Iron in ferritin or in salts (ferrous sulfate) is equally bioavailable in nonanemic women¹⁻³

Penni Davila-Hicks, Elizabeth C Theil, and Bo Lönnerdal

ABSTRACT

Background: Recent studies in humans suggest that ferritin iron in soybeans has high bioavailability. However, direct evidence for this is lacking because the soybeans were intrinsically labeled; thus, iron bound to other ligands, such as phytate, was also labeled.

Objective: The objectives of the study were to evaluate the absorption of iron from extrinsically labeled, purified ferritin (horse spleen) reconstituted with either high-phosphate iron mineral (plant-type) or low-phosphate iron mineral (animal-type) and to compare it with iron absorption from ferrous sulfate.

Design: Nonanemic, healthy young women were fed a standard breakfast meal supplemented with ⁵⁹Fe-labeled ferritin or ferrous sulfate, in randomized order. Fifteen subjects received ferritin with the low-phosphate iron mineral, and 15 subjects received ferritin with the high-phosphate iron mineral. Iron absorption was measured in a whole-body counter after 14 and 28 d and by red blood cell incorporation after 28 d.

Results: There was no significant difference in iron absorption between ferritin and ferrous sulfate: low-phosphate iron mineral ferritin (5 ± SD: 21.4 ± 14.7%) compared with ferrous sulfate (21.9 ± 14.6%), or high-phosphate iron mineral ferritin (22.2 ± 19.2%) compared with ferrous sulfate (16.7 ± 7.1%). Results obtained by using whole-body retention of iron and red blood cell incorporation differed with the type of iron, which suggests that pathways for iron uptake and utilization differed for the 2 forms.

Conclusions: Iron is equally well absorbed from ferritin and ferrous sulfate independent of the phosphate content of the ferritin iron mineral. Thus, dietary ferritin iron is likely to be a good source of iron. Am J Clin Nutr 2004;80:936–40.

KEY WORDS: Iron, iron bioavailability, ferritin, ferritin iron, iron absorption, iron mineral ferritin

INTRODUCTION

Dietary iron has many chemical forms, such as heme (iron protoporphyrin), natural iron salts (eg, iron oxalate, iron citrate, and iron phytate), supplementary iron salts (eg, Fe EDTA, Fe gluconate, and Fe sulfate), ferritin-rich soybeans (1, 2), and, in infants, lactoferrin (3). The different forms of iron available for uptake have been more completely explored in bacteria than in humans, and it is clear that each different form of iron has a specific set of iron uptake proteins encoded in genes that are regulated by the presence of the specific environmental iron source (4). In humans, by contrast, the only iron uptake protein identified is that for ferrous salts, usually called divalent metal transporter 1 (DMT1; 5). An intestinal receptor for lactoferrin has also been characterized (6). Iron in soybeans, a common form of iron in diets that contain little iron in the form of ferrous salts, was recently shown to be readily absorbed in humans (7).

Ferritin is a major form of iron in soybeans (8, 9), in which the iron is a solid ferric mineral containing thousands of iron and oxygen atoms, inside the protein (10, 11). The high content of phytic acid in soybeans, which binds many forms of supplemental iron as the relatively unavailable ferric phytate (2, 12), has led to some confusion about the bioavailability of iron in soybeans and about soybeans as a source of dietary iron for humans (13, 14). The issue was clarified recently (7) when the iron bioavailability of intrinsically labeled soybeans was shown to be very high (27%) in American women with borderline iron deficiency who were consuming a controlled diet but relatively low (2%) in iron-replete American men (14). The degree of iron repletion in each study was assessed from the values for serum ferritin, hematocrits, and absorption of a ferrous sulfate reference dose. In an earlier study of East Indian and African women who appeared to be iron deficient (according to hematocrit values and absorption of the ferrous sulfate reference dose), intrinsically labeled soybean iron was also readily available (20% absorption; 13). The iron in pure ferritin, the major form of iron in soybeans (8), can be absorbed by iron-deficient rats to correct anemia (15); this suggests that, at least in rats, the availability of ferritin iron is not influenced by the phytic acid in soybeans.

Direct evidence that the iron in ferritin can be absorbed by humans is still lacking because, in the experiments to date, the percentage of iron in ferritin of intrinsically labeled soybeans either was not measured (13, 14) or constituted ≈48% of the iron (7), possibly because the plants were grown hydroponically and did therefore not nodulate. Field-grown soybeans, however, are nodulated and thus stimulated to take up into the nodule massive amounts of iron (16, 17), which is later recycled to the seed, whereas the vast majority of the soybean seed iron is in ferritin (9). In this study, we compared the whole-body absorption of iron...
from ferrous sulfate with that from pure ferritin reconstituted to form either the animal (low-phosphate) iron mineral or the plant (high-phosphate) iron mineral, as described elsewhere (18, 19).

SUBJECTS AND METHODS

Recruitment and screening

Potential subjects were recruited by advertisements on bulletin boards at the University of California, Davis; persons who responded to the advertisements were given an explanation of the study and were scheduled for screening. Before screening, written informed consent was obtained from each subject. The screening consisted of a brief health questionnaire to detect any history of hematologic or gastrointestinal disorders, an assessment of iron status via a finger prick, and a pregnancy test. Subjects were excluded from the study if they had a history of hematologic or gastrointestinal disorders, were anemic (hemoglobin <90 g/L), were taking iron-containing supplements and refused to discontinue their use for the duration of the study, or were pregnant. Thirty women participated in the study. The study was approved by the Human Subjects Review Committee and the Radiation Use Authorization Committee at the University of California, Davis.

Iron sources

Iron was removed from horse spleen ferritin (Calzyme, San Luis Obispo, CA) by thioglycolic acid reduction and dialysis (20), and iron mineral was reconstituted with or without phosphate for study use. Reconstitution of ferritin by this procedure produces a mineral that, when measured by both extended X-ray absorption fine structure absorption spectroscopy and Mössbauer spectroscopy, is essentially indistinguishable from ferritin isolated from natural tissue (18, 19). All steps were performed at 4°C unless otherwise specified. Horse spleen ferritin was mixed with 2% thioglycolic acid at a 1:1 ratio, gently purged with nitrogen, and stored at 4°C for 1 h. This mixture was then placed in dialysis tubing [Spectra/Pore CE (MW cutoff: 10,000); Spectrum, Rancho Dominguez, CA] and placed into a dialysate of 1% thioglycolic acid and 0.05 mmol HEPES/L. The dialysate was changed every 8 h for 3 d. On day 4, the dialysate consisted of 0.05 mmol HEPES/L and 0.1 mmol NaCl/L. The iron-free ferritin protein (<10 Fe atoms/protein molecule)—often called apoferritin even though the iron is not a cofactor—was then stored at 4°C until reconstitution of the mineral inside the ferritin protein.

The iron mineral in the apoferritin was reconstituted with 59FeSO4 (27.7 mCi/mg; Perkin Elmer, Boston) to 480 Fe atoms/protein molecule, with and without iron mineral (K2HPO4), for study use (18). A quantity of iron equal to that in ferritin in the form of 59Fe-labeled ferrous sulfate was used for comparison. In a subset of subjects (n = 15), high-phosphate iron mineral was formed in the presence of K2HPO4 at a 4:1 ratio of phosphate:59Fe-labeled iron to reconstitute a ferritin iron mineral similar to that found in plants such as soybeans.

Study protocol

Subjects were randomly assigned to begin the study with either the 59Fe-labeled horse spleen ferritin (with or without phosphate) meal or the 59Fe-labeled ferrous sulfate meal, each containing 1 mg Fe. The labeled ferritin or ferrous sulfate was administered in 60 mL apple juice, which delivered ≈1 μCi 59Fe to each subject. A plain, white flour bagel and full-fat cream cheese (1 tsp) was also given to represent a realistic situation for subjects consuming a ferritin-containing meal. All meals were consumed after a 12-h overnight fast.

On day 1 of the study, the subjects arrived between 0700 and 0900 in a fasted state, and background whole-body radioactivity was measured in a whole-body counter (Institute of Toxicology and Environmental Health, University of California, Davis) equipped with two 10 × 20-cm sodium iodide crystals and a multichannel analyzer (ND-66; Nuclear Data, Schaumburg, IL). The subjects were then given the first randomized labeled meal and immediately reevaluated in the whole-body counter. Subjects were instructed to refrain from eating and from drinking any fluid except water for the next 4 h. Radioactivity was measured 14 and 28 d after consumption to assess retention of the previously consumed dose. On day 28, subjects arrived at the whole-body counter in a fasted state. After whole-body radioactivity was measured, a venous blood sample was drawn from an antecubital vein for assessment of the incorporation of 59Fe into red blood cells (RBCs). After the blood was drawn, the subjects were given the second randomized labeled meal and immediately reevaluated in the whole-body counter. The subjects were also evaluated in the whole-body counter 14 and 28 d after consumption of the second meal. A final blood sample was drawn on day 28. Blood samples were used to measure hemoglobin (HemoCue, Ängelholm, Sweden) and ferritin (Ferritin IRMA; Diagnostic Products Corporation, Los Angeles) concentrations.

Statistical analysis

SAS for WINDOWS software (version 8; SAS Institute Inc, Cary, NC) was used for the statistical analyses. Two-factor repeated-measures analysis of variance (ANOVA; with type of iron as the within-subject factor and type of diet as the between-subject factor) was used to compare ferritin, whole-body counter, and RBC-incorporation values. Three-factor repeated-measures ANOVA was used to compare the whole-body counter and RBC-incorporation methods (with method as the within-subject factor) and to compare results in the iron-deficient and the iron-replete subjects (with iron status as the between-subject factor). The ferritin and absorption variables were log transformed to conform to the assumption that the error terms are normally distributed.

RESULTS

None of the subjects were anemic at the start of the study, but iron status varied (range of serum ferritin: 3–48 μg/L). Mean hemoglobin for all subjects was 135.2 g/L (range: 120–152 g/L). The iron status of the subjects, measured as serum ferritin, did not differ significantly between treatment groups (P = 0.83; Tables 1 and 2).

There was no significant difference in iron absorption from a typical breakfast when iron was provided as ferritin or as ferrous sulfate (P = 0.90; Table 1). From a breakfast with low-phosphate (animal) mineral ferritin, 21.4 ± 14.7% of the iron was absorbed, compared with 21.9 ± 14.6% of ferrous sulfate. There was also no significant (P = 0.90) difference in iron absorption between high-phosphate (plant-type) mineral ferritin (22.2 ± 19.2%) and ferrous sulfate (16.7 ± 7.1%; Table 2). There was no significant
difference in iron absorption between dietary groups ($P = 0.74$) or an interaction between diet and type of iron ($P = 0.63$).

Repeated-measures ANOVA of log-transformed values found no significant differences in serum ferritin (main effect of iron type: $P = 0.18$; main effect of diet: $P = 0.83$; Fe type × diet interaction: $P = 0.76$). There was, however, an effect of the interaction between diet and iron type on RBC incorporation (main effect of iron type: $P = 0.042$; main effect of diet: $P = 0.11$; effect of iron type × diet interaction: $P = 0.020$). In the low-phosphate ferritin group, RBC incorporation from ferrous sulfate (47.6 ± 16.0%) differed significantly from that from ferritin (27.0 ± 17.1%), whereas, in the high-phosphate ferritin group, there was no difference (29.4 ± 5.6% and 29.2 ± 13.1%, respectively). Comparing the whole-body counting and RBC-incorporation methods by using a 3-factor ANOVA was somewhat problematic because the RBC method resulted in skewed data, whereas the whole-body counting method did not; hence, we analyzed both untransformed and log-transformed data, with

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5 ± SD 134.7 ± 10.4 17.3 ± 13.7 20.8 ± 13.5 21.9 ± 14.6 21.4 ± 14.7 47.6 ± 16.0 27.0 ± 17.1

1 RBC, red blood cell.
2 No sample because of unsuccessful blood draw.
3 Significantly different from FeSO$_4$, $P = 0.0005$.
similar results. For the untransformed data, the main effects of iron type ($P = 0.42$) and diet ($P = 0.30$) were not significant, whereas the main effect of method was ($P = 0.0157$). The interactions between iron type and diet ($P = 0.0032$) and between iron type and method ($P = 0.0249$) were significant, whereas the interaction between diet and method ($P = 0.41$) and the 3-way interaction ($P = 0.30$) were not (repeated-measures 3-way ANOVA). Bars with different letters are significantly different between groups ($P < 0.05$; Tukey-Kramer test); the 2 iron-absorption methods were significantly different ($P = 0.0005$) for ferrous sulfate.

**DISCUSSION**

Matching the most efficacious form of dietary iron to specific nutritional states is the goal of nutritional genomics for health and disease, especially with respect to such genetic diseases as hemochromatosis, sickle cell disease, and the thalassemias (1), in which iron absorption is altered by the disease. Understanding the availability of different chemical forms of iron in the diet will allow advances in diet design to ameliorate the variations in genetic background related to iron absorption. Inorganic iron salts and iron complexes found in foods with phytate, oxalate, and heme have received the most attention (21). Ferritin is, however, an abundant form of nonheme iron in many plant foods, such as legumes, that has been little considered as a nutritional iron source until recently.

Our study shows that iron from ferritin was well utilized in a group of young women with varied iron status but without anemia (Tables 1 and 2). Absorption of iron from ferritin did not differ significantly from that of iron from ferrous sulfate—a form of iron with high bioavailability when given in meals with a low content of inhibitors (21). Ferrous sulfate, however, cannot be used for iron fortification in most foods because it causes rancidity (oxidation) and discoloration (12), which make the product inedible. Thus, ferritin iron represents a form of iron that is highly bioavailable to humans and that is not likely to affect the food in which it is consumed. Further studies are needed to evaluate the effects of inhibitors and enhancers of nonheme-iron absorption on the absorption of iron from ferritin.

Plant ferritins are more likely than animal ferritins to be the source of ferritin in natural foods, and their mineral has a higher ratio of phosphate to iron (usually $\approx 4:1$) than does that of animal ferritins (usually $\approx 1:8$). The chemical difference in the plant ferritin mineral leads to a more disordered iron mineral structure (18, 19) in plant ferritins that might influence the bioavailability of iron from ferritin. However, in our study comparing both types of ferritin iron mineral, we observed no difference in iron bioavailability (Tables 1 and 2). Similar results were also obtained when comparing iron-deficient and iron-replete subjects.

We used a highly sensitive whole-body counter with very low background radiation (pre-World War II steel) and were therefore able to measure iron retention very precisely. The results for iron absorption from ferritin using the direct measure of whole-body retention or the more indirect RBC incorporation method that reflects assumptions made for blood volume and the percentage of iron incorporation into RBCs, led to the same conclusion—ie, that iron from ferritin is absorbed by humans as well as is iron from ferrous sulfate (Tables 1 and 2)—and confirmed the results obtained with intrinsically labeled soybeans, in which part of the label was in ferritin in whole soybeans (7). However, when we compared the 2 methods, the results varied with the type of iron. The RBC incorporation method showed that iron from ferritin was incorporated into hemoglobin less than was that from ferrous sulfate, and these results are similar to those obtained in an earlier study in rats (15). Thus, it appears that the metabolic fate of iron absorbed into the body (whole-body counting) may be different for ferritin iron and iron from ferrous sulfate, which potentially suggests different absorptive pathways.

A quantitative comparison of the 2 methods of measuring iron absorption, whole-body retention and RBC incorporation, found that the values for RBCs were considerably higher when ferrous sulfate was given. Thus, the RBC-incorporation method overestimates iron absorption and reflects the differentially higher uptake of absorbed iron by the erythron than by other tissues in the body. In the previous study in humans, which used intrinsically labeled soybeans, only the RBC-incorporation method was used (7). The mean absorption value from that study was lower than that in the present study, which used the same method and subjects with similar iron status. This difference is likely due to the fact that only 48% of the radiolabeled iron was bound to ferritin in the previous study and the remainder was bound to other ligands, eg, phytate, that have an inhibitory effect on iron absorption (21).

The mechanism behind iron absorption from ferritin is not yet known, whereas iron as ferrous sulfate will be absorbed via DMT1 (5). Ferritin is very stable to low pH and resists denaturation by heat (temperatures up to 85 °C), urea, and many proteolytic enzymes (10, 22). Ferritin also appears resistant to in vitro digestion (23). Furthermore, ferritin in seeds is inside plastids that are inside plant cells, which makes the ferritin even more...
stable. These observations suggest that ferritin iron (Fe^{3+} solid mineral) enters the mucosal cell from food intact and that the iron will be absorbed by a pathway other than the DMT1 pathway for Fe^{2+} (5). The hypothesis that, as a required step in iron absorption, ferric iron is reduced to ferrous iron in the gut by enzymes such as duodenal cytochrome b, (24) is unlikely to apply to iron in ferritin, where it is inaccessible to reduction because the gated pores in the ferritin protein (22) are usually closed. Other evidence that mechanisms of iron absorption for the Fe(III) solid mineral in ferritin may differ from those for Fe(II)SO₄ includes observations on whole body counting and RBC incorporation of iron from ferritin at 14 d in rats (15). The potential for multiple nonheme-iron absorption mechanisms is emphasized by the identification of the lactoferrin receptor (6).

The reason or reasons for the differences in results in the 2 forms of iron obtained by the 2 methods used for assessment of iron absorption are not known. It appears that, whereas net iron absorption (whole-body counting) is similar for the 2 forms of iron, relatively less iron was incorporated into RBCs from ferritin. In the previous rat study (15), it was found that relatively more iron from ferritin was incorporated into the liver, presumably as iron stores. Because iron stores, reflected by serum ferritin, are regulated by hepcidin (a low-molecular-weight peptide synthesized by the liver, but secreted into the bloodstream), which signals the iron status to the small intestine (25), hepcidin may respond differently to iron absorbed from ferritin than to that absorbed from ferrous sulfate. In the enterocyte, hepcidin appears to regulate iron absorption (via ferroportin or HFE, or both) primarily on the basolateral side (26), and this indicates that iron entering the enterocytes in 2 different forms and possibly by 2 different mechanisms enters a convergent pathway. Such an idea is supported by the observations that iron exported from Caco-2 cells is independent of the form of the iron at entry (ferritin or ferrous sulfate; 23). The idea is also supported by the results from studies on iron trafficking in microorganisms (4, 27), in which multiple cell surface receptors, matched to different forms of environmental iron, fed the iron into a common intracellular trafficking pathway. Such a processing “system” could easily exist in the enterocyte, with surface receptors matched to the iron donor—eg, ferrous iron or iron in lactoferrin, heme, or food ferritin—and feeding the iron into a common pathway (eg, enterocyte ferritin or ferroportin) through the cell to the basolateral membrane. Whereas a few of the human intestinal iron transporters or chaperones are already known, many more may remain to be discovered, on the basis of analogies to what has been unraveled regarding iron uptake in microorganisms. The similarity of iron utilization from a simple salt and from ferritin in normal subjects, with or without iron deficiency, indicates the potential of using naturally ferritin-rich foods for preventing iron deficiency. Whether there will be nutritional genomic effects related to diseases with inherited defects in iron metabolism, such as sickle cell disease, hemochromatosis or the thalassemias, should be investigated.

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BL and ECT planned the study, PD-H and BL performed the experiments, and all 3 authors wrote the manuscript. None of the authors had a personal or financial conflict of interest.

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