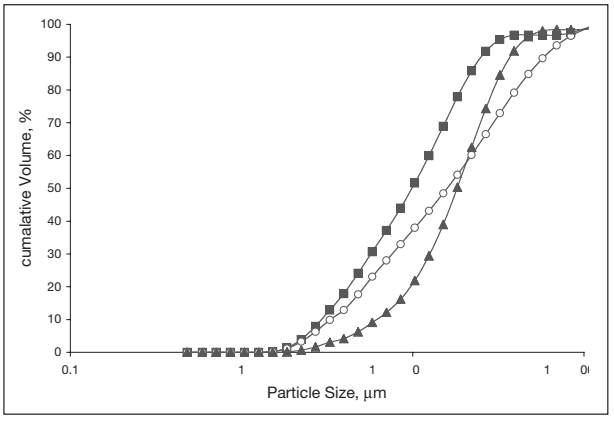

Figure 1: Particle size distribution shown as cumulative volume percentage frequency curve of fine (-■-), non-separated (-○-), and coarse (-▲-) ferric pyrophosphate fractions measured by laser diffraction (Mastersizer X, Malvern Instruments Ltd., Malvern, UK).

sidered to be the most appropriate parameter to describe average particle size [16, 17].

Quantification of iron isotopes in labeled iron fortificants

Isotope dilution mass spectrometry was used to determine the concentration of ^{57}Fe and ^{58}Fe stable isotopes in the ferric pyrophosphate and ferrous sulfate solutions. For analysis, [^{57}Fe]ferric pyrophosphate was dissolved in concentrated nitric acid. An accurately measured amount of iron of natural isotopic composition was added to aliquots taken from the prepared solutions of labeled iron fortificants. The iron 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 ionization mass spectrometry (NTI-MS). Iron concentrations of each labeled iron fortificant were calculated based on the shift in iron isotopic abundances, the determined isotopic abundances of the isotopic labels, and the natural iron isotopic abundances [18, 19].

Iron status measurements

Venous blood samples (7 mL) were drawn in ethylenediaminetetracetic acid (EDTA)-treated tubes at each sampling. Samples were analyzed for iron status indices (Hb, plasma ferritin) and for incorporation of ^{57}Fe and ^{58}Fe into erythrocytes (day 16 and day 32). Whole blood samples were aliquoted for analysis of Hb and isotopic composition and plasma was separated, aliquoted, and frozen for later analysis of plasma ferritin. Hb was measured by the cyanmethemoglobin method (Sigma kit, Sigma, St. Louis, MO) and plasma ferritin by enzyme-linked immunosorbent assay (ELISA) (Ramco Laboratories, Houston, Texas). Commercial quality control materials (DiaMed, Cressier sur Morat, Switzerland and Ramco Laboratories) were analyzed together with samples analyzed for Hb and plasma ferritin, respectively.

Quantification of iron isotope in blood

Each isotopically enriched blood sample was analyzed in duplicate for its iron isotopic composition as previously described by Walczyk *et al* [19]. The blood samples were mineralized by microwave digestion using a mixture of nitric acid and hydrogen peroxide. Iron 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 ionization mass spectroscopy (NTI-MS) [18].

Calculation of iron absorption

The amounts of ^{57}Fe and ^{58}Fe isotopic labels in blood 14 days after test meal administrations were calculated based on the shift in iron isotope ratios and on the amount of iron circulating in the body. The calculations were based on the principles of isotope dilution and took into account that the iron isotopic labels were not monoisotopic [19]. Circulating iron was calculated based on blood volume and Hb concentration [20]. Blood volume calculations were based on height and weight according to Brown *et al* [21]. For calculations of fractional iron absorption, 80% incorporation of the absorbed iron into erythrocytes was assumed [22].

Food analysis

Cereal and milk powder were analyzed for iron and calcium by electrothermal/flame atomic absorption spectroscopy (SpectrAA 400, Varian, Mulgrave, Australia) after mineralization by microwave digestion (MLS 1200) in a HNO_3/H_2O_2 mixture and using standard addition technique to minimize matrix effects. Phytic acid in the infant cereal was determined by a modification of the Makower method [23] in which cerium replaced iron in the precipitation step.

Statistics

Fractional iron absorption values are presented as geometric means (–1SD, +1SD). Student's paired *t*-test was used to compare absorption data within each study. Student's unpaired *t*-test was used to compare iron absorption ratios between studies. Absorption values were logarithmically transformed before statistical analysis (Excel 2002, Microsoft Corporation, Redmond, WA, USA).

Results

None of the women participating in the study were anemic (hemoglobin concentration < 120 g/L). However, eight women had low iron stores as indicated by low plasma ferritin values (< 12 µg/L).

The test meals contained 0.6 mg native iron (1.1 mg iron/100 g infant cereal, 0.15 mg iron/100 g milk powder), 167 mg calcium (148 mg calcium/100 g infant cereal, 1159 mg calcium/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.

The results of the iron absorption studies are presented in Table I as geometric mean iron absorption and as rela-

tive bioavailability values (RBV) (RBV of ferrous sulfate = 100%). In study 1, the expected low bioavailability of ferric pyrophosphate was confirmed by the direct comparison of iron absorption from ferric pyrophosphate- (un-separated) and ferrous sulfate-fortified infant cereal (geometric mean 0.9 vs. 2.6%, respectively, $p < 0.0001$, RBV 36%). When ascorbic acid was added to the test meals, iron absorption from ferric pyrophosphate (un-separated) and ferrous sulfate increased 2.6-fold and 3.7-fold, respectively (geometric mean 2.3 vs. 0.9%, $p=0.01$ and 9.7 vs. 2.6%, $p < 0.001$, respectively, RBV 23%). RBV from fine (study 2) and coarse (study 3) fractions of ferric pyrophosphate was 52 and 42%, respectively (1.2% (fine) vs. 2.3% (ferrous sulfate), $p = 0.004$ and 2.0% (coarse) vs. 4.9% (ferrous sulfate), $p < 0.001$). Decreasing mean particle size from 8.5 to 6.7 μm did not affect iron absorption significantly (RBV 36 vs. 52%, $p = 0.09$). Further, increasing the particle size from 8.5 to 12.5 μm had no significant effect on RBV (36 vs. 42%, $p = 0.47$).

Discussion

Ferric pyrophosphate is currently used by European food companies to fortify infant cereals and chocolate drink powders as this fortificant does not cause unacceptable changes to the color and flavor [24]. However, until now only few human iron absorption studies have been made with this fortificant. This is primarily due to the technical difficulties involved in producing labeled iron fortificants. While the preparation of labeled ferrous sulfate is relatively easy, special care has to be taken when preparing labeled water-insoluble iron fortificants, such as ferric pyrophosphate, so as to ensure that the labeled fortificant is similar to the commercial iron fortificant. Besides the technical difficulties associated with using a down-scaled production method, the high cost involved in the preparation of such fortificants is a limiting factor, especially when stable iron isotopes are used. In our study, the labeled ferric pyrophosphate (un-separated) was produced in collaboration with a manufacturer of commercial ferric pyrophosphate and had a similar solubility to commercial ferric pyrophosphate in dilute acid [5].

The importance of evaluating iron absorption from fortificants besides ferrous sulfate has been emphasized by the results of recent studies showing that iron absorption enhancing effects demonstrated with ferrous sulfate cannot necessarily be extrapolated to water-insoluble iron fortificants. For example, although the enhancing effect of Na_2EDTA has been repeatedly demonstrated on iron absorption from ferrous sulfate in humans [4, 25, 26], the addition of Na_2EDTA to a ferric pyrophosphate-fortified infant cereal had no effect on iron absorption [4]. Until now, the effect of ascorbic acid on iron absorption from ferric pyrophosphate had not been evaluated.

The results from the present study show that ascorbic acid added to infant cereal at a 4:1 molar ratio relative to the fortification iron enhances iron absorption 2.6-fold from ferric pyrophosphate (un-separated). These results therefore support previous evidence that ascorbic acid has an enhancing effect on iron absorption from water-insoluble iron fortificants such as ferric orthophosphate and electrolytic iron [10]. However, the magnitude of the enhancing effect of ascorbic acid on iron absorption was slightly higher from test meals fortified with ferrous sulfate (3.7-fold) than with ferric pyrophosphate (un-separated) (2.6-fold). This resulted in a significantly lower RBV for ferric pyrophosphate (un-separated) in the presence of ascorbic acid as compared to the absence of ascorbic acid (23 vs. 36%, respectively, $p < 0.05$). Forbes *et al* [10] similarly reported a greater enhancing effect of ascorbic acid (5:1 molar ratio relative to iron) on iron absorption from ferrous sulfate than from electrolytic iron (3-fold vs. 2-fold increase in iron absorption, $p < 0.007$). Based on these studies, it seems possible that more ascorbic acid needs to be added to foods fortified with water-insoluble iron fortificants than water-soluble iron fortificants to get an equivalent increase in iron absorption.

The influence of ferric pyrophosphate particle size on iron absorption was also evaluated in the present study. For studies 2 and 3, the same labeled ferric pyrophosphate that was used in study 1 was separated into two fractions – fine and coarse with a 2-fold difference in particle size (6.7 μm vs. 12.5 μm). By using the same batch of labeled ferric pyrophosphate in all three studies, the influence of other potentially important factors such as particle density or porosity as well as heat treatment [3, 27, 28] was minimized.

Our results show that RBV of ferric pyrophosphate is not affected by mean particle size within the range of 6.7 to 12.5 μm. Thus, small differences in the particle size of commercial ferric pyrophosphate will not influence iron absorption significantly from this fortificant. In an earlier study, RBV of micronized, dispersible ferric pyrophosphate (average particle size of 0.3 μm) added to the same infant cereal as used in the present study was 83%, which was not significantly different from ferrous sulfate [15]. At the present time, no conclusion can be drawn as to what extent mean particle size should be decreased from its current approximately 8 μm to achieve a significant increase in iron absorption from ferric pyrophosphate. With conventional grinding procedures, however, it is difficult to decrease mean particle size below 2–3 μm. Particle size below 1 μm can be produced with special technology which includes generating ferric pyrophosphate particles

in aqueous solutions and adding emulsifier to prevent agglomeration [29]. However, this procedure markedly increases the cost of the iron fortificant.

By evaluating the results of the present study together with those of Cook *et al* [12] the influence of particle size on iron absorption from H-reduced elemental iron powder and from ferric pyrophosphate can be compared. In this earlier human study [12], iron absorption from H-reduced elemental iron powder, with particle sizes between 5–10 µm, was found to be equivalent to ferrous sulfate. In the present study iron from ferric pyrophosphate with average particle sizes of 8.5 and 6.7 µm was only 40–50% as available as from ferrous sulfate. Therefore, it is clearly not possible to extrapolate effects of particle size from one water-insoluble iron fortificant to another.

However, particle size is presumably not the only physico-chemical property that influences iron absorption from ferric pyrophosphate. In earlier studies, sterilization of ferric pyrophosphate-fortified infant formula was shown to increase RBV of ferric pyrophosphate in rats [27, 28]. On the contrary, Hurrell *et al* [3] reported that RBV of ferric pyrophosphate decreased from 75 to 21% in adult human subjects as a result of heat treatment (vacuum drying of a chocolate drink powder at 100°C). Furthermore, when the same labeled ferric pyrophosphate was evaluated several years later in an infant cereal, to which it was added after processing, RBV was only 15% [4]. Although a direct comparison of these studies is not possible due to the difference in test meal composition, it is noteworthy that the RBV of the same labeled batch of ferric pyrophosphate has ranged from 15% (infant cereal) to 75% (chocolate drink powder). This indicates that heat treatment and perhaps also storage duration and/or conditions influence absorption of ferric pyrophosphate.

In conclusion, the present studies showed that iron absorption from ferric pyrophosphate can be increased significantly by adding ascorbic acid. Food manufacturers should therefore ensure that adequate amounts of ascorbic acid are added to ferric pyrophosphate-fortified foods. Decreasing the particle size of ferric pyrophosphate by 25% did not increase iron absorption significantly. However, the results of the present study and previous studies indicate that iron absorption from ferric pyrophosphate is dependent on particle size. Further studies are needed to investigate to what extent the particle size must be decreased to significantly increase iron absorption from ferric pyrophosphate and whether other factors such as density or surface area influence dissolution in the gastric juice and thus absorption.

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