The Journal of Nutrition
Nutrient Physiology, Metabolism, and Nutrient-Nutrient Interactions

Electrolytic Iron or Ferrous Sulfate Increase Body Iron in Women with Moderate to Low Iron Stores[1,2]

James H. Swain,[3] LuAnn K. Johnson, and Janet R. Hunt*

USDA, Agricultural Research Service, Grand Forks Human Nutrition Research Center, Grand Forks, ND 58202

Abstract

Commercial elemental iron powders (electrolytic and reduced iron), as well as heme iron supplements, were tested for efficacy in improving the iron status of women. In a randomized, double-blind trial, 51 women with moderate to low iron stores received daily for 12 wk: *1*) placebo, *2*) 5 mg iron as heme iron or 50 mg iron as *3*) electrolytic iron, *4*) reduced iron, or *5*) $FeSO_4$. Treatments were provided in 2 capsules (heme carrier) and 3 wheat rolls (other iron sources). Differences in iron status, food nonheme iron absorption, and fecal properties were evaluated. Body iron, assessed from the serum transferrin receptor:ferritin ratio, increased significantly more in subjects administered $FeSO_4$ (127 ± 29 mg; mean ± SEM) and electrolytic (115 ± 37 mg), but not the reduced (74 ± 32 mg) or heme (65 ± 26 mg) iron forms, compared with those given placebo (2 ± 19 mg). Based on body iron determinations, retention of the added iron was estimated as 3.0, 2.7, 1.8, and 15.5%, in the 4 iron-treated groups, respectively. Iron treatments did not affect food iron absorption. The 50 mg/d iron treatments increased fecal iron and free radical–generating capacity in vitro, but did not affect fecal water cytotoxicity. In subjects administered $FeSO_4$, fecal water content was increased slightly but significantly more than in the placebo group. In conclusion, electrolytic iron was ~86% as efficacious as $FeSO_4$ for improving body iron, but the power of this study was insufficient to detect any efficacy of the reduced or heme iron within 12 wk. With modification, this methodology of testing higher levels of food fortification for several weeks in healthy women with low iron stores has the potential for economically assessing the efficiency of iron compounds to improve iron status. J. Nutr. 137: 620–627, 2007.

Introduction

Effective iron supplementation and food fortification strategies are needed to combat the global problem of iron deficiency and its associated anemia. Although ferrous sulfate is a well-absorbed form of iron for dietary fortification or supplementation, it is associated with poor compliance in supplemental amounts and with discoloration and a reduced shelf life in fortified grain products (1). Commonly used elemental iron powders are economical and do not adversely affect fortified food. These powders are generally characterized by production method as carbonyl, electrolytic, or reduced iron, and are composed of relatively pure metallic iron (2). Their nutritional bioavailability is influenced by particle size, density, surface area, and shape (3–6), which differ according to production method and supplier. Six elemental iron powders commercially available in 2001 were 21 to 64% as bioavailable as ferrous sulfate, and differed significantly from each other when assessed by the rat hemoglobin repletion method (6). In vitro methods for assessing the iron powders have not been verified with in vivo studies in animals or humans, and therefore cannot be used to assess the bioavailability of elemental iron powders (2). The human absorption of iron from such powders has been difficult to assess because powders isotopically prepared for research do not match the physical characteristics of their commercial counterparts (7–15). Human efficacy studies, testing for improvements in iron status with the elemental iron powders, have been limited, and showed benefits of carbonyl iron in supplemental doses (16–19) and of electrolytic iron (~14–17 mg/d) provided to infants in cereal (20) but yielded mixed results with reduced iron added to bread flour (21).

Supplementation with heme iron is of increasing interest because it is generally better absorbed than nonheme forms of iron, and heme iron absorption is unaffected by phytic acid, which is present in many grains and legumes. Heme iron may result in less gastrointestinal discomfort and oxidative stress than the ionic forms of iron (22–24). In supplemental amounts,

[1] Funded by USDA-CREES grant 2002-35200-12222, and the USDA Agricultural Research Service.
[2] Electrolytic iron, reduced iron, and ferrous sulfate were obtained from commercial suppliers in 2001 by SUSTAIN (Sharing Science and Technology to Aid in the Improvement of Nutrition; Washington, DC). This research was conducted collaboratively with SUSTAIN and its partners as part of its overall review of the bioavailability of elemental iron powders. Heme iron supplements and placebos were provided by Proliant, Ames, IA. Mention of a trademark or proprietary product does not constitute a guarantee of or warranty for the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.
[3] Present address: Department of Nutrition, Case Western Reserve University, Cleveland, OH 44106.
* To whom correspondence should be addressed. E-mail: jhunt@gfhnrc.ars.usda.gov.

Manuscript received 7 September 2006. Initial review completed 28 October 2006. Revision accepted 20 December 2006.

heme iron is also less likely than nonheme iron to competitively interfere with absorption of other trace elements such as zinc and copper, because heme iron is absorbed within an intact porphyrin complex (25). However, the amount that can be used as a supplement in a practical number of capsules is limited by the large molecular size of heme iron.

In this study, the nutritional efficacy of iron from reduced and electrolytic elemental iron powders and supplemental heme iron was determined by measuring the change in iron status in women during a 12-wk iron treatment. This controlled trial provided the opportunity to test secondary outcomes hypothesized to be influenced by increasing iron intake, including the absorption of nonheme iron from a test menu of radiolabeled foods, and the soluble and chelatable iron, free radical-generating capacity, and cytotoxicity of feces (22).

Subjects and Methods

General protocol. Healthy women of child-bearing age with moderate to low iron stores were recruited to participate in this randomized, double-blind, controlled efficacy trial. The women's iron status was assessed and modified as needed by phlebotomy in the first 5 wk to achieve reduced iron stores without iron deficiency. Subjects then began a 12-wk iron treatment period. Treatments were assigned randomly with blocking based on whether or not the subject donated a unit of blood in the first wk (see below). The 5 treatments were: *1*) placebo, *2*) 5 mg iron as heme iron, or 50 mg iron as *3*) electrolytic iron, *4*) reduced iron, or *5*) bakery-grade ferrous sulfate. All subjects were provided with both capsules and wheat rolls; the heme iron (or placebo) was consumed in 2 capsules/d and the other iron sources (or a placebo of no added iron) were consumed in 3 wheat rolls/d. The efficacy of the iron sources was evaluated by measuring the change in blood indices of iron status and other variables at the beginning and end of treatment.

Subjects. The study was approved by the Institutional Review Board and Radioactive Drug Research Committee at the University of North Dakota and by the USDA Human Studies Review and Radiological Safety Committees. All participants gave informed consent. Fifty-one women aged 40 ± 1 y (range: 21–51 y), and a BMI (kg/m^2) of 26.0 ± 0.5 (range: 18.4–35.0) completed the study. The participants were recruited locally through public advertising. They were apparently healthy, menstruating, not pregnant or breast-feeding, and not using medication (except possibly hormonal contraceptives used for ≥6 mo).

Subjects lived at home and consumed self-selected diets, but were instructed not to use dietary supplements, consume fortified cereals, or use highly fortified liquid meals. A daily multivitamin without minerals (One-A-Day Essential; Bayer) was provided. This multivitamin contained 60 mg ascorbic acid, and subjects were instructed to consume it along with the iron capsules (heme or placebo, see below) at a consistent meal time.

Two subjects treated with ferrous sulfate experienced difficulties with irritable bowel syndrome; 1 had failed to reveal this as a preexisting condition, the other was newly diagnosed after 10 wk of treatment. A 3rd subject who was treated with heme iron and who had a history of gestational hemorrhoids experienced blood in her stools. All 3 of these subjects were eliminated from further analyses.

Initial iron status and phlebotomy. A blood sample prior to admission established that subjects had normal hemoglobin and plasma iron binding capacity. Unfortunately, we retrospectively discovered that plans to admit subjects with serum ferritin ≤30 μg/L and to further lower that ferritin to ≤15 μg/L by phlebotomy were not met because of unexpectedly low and variable measurements from a commercial ferritin assay. Subsequent use of a different assay (Immulite ferritin, which was used for all data in this report) established that serum ferritin concentrations were ≤71 μg/L on admission (wk 0) and, through phlebotomy, were reduced to ≤53 μg/L at baseline (wk 5) (see also **Table 1**), while hemoglobin concentrations were maintained at ≥120 g/L.

In the attempt to reduce body iron by phlebotomy during the first 5 wk, 65–820 mL total blood was drawn per subject. The greatest phlebotomy volumes were obtained from 9 of 51 women, who were initially assessed to have the highest iron stores, and who each donated 1 unit (475 mL) of blood during the 1st wk. Although no additional time was allowed for possible stabilization between the 5 wk that included phlebotomy and the 12 wk of experimental treatments, most of the women had minimal phlebotomy (<65 mL/5 wk for 34 of 51 women)

TABLE 1 Women's serum ferritin concentrations at admission, baseline iron status indices, and changes with 12-wk iron treatment[1]

	Placebo	Heme	Reduced	Electrolytic	$FeSO_4$
n	12	9	10	12	8
Admission serum ferritin,[2] *μg/L*	28 (24,32)^{ab}	34 (31,38)^{a}	25 (21,31)^{ab}	26 (21,32)^{ab}	13 (10,17)^{b}
Baseline serum ferritin,[2] *μg/L*	19 (17,22)^{ab}	23 (20,27)^{a}	20 (17,23)^{ab}	20 (17,23)^{ab}	12 (11,15)^{b}
Δ Serum ferritin,[2] *μg/L*	0.5 ± 1.3	4.6 ± 2.3	7.9 ± 3.4	9.9 ± 2.9*	6.4 ± 1.9
Baseline transferrin receptor,[3] *nmol/L*	19.8 ± 1.3^{ab}	18.0 ± 1.5^{a}	19.0 ± 1.5^{ab}	18.7 ± 1.3^{a}	25.2 ± 1.6^{b}
Δ Transferrin receptor, *nmol/L*	−0.02 ± 0.7	−1.6 ± 0.5	−1.3 ± 0.8	−0.4 ± 0.7	−3.2 ± 0.7*
Baseline body iron[3,4] *mg/kg body weight*	2.9 ± 0.6	3.8 ± 0.5	3.1 ± 0.7	3.2 ± 0.6	0.4 ± 1.0
Δ Body iron, *mg/kg*	0.1 ± 0.3	1.0 ± 0.4	1.1 ± 0.4	1.8 ± 0.6	2.1 ± 0.5*
Baseline body iron,[3,4] *mg*	199 ± 49	262 ± 40	226 ± 54	230 ± 53	22 ± 69
Δ Body iron, *mg*	2 ± 19	65 ± 26	74 ± 32	115 ± 37*	127 ± 29*
Baseline total iron binding capacity, *μmol/L*	53 ± 2	53 ± 2	55 ± 2	56 ± 2	56 ± 2
Δ Total iron binding capacity, *μmol/L*	2.9 ± 0.8	0.5 ± 2.2	−0.9 ± 1.3	0.9 ± 1.0	−2.6 ± 0.9*
Baseline RBC zinc protoporphyrin, *μg/L*	36 ± 11	39 ± 13	65 ± 12	43 ± 11	69 ± 14
Δ RBC zinc protoporphyrin, *μg/L*	16 ± 9	6 ± 11	−21 ± 11	−3 ± 10	−23 ± 9*
Baseline nonheme Fe absorption from radiolabeled food, %	12.4 (9.9, 15.5)	8.9 (6.9, 11.6)	9.6 (7.5, 12.3)	13.6 (10.7, 17.2)	12.5 (9.4, 16.4)
Δ nonheme Fe absorption, *percentage points*	−2.9 ± 1.9	−2.5 ± 2.1	−6.5 ± 2.0	−5.4 ± 1.9	−5.9 ± 2.3

[1] Values are means ± SEM or geometric means (−1 SEM, +1 SEM). For admission (wk 0) or baseline (wk 5) data, means in a row with superscripts without a common letter differ, $P < 0.05$ (Tukey-Kramer contrasts). *Change (Δ = wk 17 minus wk 5) differed from that in the placebo group, $P < 0.05$ (Dunnett's contrasts). Neither hemoglobin concentration nor transferrin saturation indices differed between groups at baseline or changed relative to the placebo group after iron treatment (data not shown).

[2] Serum ferritin was measured using the Immulite assay (Diagnostic Products).

[3] Transferrin receptor concentrations were measured using the R & D Systems assay, but were mathematically converted to values associated with the Ramco assay method to report body iron, which is commonly reported in mg (31).

[4] To convert body iron from mg/kg to μmol/kg or from mg to μmol, multiply by 17.91.

and the remaining women had most of the blood drawn early in the 5 wk period. Phlebotomy volumes did not differ among the iron treatment groups (**Table 2**).

Iron treatment. During the iron treatment period (wk 6–17), subjects visited the research center biweekly to receive rolls and supplements and to provide general health and compliance information. All subjects were provided with both capsules and wheat rolls, and placebo versions of each were used as appropriate.

Wheat rolls were fortified with electrolytic iron [A-131, SCM Metal Products (now N.A. Höganäs High Alloys, LLC), 99% iron]; reduced iron (ATOMET 95SP, Quebec Metal Powders, 99% iron); or bakery-grade ferrous sulfate (ferrous sulfate monohydrate USP/FCC, Crown Technology). The elemental iron powders and ferrous sulfate were mixed into unenriched wheat flour (North Dakota Mill), baked into rolls at the University of North Dakota campus bakery, and frozen until use. Subjects consumed 3 rolls/d [16.7 mg iron/roll (0.3 ± 0.03 mg/roll for unfortified rolls)] to obtain a total of 50 mg iron/d. They were instructed to consume the rolls with their meals in substitution for other carbohydrates and to consume missed rolls as soon as they remembered.

The heme iron supplements provided 5 mg iron/d in 2 gelatin capsules, each containing 2.6 mg iron in 190 mg modified hemoglobin (Vitaheme, Proliant). The hemoglobin was commercially prepared from bovine hemoglobin; partial enzyme hydrolysis of the hemoglobin resulted in a heme-iron polypeptide as the final product containing globin derived peptides but no intact globin. Placebo capsules contained maltodextrin. Subjects were instructed to take the 2 capsules with the same meal each day, replacing any missed capsules by allowing ~8 h between doses.

To enhance and monitor compliance, the capsules were provided in numbered blister packs. Forms were provided to subjects for recording their consumption of rolls or capsules and indicating any delays. These tracking forms and capsule packaging (with any missed capsules) were returned and discussed with center staff during the biweekly visits, when subjects also completed questionnaires concerning possible illness or use of medications. Self-reports indicated that ~6% of the wheat rolls and 3% of the capsules were not consumed. Compliance did not differ significantly among the treatment groups.

Dietary nonheme iron absorption. To determine whether the iron treatments changed the efficiency of nonheme iron absorption, nonheme iron absorption from a 1-d menu (3 meals) was measured with a radiotracer during wk 6 and 15. None of the iron treatments were administered on these test days. Weighed foods were extrinsically labeled with 7.4 kBq $^{59}FeCl_3$ in ≤0.06 μg (≤1 nmol) iron (NEN Life Science Products) and distributed in proportion to the nonheme iron content of the meals (constant specific activity). The menu included chicken and shrimp and had a relatively high bioavailable iron profile. It contained 9.4 MJ (2250 kcal) energy, 88 g protein, 235 mg ascorbic acid, 1224 μg retinol equivalents vitamin A [calculated (26)], 380 mg phytic acid [calculated (27)], 609 ± 4 mg calcium, 6.2 ± 0.1 mg zinc, and 11.7 ± 0.2 mg iron (0.2 mg heme iron and 11.5 mg nonheme iron). The absorption of nonheme iron was determined from radioiron retention after 15 d, measured in a whole-body scintillation counter (28) as previously described (29).

Fecal analyses. Subjects collected all feces for 1 wk before (wk 5) and near the end of iron treatment (wk 15). Water soluble and EDTA-chelatable iron concentrations in 1 wk composites were measured as described by Lund et al. (22), with iron determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES).

The influence of iron treatment on in vitro free radical–generating capacity was measured as described previously (22,30). Pertinent to the interpretation of the present results, this method includes protein denaturation by HCl acidification at pH 1.0 for 10 min.

The cytotoxicity of the soluble portion of the feces was evaluated by exposing a human tumor adenocarcinoma cell line (HT-29) to 8 graduated volumes of fecal water, prepared as described by Davis (30). This assay was excluded for the electrolytic iron treatment group because of limited technical resources.

Blood and chemical analyses. Hemoglobin was measured using a CELL-DYN 3500 System (Abbott Diagnostic Division). Serum iron was determined colorimetrically with a commercial chromagen (Ferene, Raichem Division of Hemagen Diagnostics). Iron binding capacity was similarly determined after adding a known amount of ferrous iron under alkaline conditions. Percentage transferrin saturation was calculated from serum iron and total iron binding capacity. Zinc protoporphyrin was measured fluorometrically [ZnP Model 4000 Hematofluorometer, Environmental Sciences Associates]. Transferrin receptor was measured by enzyme-linked immunoabsorbent assay (Quantikin Human Transferrin Receptor Immunoassay, R & D Systems). Body iron was calculated using the serum transferrin receptor:serum ferritin ratio (31). This method, published while the present study was in progress, was developed using assay materials from Ramco. Commercial methods for transferrin receptor have not been standardized, but results from R & D Systems correlate well with those from Ramco ($R^2 = 0.86$) (32) with consistent regression coefficients in 2 publications (32,33) as well as our own unpublished observations. Accordingly, conversion factors (32) were used to adjust the R & D transferrin receptor analyses and allowed comparison with the body iron method of Cook et al. (31). The resulting body iron estimations were consistent with those reported by Cook et al. (31). Because initial serum ferritin measures (Ferritin Enzyme Immunoassay, American Laboratory Products; standardized using WHO Standard 80/602) were much lower than expected, we remeasured all samples using another immunoassay (Immulite ferritin, Diagnostic Products; standardized using WHO Standard 80/578). The Immulite method produced a broader range of values, consistent with other studies, and was also more sensitive to differences between treatment groups. Only values from the Immulite ferritin assay were used in data analyses and reported here. C-reactive protein was measured by nephelometry (Behring Diagnostics) to help detect increases in serum ferritin concentration related to inflammation. A single serum ferritin value was excluded based on the arbitrary criteria that serum C-reactive protein was >6 mg/L and the corresponding ferritin value was 50% more than the preceding measurement. Plasma zinc and copper were measured by ICP-OES with precautions to avoid contamination of supplies and equipment used for phlebotomy and processing. Changes in iron, copper, and zinc status measures were calculated from the mean of 2 subsamples at wk 17 minus the values at wk 5.

TABLE 2 Phlebotomy volumes during the first 5 wk of the study in women in the 5 groups

	Placebo	Heme	Reduced	Electrolytic	$FeSO_4$
Total phlebotomy volume,[1] *mL/5wk*	198 ± 60 (65–540)	148 ± 72 (65–720)	169 ± 70 (65–630)	228 ± 68 (65–820)	124 ± 59 (65–540)
Phlebotomy volumes,[2] *n*					
530–820 mL/5 wk	3	1	2	2	1
155–335 mL/5 wk	2	1	0	5	0
65 mL/5 wk	7	7	8	5	7

[1] Values are means ± SEM and (range). Phlebotomy volumes did not differ (ANOVA).
[2] Number of subjects in 3 categories of phlebotomy volumes, by subsequent treatment group. Subjects in the highest phlebotomy category donated 1 unit (475 mL) of blood during wk 1 of the study.

The iron content of the wheat rolls, heme iron supplements, and fecal samples, as well as mineral content of the test diet, were determined by ICP-OES after digestion with concentrated nitric acid and 70% perchloric acids by method (II)A of the Analytical Methods Committee (34). Iron values for Typical Diet Reference Material (1548a) were 98 ± 7% of those certified by the National Institute of Standards and Technology. Nonheme iron in the chicken and shrimp of the test diet was extracted (35) for analysis, and heme iron was calculated by difference.

Statistical and power analyses. The initial power analysis based on previously observed changes in serum ferritin (36) indicated that 10–12 subjects per treatment would provide 85–95% power to detect a 50% difference ($\alpha = 0.05$) between groups that were provided a test iron (electrolytic, reduced, or heme) and either ferrous sulfate (positive control) or placebo (negative control). This statistical power was reduced because the baseline iron status was greater than planned (resulting from the problems with the ferritin assay) and because dropouts were greater than anticipated. The study was not designed to detect differences between electrolytic, reduced, and heme iron.

The data for nonheme iron absorption, serum ferritin, and fecal total, soluble, and chelatable iron, and free radical-generating capacity were logarithmically transformed to meet the conditions of a normal distribution and geometric means are reported for these variables. When these variables were expressed as a change (wk 17 minus wk 5), the distributions were not skewed or influenced by kurtosis; therefore, transformation was eliminated and arithmetic means were reported. SAS, version 9.1 software (SAS Institute) was used for statistical analysis. Iron treatment effects were determined by ANOVA. Because of the heterogeneity of the variances of the change scores, separate variance estimates were computed for each group using the Satterthwaite method provided in Proc Mixed of SAS (37). When the ANOVA was significant, baseline differences were tested with Tukey-Kramer contrasts between pairs of means, and change scores were tested with Dunnett's contrasts for multiple comparisons to placebo. Differences were considered significant at $P < 0.05$ (two-tailed). Values in text are expressed as means ± SEM.

Results

Blood indices of iron and mineral status. Although treatments were randomly assigned, the pattern of dropouts and eliminations, especially in the ferrous sulfate group, resulted in a lower baseline (wk 5) iron status for this group. Baseline serum ferritin and transferrin receptor concentrations indicated a lower iron status in the ferrous sulfate group compared with some of the other iron treatment groups (Table 1), although the ferrous sulfate group did not differ from the placebo group and the other iron-treated groups did not differ from one another. Body iron, total iron binding capacity, transferrin saturation, RBC zinc protoporphyrin, and hemoglobin were not different among the groups at baseline (Table 1).

Both ferrous sulfate and electrolytic iron improved subjects' iron status. Ferrous sulfate improved iron status significantly compared to the placebo group, based on differences in changes in transferrin receptor and body iron concentrations, total iron binding capacity, and RBC zinc protoporphyrin, as well as a tendency for a greater change in serum ferritin ($P < 0.1$, Table 1). The electrolytic iron also improved iron status significantly in comparison to the placebo group, as indicated by greater increases in serum ferritin and total body iron. Although the increase in serum ferritin with reduced iron was as great as for ferrous sulfate and electrolytic iron, this increase was not different from that in the placebo group, because of the greater variation associated with reduced iron. Thus, reduced and heme iron, the latter tested at one-tenth the dose of the other iron sources, did not differ from the placebo in improving iron status (Table 1). Because statistical power was limited, it may be noteworthy that changes in body iron (expressed either as mg/kg or mg) for all the iron sources, but not the placebo group, were significantly greater than zero.

Body iron, which is based on both transferrin receptor and ferritin measurements, was the only iron status indicator measured that is sensitive across a broad range of iron status, including functional as well as storage iron. This is useful in considering the results observed in the ferrous sulfate group, which began with a lower baseline iron status (Table 1) and, in comparison to the placebo group, improved significantly in functional indicators of iron status such as transferrin receptor concentration, iron binding capacity, and RBC zinc protoporphyrin and tended to have a greater increase in serum ferritin ($P < 0.1$). In comparison with the other iron treatment groups, the lower initial iron status of the ferrous sulfate group likely resulted in a greater distribution of retained iron into functional body iron pools (influencing total iron binding capacity) and less into storage iron pools (influencing serum ferritin). Accordingly, we considered the change in body iron to be the best measure of change in iron status for comparing the iron treatments to the placebo.

The total amounts of iron retained with each iron treatment were estimated by multiplying body iron concentrations by the subjects' body weights to express the body iron results as total mg iron/person (Table 1, **Fig. 1**). Although only the ferrous sulfate and electrolytic iron treatments differed significantly from the placebo treatment, the change in iron was significantly different from zero for all 4 iron treatments. Dividing these iron retention estimates by the amounts of supplemental iron administered (5 mg/d as heme and 50 mg/d for the other iron sources) for 12 wk resulted in estimates of absorptive efficiency of 15.5, 1.8, 2.7 and 3.0% for iron from heme, reduced, electrolytic and ferrous sulfate sources, respectively.

An older estimate of body iron based on an algorithm using multiple measures (hemoglobin, serum ferritin, transferrin saturation, and for which we substituted Zn protoporphyrin for erythrocyte protoporphyrin) (38) correlated well with the newer method (31) ($R^2 = 0.78$, $P < 0.001$) but suggested that the increase in body iron was greatest in subjects given electrolytic iron rather than ferrous sulfate (emphasizing the change in ferritin). The increases in body iron stores with placebo, heme, reduced iron, electrolytic iron, and ferrous sulfate treatments, respectively, were 2, 65, 74, 115, and 127 mg/12 wk with the

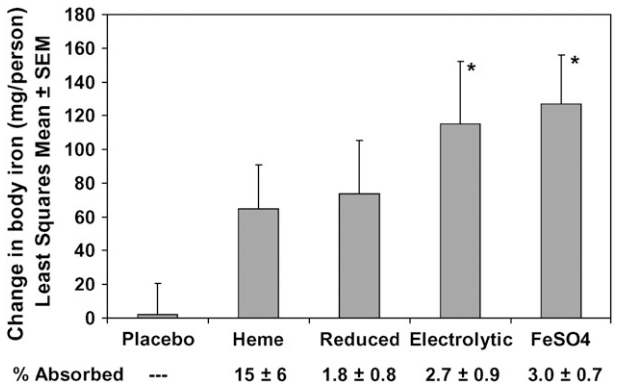

Figure 1 Changes in women's body iron due to treatment with 50 mg/d iron as reduced iron, electrolytic iron, or ferrous sulfate, or 5 mg/d as heme iron. The absorption of each form of iron was estimated from the change in body iron expressed as a percentage of the total additional iron ingested in 12 wk. Values are means ± SEM, $n = 8$–12. *Different from placebo, $P < 0.05$ (Dunnett's contrasts). To convert body iron from mg to μmol, multiply by 17.91.

newer method (31) and 17, 70, 92, 179, and 143 mg/12 wk with the older method (38).

We hypothesized that plasma zinc and copper would be negatively affected by iron administered in these amounts, possibly because of competitive transport mechanisms. However, plasma zinc and copper did not differ among groups at baseline, and were unaffected by iron treatment (data not shown).

Efficiency of dietary nonheme iron absorption. Dietary treatment did not affect the subjects' efficiency of nonheme iron absorption as measured with a radiolabeled test diet (Table 1). However, the change in nonheme iron absorption from food was significantly greater than zero for all groups, suggesting a general improvement in iron status, even in the placebo group, by this criteria. Although the ferrous sulfate group had a lower baseline iron status than the other treatment groups (Table 1), this group did not appear to absorb dietary iron more efficiently.

Fecal measurements. None of the fecal variables differed among groups at baseline (**Table 3**). Whereas subjects did not noticeably complain of loose stools, the ferrous sulfate treatment, but none of the others, decreased dry matter in feces between wk 5 and wk 15 by ~6 percentage points more ($P < 0.05$) than in the placebo group (Table 3).

Ferrous sulfate, electrolytic iron, and reduced iron, administered at 50 mg iron/d each, but not heme iron provided at a lower dose, increased the content of iron in feces significantly more than did the placebo (Table 3). Approximately 1–2% of the fecal iron was soluble and an additional 1–2% was chelatable. The changes in soluble iron were highly variable and did not differ significantly from the placebo for any of the iron treatments. Fecal chelatable iron increased significantly more than placebo with reduced and electrolytic iron, but a similar degree of increase with ferrous sulfate was not significantly different from placebo because of high variation in the data for that group (Table 3). In contrast to placebo, ferrous sulfate, reduced iron, and electrolytic iron, but not heme iron, significantly increased the free radical–generating capacity of the feces in vitro.

The evaluation of the cytotoxicity of fecal water yielded dose-response patterns that were remarkably reproducible within subjects but these patterns varied considerably between subjects and did not enable the consistent modeling of a cytotoxicity threshold. An ANOVA, including all tested volumes of fecal water, indicated significant toxicity with increasing volume but no interaction between the iron treatment and the week of fecal collection (5 wk vs. 15 wk). Thus, iron treatment did not affect fecal water cytotoxicity (data not shown).

Discussion

This study was designed to investigate the efficacy of using elemental and heme forms of iron as fortificants and supplements, along with positive and negative controls (bakery-grade ferrous sulfate and placebo, respectively), rather than to detect differences between the heme, reduced, and electrolytic iron sources. Although the changes in body iron were significantly different from the placebo group only in subjects administered ferrous sulfate and electrolytic iron, the changes were positive compared with placebo for all the iron treatments (Table 1). Iron status responds gradually to treatment, and to test the efficacy of these products in a limited time period (12 wk), a supplemental dose (50 mg/d in 3 portions) of the nonheme iron treatments was administered. The estimated efficiency of absorption of 1.8, 2.7, and 3.0% for reduced, electrolytic, and ferrous sulfate, respectively, (Fig. 1) could be ~1.5 to 2 times these values when using the lower doses associated with the fortification of staple cereal grains (39,40).

Expressing the change in body iron relative to the results for ferrous sulfate, the reduced and electrolytic iron were ~52 and 86% as bioavailable as ferrous sulfate. The latter figure compares well with the 77% relative bioavailability observed with the same electrolytic iron powder (assembled by SUSTAIN for bioavailability testing) recently tested in a randomized, double-blind, controlled, 35-wk trial with Thai women (41). The greater length and number of subjects in the Thai trial provided sufficient statistical power to show that the electrolytic iron differed significantly from ferrous sulfate as well as from the placebo, based on the change in body iron. However, the similarity of relative bioavailability results between the Thai study and the present study suggest that the present model of using relatively high iron doses for a short time (50 mg/d for 12 wk) may be useful for such evaluations if the statistical power is increased by including more subjects. The present relative

TABLE 3 Women's baseline data and changes with iron treatment in fecal total, soluble, and chelatable iron fractions and free radical–generating capacity values[1]

Fecal measurements, *wet wt*	Placebo	Heme	Reduced	Electrolytic	$FeSO_4$
n	11–12	8	10	10–11	8
Baseline fecal dry matter, %	29 ± 2	28 ± 2	27 ± 2	27 ± 2	28 ± 2
Δ Fecal dry matter, %	1 ± 1	0.2 ± 2	1 ± 1	−1 ± 1	−5 ± 1*
Baseline total fecal iron, *μmol/g*	2.2 (1.9, 2.5)	2.1 (1.8, 2.4)	1.7 (1.5, 2.0)	1.8 (1.6, 2.1)	2.1 (1.9, 2.5)
Δ Total fecal iron, *μmol/g*	0.44 ± 0.26	0.82 ± 0.22	6.39 ± 0.70*	4.93 ± 0.85*	4.13 ± 1.15*
Baseline soluble fecal iron, *μmol/kg*	48 (42, 54)	58 (50, 67)	45 (40,51)	68 (60, 77)	49 (42, 56)
Δ Soluble fecal iron, *μmol/kg*	7.9 ± 7.1	13.0 ± 10.1	51.4 ± 15.4	107.4 ± 43.0	46.9 ± 19.9
Baseline chelatable fecal iron, *μmol/kg*	26 (21,32)	22 (17,29)	14 (11,18)	23 (19,30)	30 (23,39)
Δ Chelatable fecal iron, *μmol/kg*	9.9 ± 7.4	42.8 ± 27.7	136.4 ± 36.6*	100.7 ± 26.8*	120.3 ± 44.5
Baseline free radical–generating capacity, *mmol methanesulfinic acid/g feces wet wt*	1.5 (1.3, 1.7)	1.1 (0.9, 1.3)	0.9 (0.8, 1.1)	1.2 (1.0, 1.4)	1.0 (0.9, 1.2)
Δ Free radical–generating capacity, *mmol methanesulfinic acid/g feces wet wt*	−0.44 ± 0.14	−0.08 ± 0.31	1.25 ± 0.35*	1.28 ± 0.44*	0.85 ± 0.33*

[1] Values are means ± SEM or geometric means (−1 SEM, +1 SEM). *Change (Δ = wk 15 minus wk 5), differed from the placebo group, $P < 0.05$ (Dunnett's contrasts).

bioavailability results of 52 and 86%, for reduced and electrolytic iron, are somewhat greater than the 24 and 54%, respectively, observed when the same iron powders (also from SUSTAIN) were tested with a rat hemoglobin repletion assay, in which the 2 products differed significantly from ferrous sulfate and from each other (6).

The electrolytic iron tested in the present study is the same type that significantly reduced the incidence of iron deficiency anemia when used to fortify the cereal of Chilean infants (20), but it did not reduce the incidence of anemia when used to fortify the flour of children and nonpregnant women in Sri Lanka (42). The latter negative results may have occurred if the subjects' anemia was not primarily caused by iron deficiency. Consumed in a meal of wheat farina and milk, a similar, but not identical, isotopically labeled electrolytic iron powder was 3.4% absorbed by adult subjects, making it ~75% as bioavailable as ferrous sulfate tested in that same study (8). These data are consistent with the improvement in body iron and ~86% relative bioavailability in the present study.

Although it has been widely used in North America, the reduced iron powder in this study has not been previously tested for absorption or nutritional efficacy in humans. This reduced iron powder, which has also been referred to as "atomized" iron, is produced differently than the hydrogen-reduced or carbon monoxide-reduced iron (2). Compared with ~52% relative bioavailability in the present experiment, by rat hemoglobin repletion assay, the relative bioavailability of this reduced "atomized" iron was 24%, which was similar to that of a carbon monoxide-reduced iron powder (21%) but significantly less than that of a hydrogen-reduced iron powder (42%) (6). The hydrogen-reduced iron (from SUSTAIN) with 42% relative bioavailability in rats (6) also had a bioavailability of 42% in the recent Thai trial, which was significantly less than that of ferrous sulfate or electrolytic iron and greater than placebo, based on body iron, but not significantly better than placebo in reducing the incidence of anemia or iron deficiency (41). The reduced iron tested in the present study is not directly comparable to an earlier reduced iron product that, administered at ~80 mg iron/d in bread for 6 mo, was indistinguishable from a placebo, and less effective than ferrous gluconate for improving the hemoglobin of anemic women (21). The present test of reduced "atomized" iron, although suggesting a relative bioavailability as much as 52% that of ferrous sulfate, also did not significantly distinguish the product from the placebo.

Based on the change in body iron, the absorption of heme iron by subjects receiving 5 mg iron/d in the heme form was ~15% (Fig. 1). This is consistent with a high absorption (20–40%) of heme iron (36,43–45) reduced by about half because of the relatively large 5-mg iron dose administered (46). The heme-iron polypeptide product in the present study contained globin-derived peptides, but no intact globin. Purified heme is poorly absorbed because of polymerization at low gastric pH; peptides produced by digestion of globin prevent heme polymerization and enhance absorption of the intact metalloporphyrin molecule (47–49). Although the present results suggest that supplementation with 5 mg iron in this heme form may increase body iron about half as effectively as 50 mg iron from ferrous sulfate, this was not sufficient to distinguish it from the placebo.

We hypothesized that nonheme iron absorption from the diet would be reduced by iron treatment, consistent with the reduction of nonheme iron absorption from food associated with iron supplementation (36) or high dietary iron bioavailability (50,51). However, the reduction in nonheme iron absorption during the treatment period (Table 1) was not affected by the dietary treatment, and occurred, perhaps to a lesser degree, in the placebo group as well. Because nonheme iron absorption was unaffected by placebo in a previous study (36), the change observed in the present study seemed at least partially attributable to recovery from the iron depletion by phlebotomy experienced by all treatment groups. The similar absorption of nonheme iron by the ferrous sulfate group, in comparison with the other treatment groups (Table 1), suggests that the greater increase in body iron with ferrous sulfate treatment (Table 1, Fig. 1) was not attributable to a greater efficiency of iron absorption in this group, despite their slightly lower baseline iron status.

The increased fecal free radical–generating capacity in vitro for the ferrous sulfate group confirms the results of Lund et al. (22) who observed a similar increase, as well as increases in soluble and chelatable fecal iron, when subjects were supplemented with 100 mg ferrous sulfate daily. However, there was no clear indication that the unabsorbed elemental iron powders, which were expected to remain partially particulate, were any less soluble, chelatable, or reactive in the feces, compared with iron from ferrous sulfate. The use of acid to precipitate protein in the free radical assay may have made the elemental iron powders more soluble and reactive in vitro than in vivo. However, fractions of soluble or chelatable iron from fecal samples not treated with acid were similarly high from subjects treated with reduced iron, electrolytic iron, or ferrous sulfate (although chelatable iron was more variable and not significantly different from placebo in the ferrous sulfate group) (Table 3). The unabsorbed elemental iron powders may have already become fully soluble in the colon. Thus, these fecal measurements are consistent with previously observed in vitro effects of ferrous sulfate administered at a higher dose, but do not suggest less fecal reactivity of the iron from the elemental powders compared with ferrous sulfate. Furthermore, the fecal iron results do not necessarily indicate increased free radical reactions in vivo with any of the iron treatments.

This experiment tested new methodology for assessing the efficacy of iron compounds in improving iron status. The experiment was underpowered because of the pattern of subject dropouts and because faulty ferritin analyses were not detected before admitting subjects with higher serum ferritin than planned. However, as discussed above, the estimates of absorption efficiency and relative bioavailability of the compounds tested were consistent with results using other methods. For future studies of this kind, we would not use phlebotomy to reduce body iron stores but would either screen additional applicants to admit only those with low body iron stores, or increase the number of subjects to allow for greater variation in response by women with varying iron stores. The results suggest that this methodology, testing healthy women for changes in body iron in response to iron compounds provided in supplemental doses for several weeks (eliminating preparatory phlebotomy, radiotracers, and stool collections), may be a useful way to evaluate iron fortificants.

In conclusion, both ferrous sulfate and electrolytic iron improved body iron, but the power of this study was not sufficient to detect the efficacy of reduced iron administered at 50 mg/d, or of heme iron at 5 mg/d for 12 wk. The results suggest that electrolytic iron, advantageous as a food fortificant because of relatively low cost and high food stability, is an effective form of iron for food fortification.

Acknowledgments

We gratefully acknowledge the contributions of members of our human studies research team: Emily Nielsen managed volunteer

recruitment and scheduling, Angela Scheet met with volunteers and assessed food diaries, and Sandy Gallagher supervised clinical laboratory analyses. Dr. Cindy Davis helped plan and Laura Idso helped implement fecal measurements, including free radical–producing capacity and fecal cytotoxicity. We thank Lynette Perkinson for supervising preparation of the iron-fortified rolls.

Literature Cited

1. SUSTAIN. Sharing United States Technology to Aid in the Improvement of Nutrition. Guidelines for Iron Fortification of Cereal Food Staples. 2001 [cited 2005 Dec 6]; Available from: http://www.micronutrient.org/resources/publications/fe_guide.pdf#search='Guidelines%20for%20Iron%20Fortification%20of%20Cereal%20Food%20Staples%20SUSTAIN'
2. Hurrell R, Bothwell T, Cook JD, Dary O, Davidsson L, Fairweather-Tait S, Hallberg L, Lynch S, Rosado J, et al. The usefulness of elemental iron for cereal flour fortification: a SUSTAIN task force report. Sharing United States Technology to Aid in the Improvement of Nutrition. Nutr Rev. 2002;60:391–406.
3. Fritz JC. Bioavailability of mineral nutrients. Chemtech. 1976;6:643–8.
4. Shah BG, Giroux A, Belonje B. Specifications for reduced iron as a food additive. J Agric Food Chem. 1977;25:592–4.
5. Motzok I, Verma RS, Chen VS, Rasper J, Hancock RGV, Ross HU. Bioavailability, in vitro solubility, and physical and chemical properties of elemental iron powders. J Assoc Off Anal Chem. 1978;61:887–93.
6. Swain JH, Newman SM, Hunt JR. Bioavailability of elemental iron powders to rats is less than bakery-grade ferrous sulfate and predicted by iron solubility and particle surface area. J Nutr. 2003;133:3546–52.
7. Cook JD, Minnich V, Moore CV, Rasmussen A, Bradley WB, Finch CA. Absorption of fortification iron in bread. Am J Clin Nutr. 1973;26:861–72.
8. Forbes AL, Adams CE, Arnaud MJ, Chichester CO, Cook JD, Harrison BN, Hurrell RF, Kahn SG, Morris ER, et al. Comparison of in vitro, animal and clinical determinations of iron bioavailability: international nutritional anemia consultative group task force report on iron bioavailability, bioavailability of iron used in fortification and enrichment, electrolytic iron, ferric orthophosphate, ferrous sulfate. Am J Clin Nutr. 1989;49:225–38.
9. Hallberg L, Brune M, Rossander L. Low bioavailability of carbonyl iron in man: studies on iron fortification of wheat flour. Am J Clin Nutr. 1986;43:59–67.
10. Hoglund S, Reizenstein P. Studies on iron absorption. V. Effect of gastrointestinal factors on iron absorption. Blood. 1969;34:486–504.
11. Björn-Rasmussen E, Hallberg L, Rossander L. Absorption of 'fortification' iron. Bioavailability in man of different samples of reduced Fe, and prediction of the effects of Fe fortification. Br J Nutr. 1977;37:375–88.
12. Roe MA, Fairweather-Tait SJ. High bioavailability of reduced iron added to UK flour. Lancet. 1999;353:1938–9.
13. Fairweather-Tait SJ, Wortley GM, Teucher B, Dainty J. Iron absorption from a breakfast cereal: effects of EDTA compounds and ascorbic acid. Int J Vitam Nutr Res. 2000;71:117–22.
14. Walter T, Pizarro F, Abrams SA, Boy E. Bioavailability of elemental iron powder in white wheat bread. Eur J Clin Nutr. 2004;58:555–8.
15. Walter T, Pizarro F, Boy E, Abrams SA. The poor bioavailability of elemental iron in corn masa flour is not affected by disodium EDTA. J Nutr. 2004;134:380–3.
16. Gordeuk VR, Brittenham GM, Hughes MA, Keating LJ, Opplt JJ. High dose carbonyl iron for iron deficiency anemia: a randomized, double-blind trial. Am J Clin Nutr. 1987;46:1029–34.
17. Gordeuk VR, Brittenham GM, Hughes MA, Keating LJ. Carbonyl iron for short-term supplementation in female blood donors. Transfusion. 1987;27:80–5.
18. Gordeuk VR, Brittenham GM, Bravo J, Hughes MA, Keating LJ. Prevention of iron deficiency with carbonyl iron in female blood donors. Transfusion. 1990;30:239–45.
19. Devasthali SD, Gordeuk VR, Brittenham GM. Bravo-J-R, A. HM, Keating LJ. Bioavailability of carbonyl iron: a randomized, double-blind study. Eur J Haematol. 1991;46:272–8.
20. Walter T, Dallman PR, Pizarro F, Velozo L, Pena G, Bartholmey SJ, Hertrampf E, Olivares M, Letelier A, Arredondo M. Effectiveness of iron-fortified infant cereal in prevention of iron deficiency anemia. Pediatrics 1993;91:976–82.
21. Elwood PC. A clinical trial of iron-fortified bread. BMJ. 1963;4:224–7.
22. Lund EK, Wharf SG, Fairweather-Tait SJ, Johnson IT. Oral ferrous sulfate supplements increase the free radical-generating capacity of feces from healthy volunteers. Am J Clin Nutr. 1999;69:250–5.
23. Frykman E, Bystrom M, Jansson U, Edberg A, Hansen T. Side effects of iron supplements in blood donors: superior tolerance of heme iron. J Lab Clin Med. 1994;123:561–4.
24. Hallberg L, Hogdahl AM, Nilsson L, Rybo G. Menstrual blood loss and iron deficiency. Acta Med Scand. 1966;180:639–50.
25. Wessling-Resnick M. Iron transport. Annu Rev Nutr. 2000;20:129–51.
26. USDA HNIS. Nutrient database for standard reference, release 14. Springfield (VA): National Technical Information Service; 2001.
27. Harland BF, Oberleas D. Phytate in foods. World Rev Nutr Diet. 1987;52:235–59.
28. Lykken GI, Ong HS, Alkhatib HA, Harris TR, Momcilovc B, Penland JG. Perquisite spin-off from twenty-two years of measuring background in the whole body counter steel room. Ann N Y Acad Sci. 2000;904:267–70.
29. Hunt JR, Roughead ZK. Nonheme iron absorption, fecal ferritin excretion, and blood indexes of iron status in women consuming controlled lactoovovegetarian diets for 8 wk. Am J Clin Nutr. 1999;69:944–52.
30. Davis CD. Low dietary copper increases fecal free radical production, fecal water alkaline phosphatase activity and cytotoxicity in healthy men. J Nutr. 2003;133:522–7.
31. Cook JD, Flowers CH, Skikne BS. The quantitative assessment of body iron. Blood. 2003;101:3359–63.
32. Wians FH, Jr., Urban JE, Kroft SH, Keffer JH. Soluble transferrin receptor (sTfR) concentration quantified using two sTfR kits: analytical and clinical performance characteristics. Clin Chim Acta. 2001;303:75–81.
33. Akesson A, Bjellerup P, Vahter M. Evaluation of kits for measurement of the soluble transferrin receptor. Scand J Clin Lab Invest. 1999;59:77–81.
34. Analytical Methods Committee. Methods of destruction of organic matter. Analyst. 1960;85:643–56.
35. Rhee KS, Ziprin YA. Modification of the Schricker nonheme iron method to minimize pigment effects for red meats. J Food Sci. 1987;52:1174–6.
36. Roughead ZK, Hunt JR. Adaptation in iron absorption: iron supplementation reduces nonheme- iron but not heme-iron absorption from food. Am J Clin Nutr. 2000;72:982–9.
37. SAS/STAT user's guide, version 9.1. Cary, NC: SAS Institute; 1999.
38. Cook JD, Skikne BS, Lynch SR, Reusser ME. Estimates of iron sufficiency in the US population. Blood. 1986;68:726–31.
39. Werner E, Roth P, Kaltwasser JP. Relationship between the dose administered and the intestinal absorption of iron from ferrous sulphate in humans. In: Saltman P, Hegenauer J, editors. The biochemistry and physiology of iron. The Netherlands: Elsevier North Holland; 1982. p. 821–3.
40. Pizarro F, Olivares M, Hertrampf E, Mazariegos DI, Arredondo M, Letelier A, Gidi V. Iron bis-glycine chelate competes for the nonheme-iron absorption pathway. Am J Clin Nutr. 2002;76:577–81.
41. Zimmermann MB, Winichagoon P, Gowachirapant S, Hess SY, Harrington M, Chavasit V, Lynch SR, Hurrell RF. Comparison of the efficacy of wheat-based snacks fortified with ferrous sulfate, electrolytic iron, or hydrogen-reduced elemental iron: randomized, double-blind, controlled trial in Thai women. Am J Clin Nutr. 2005;82:1276–82.
42. Nestel P, Nalubola R, Sivakaneshand R, Wickramasinghe AR, Atukorala S, Wickramanayake T. The use of iron-fortified wheat flour to reduce anemia among the estate population in Sri Lanka. Int J Vitam Nutr Res. 2004;74:35–51.
43. Taylor P, Martinez-Torres C, Leets I, Ramirez J, Garcia-Casal MN, Layrisse M. Relationships among iron absorption, percent saturation of plasma transferrin and serum ferritin concentration in humans. J Nutr. 1988;118:1110–5.
44. Lynch SR, Skikne BS, Cook JD. Food iron absorption in idiopathic hemochromatosis. Blood. 1989;74:2187–93.

45. Hallberg L, Hulten L, Gramatkovski E. Iron absorption from the whole diet in men: how effective is the regulation of iron absorption? Am J Clin Nutr. 1997;66:347–56.
46. Pizarro F, Olivares M, Hertrampf E, Mazariegos DI, Arredondo M. Heme-iron absorption is saturable by heme-iron dose in women. J Nutr. 2003;133:2214–7.
47. Conrad ME, Cortell S, Williams HL, Foy AL. Polymerization and intraluminal factors in the absorption of hemoglobin-iron. J Lab Clin Med. 1966;68:659–68.
48. Vaghefi N, Nedjaoum F, Guillochon D, Bureau F, Arhan P, Bougle D. Influence of the extent of hemoglobin hydrolysis on the digestive absorption of heme iron. An in vitro study. J Agric Food Chem. 2002;50:4969–73.
49. Turnbull A, Cleton F, Finch CA. Iron absorption. IV. The absorption of hemoglobin iron. J Clin Invest. 1962;41:1897–907.
50. Hunt JR, Roughead ZK. Adaptation of iron absorption in men consuming diets with high or low iron bioavailability. Am J Clin Nutr. 2000;71:94–102.
51. Hunt JR. High-, but not low-bioavailable diets enable substantial control of women's iron absorption in relation to body iron stores, with minimal adaptation within several weeks. Am J Clin Nutr. 2003;78: 1168–77.