Iron Absorption from Iron-Enriched *Aspergillus oryzae* Is Similar to Ferrous Sulfate in Healthy Female Subjects

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Abstract

Background: Iron deficiency anemia (IDA) remains a global health issue, affecting mainly children and adolescent and pregnant women. Because of problems associated with current iron compounds used in both supplementation and fortification areas, there is an emerging interest in new natural iron sources to combat IDA.

Objective: The objective of this study was to compare the iron absorption of iron-enriched *Aspergillus oryzae* [Aspiron (ASP)] with $FeSO_4$ in humans.

Methods: Iron absorption was assessed using stable isotope and serum iron response methods after oral intake of iron by healthy women in 2 separate studies. In the first study, ASP was intrinsically labelled with ^{58}Fe into a dry form containing 8% iron. Subjects ($n = 16$, 18–35 y) were randomly assigned to consume liquid semipurified meals labelled with 2 stable iron isotopes, $^{57}FeSO_4$ (10 mg) and ASP containing 2 mg ^{58}Fe and 8 mg natural abundance iron, in 2 visits. Isotope enrichment was measured 2 wk after the last meal was eaten. In the second study, 17 subjects were randomly assigned to consume a test meal with 3 iron supplements during 3 separate visits: $FeSO_4$, 10 mg Fe, and ASP in 2 iron doses, 10 mg and 20 mg. Changes in serum iron were measured at regular intervals for 4 h after supplementation.

Results: The first study showed that the difference in iron absorption from $FeSO_4$ and ASP was not significant (17.18% ± 14.2% compared to 15.14% ± 12.3%; $P = 0.07$). The results of the second study suggested that the iron from ASP was released slowly compared to $FeSO_4$ and the area under the curve did not reflect the absorption of ASP iron, but rather the rate of iron release.

Conclusions: Iron-enriched *A. oryzae* has high relative bioavailability and may cause lower iron surges into the blood compared to $FeSO_4$.   *Curr Dev Nutr* 2018;2:nzy004.

Introduction

Iron plays a crucial role in many physiologic functions, such as oxygen transport, ATP synthesis, and as a cofactor for many enzymes (1). Iron deficiency anemia (IDA) is the most common nutritional problem in the world, especially among women and children in developing countries. Negative consequences of IDA include poor pregnancy outcome, impaired cognitive and physical development of children, and decreased performance and work productivity in adults (2–4).

Food fortification and supplementation are the most common strategies for combating IDA. Although food fortification is a sustainable and cost-effective approach, adding iron to foods is problematic. The soluble iron forms that are highly bioavailable can interact with food and cause unacceptable organoleptic changes. Insoluble iron compounds react less with the food matrix but their bioavailability is very low compared to soluble iron salts (5–7). Ferrous sulfate is absorbed very efficiently and its absorption is commonly used to calculate the relative bioavailability value (RBV) of other iron compounds. However, addition of ferrous sulfate can cause rancidity, unacceptable color, and flavor changes in the food (6). Also, incompletely absorbed iron from iron sulfate has been shown to alter the gut microflora and induce oxidative stress in the colon (8).

The bioavailability of elemental iron powders is low and varies based on the particle size, solubility, surface area, and manufacturing practices but has less reactivity with food (9). Ferric pyrophosphate is another nonsoluble iron salt that is used to fortify food but with low bioavailability. Its bioavailability was shown to be improved with reducing particle size even to nanoparticle dimensions (10). Synthetic organic-ligand ferrihydrite nanoparticles have also been developed as potential alternatives to high doses of ferrous sulfate (11). However, nanoparticle use is of concern because of potential local and systemic toxicities in the heart, liver, and lungs (12, 13). Thus, many efforts have been made to increase the iron bioavailability for inorganic iron, but significant problems with fortification remain.

The negative effect of iron supplementation is not only associated with gastric distress (14) but is also a major concern in populations where malaria infection is common (7, 15), because it increases the severity of the infection. Iron supplementation may also induce oxidative stress caused by a rate of iron influx into plasma that exceeds the plasma transferrin binding capacity, likely forming nontransferrin-bound iron (NTBI) as a result (15). It has been reported in humans that high doses of oral iron supplements can induce NTBI even when transferrin saturation is far from being reached, indicating the importance of rate of influx (16, 17).

Aspergillus oryzae, also known as koji culture, is an FDA (USA) Generally Recognized as Safe (GRAS) fungus. It is widely used to produce food-grade enzymes, rice vinegars, miso, and soy sauce and is consumed by a wide range of the population (18, 19). Most fungi have the ability to acquire small amounts of iron for their own survival, sequester iron mostly in vacuoles (20), and later mobilize it for cellular usage (21). However, *A. oryzae* can take up significantly higher amounts of iron than other fungi, providing a superior alternative to yeast for food fortification (22). Iron in yeast was shown to be better absorbed (23) but its low iron content limits its use. Although *A. oryzae*'s use in livestock and poultry has been reported (24, 25) to date, no studies, to our knowledge, have examined this organism for iron food fortification or supplementation.

The objective of this study was to determine the iron absorption from iron-enriched *A. oryzae* (Aspiron, referred to as ASP in the manuscript) in humans using a well-established stable isotope methodology (26, 27) and to compare the rate of appearance of orally ingested iron in serum between ASP iron and $FeSO_4$ using serum iron response curves (28, 29). Our hypothesis was that ASP iron would be as bioavailable as $FeSO_4$ and the absorption of the 2 iron sources would show a similar inverse relation with serum ferritin and hepcidin levels.

Methods

Both study protocols, described as follows, were approved by the Institutional Review Board at Iowa State University (ISU). Signed informed consent was obtained from subjects for both the studies.

Study 1: stable isotope absorption
Subjects. Healthy women were recruited from the student and staff population using a mass e-mail system at ISU. A total of 44 subjects responded to the e-mails of which only 24 participated in the initial screening. To be eligible for the study, subjects had to be women; have normal BMI [(kg/m^2) 18.5–24.9]; and have serum ferritin

concentrations of <40 µg/L. In addition, all subjects were nonsmokers, denied taking any drugs that affected iron absorption, and had no history of any gastrointestinal disorders. None of the women were pregnant or lactating. Subjects had to discontinue the use of any vitamin or mineral supplement and were not allowed to donate blood ≥2 wk before and during the study period. A sample size of 16 was needed to detect a 30% difference in fractional iron absorption between the 2 iron sources as statistically significant with a power of 80% and at an α level of 0.05 assuming a within-subject SD of 0.2 after log transformation (30). Of the 24 individuals screened, 16 subjects were eligible and recruited into the study.

Study design. This controlled randomized study was single-blinded with a cross-over design. On the morning of the study, subjects came to the Nutrition and Wellness Research Center (NWRC) at ISU after a 10-h overnight fast. On the first day of the study, women's height and weight were measured and an initial, baseline-blood sample was collected for measuring hemoglobin concentration. Ferritin, C-reactive protein (CRP), and hepcidin concentrations were analyzed in the serum. After the initial blood draw, subjects were randomly assigned (using the RAND function in Microsoft Excel, by a research scientist who was not involved with the intervention) to receive either $^{57}FeSO_4$ (10 mg elemental Fe) or $^{58}FeASP$ (10 mg elemental Fe: 2 mg of ^{58}Fe tracer and 8 mg natural abundance iron) with a liquid meal containing semi-purified components. This meal was used in previous studies (31) and consisted of 17.6 g egg albumin (Oskaloosa Food Products Corp, Oskaloosa, IA), 45 g corn syrup solids (Maltrin M250, Grain Processing Corporation, Muscatine, IA), 17.5 g corn oil (Mazola, ACH Food Companies, Inc., Cordova, TN), 6 mL vanilla extract (Tone's, ACH Food Companies, Inc., Cordova, TN), and 100 mL distilled water. The $FeSO_4$ (natural abundance of iron) was purchased from Fisher Scientific (Pittsburgh, PA). For the $^{57}FeSO_4$ treatment, the iron source was added to the meal directly since the preparation of the stable isotope yielded a liquid product and it is a normal practice of labelling meals with soluble iron salts (31). For the $^{58}FeASP$ treatment, capsules containing $^{58}FeASP$ were taken in the middle of consuming the meal and it should be noted that both iron sources were taken with the meal, which was consumed in a short period of time. Since the ASP iron is not water soluble, to avoid losses in the container, we chose to give it in the form of capsules. ASP in capsules was shown to be released very quickly under in vitro conditions (data not shown). To be consistent, subjects consumed empty capsules in the middle of the meal when ferrous sulfate was taken. They were not allowed to eat or drink any food or beverage, except water, for 3 h after consuming the test meal. This study was designed to assess iron absorption from supplemental iron taken with food. After consuming the meals and capsules, each participant was required to rinse the cup twice with water and drink the water. The 2 iron-isotope treatments were fed in a cross-over design on 2 consecutive days after overnight fasting. Two weeks after the second iron tracer was administered, the participants returned after an overnight fast for a final blood draw. These samples were shipped on ice to Cornell University for analysis of the stable isotope iron enrichment.

Blood sample analysis. Serum was collected and kept frozen at –20°C for later analysis. A blood chemistry profile on the whole blood was performed by a certified clinical laboratory (Quest Diagnostics, Lenexa,

KS). Aprotinin was added to blood samples prior to hepcidin analysis according to the manufacturer's instructions. Commercially available kits were used to measure serum hepcidin (Hepcidin-25 EIA, Peninsula Laboratories International Inc., San Carlos, CA), serum ferritin (S-22 ferritin kit, Ramco Laboratories, Inc., Stafford, TX), and serum CRP (CRP ELISA, American Laboratory Products Company, Salem, NH).

Isotope preparation and sample analysis. Two Fe stable isotopes (^{57}Fe at 95.93% enrichment and ^{58}Fe at 93.23% enrichment) were purchased as metal from Trace Sciences International (Richmond Hill, Ontario, Canada). Both ^{57}Fe and ^{58}Fe isotopes were converted into $FeSO_4$ solution as previously described (27). Briefly, the iron isotopes were prepared by dissolving the metals in ultrapure nitric acid and sulfuric acid, then dried and reconstituted with sulfuric acid and water to get the desired concentration. To prepare intrinsically labelled ASP, *A. oryzae* was grown, under food-grade conditions, in media containing $^{58}FeSO_4$ using a proprietary methodology (22), harvested, oven dried, and ground to 100 mesh size powder. Total iron content was measured in both $FeSO_4$ solutions and ^{58}FeASP using atomic absorption spectroscopy (Perkin Elmer, Shelton, CT). The isotopic composition of the ^{57}Fe tracer solution and ^{58}FeASP supplement was analyzed by magnetic sector thermal ionization mass spectrometry (Thermo Scientific, Waltham, MA) at Cornell University. The ^{58}FeASP contained 24.2 mg ^{58}Fe/g and the ferrous sulfate solution contained 3.9 mg ^{57}Fe/mL. From the whole-blood samples that were sent to Cornell University, iron was extracted by the modified anion exchange chromatography method (26). Isotopic ratios of ^{57}Fe to ^{56}Fe ($^{57/56}$Fe) and ^{58}Fe to ^{56}Fe ($^{58/56}$Fe) were measured and corrected for isotopic fractionation using the $^{54/56}$Fe ratio. The fractional abundance values used were 0.02317 for ^{57}Fe and 0.00308 for ^{58}Fe.

Isotope calculations. Fractional iron absorption of ASP and ferrous sulfate was determined based on the erythrocyte incorporation method (26, 27). Erythrocyte incorporation of ^{57}Fe and ^{58}Fe was determined using an estimated blood volume for adult women of 70 mL/kg, an iron content of hemoglobin of 3.47 g/kg, and the assumption that 80% of the absorbed iron was incorporated into erythrocytes (27).

Study 2: serum iron response

Subjects. Healthy women, 18–35 y of age, were recruited at ISU out of 91 subjects that responded using the same methodology as described for study 1. Eligibility criteria included nonsmoking, nonlactating, nonpregnant, not taking vitamins, mineral supplements, or any drug that interferes with iron absorption, and having no gastrointestinal conditions. Out of 65 subjects that were screened, only 20 met the inclusion criteria and because of time conflicts with 1 subject and difficulty with the multiple blood draws with another 2, only 17 subjects (18–31 y of age) participated in the study. One of the subjects in the 20 mg ASP treatment dropped out for personal reasons, resulting in a final sample size of 16 subjects in the ASP group. A sample size of 14 subjects in each group was needed to determine a 30% difference in AUC between treatment groups as significant with 80% power and at an α level of 0.05 (28, 29).

Study design. In a double-blind, cross-over experimental design, 17 subjects were randomly given (using the RAND function in Microsoft Excel to assign the sequence of treatment, by an independent research scientist) 3 iron supplements with a meal during 3 separate study visits. The 3 supplements were all provided as capsules, being either *1*) $FeSO_4$ containing 10 mg elemental Fe, *2*) ASP providing a total of 10 mg Fe (10-mg ASP), or *3*) ASP providing a total of 20 mg Fe (20-mg ASP). $FeSO_4$ was purchased commercially from Fisher Scientific and used to grow ASP as described earlier in the isotope study. After a 10-h overnight fast, a 10-mL blood sample was obtained from each subject to measure serum iron concentrations (T_0) and CRP. The latter was used as a marker of inflammation, a process which can alter serum ferritin levels, intestinal iron absorption, and body iron trafficking. Serum ferritin and hepcidin were measured only in the blood collected before ingesting the first meal. Each subject consumed a meal with 1 of the 3 iron supplements in a random order and all participants consumed the 3 supplements. The test meal was composed of 15 g cabbage, 30 g green peas, 40 g green beans, 20 g soy sauce, 14 g peanut oil, 40 g baby carrots, 100 g steamed rice, and 125 g orange juice. We needed to use this enhancing meal to ensure that the serum iron change was in response to the iron supplementation. After the meal, a 5-mL blood sample was collected by a certified nurse every 30 min for 4 h using an indwelling catheter. We chose to collect blood only for 4 h based on a previous study (28), to avoid keeping the subjects fasting for a longer period of time. This process was repeated 2 wk apart with a different iron supplement consumed each time.

Blood sample analysis. The serum iron concentration of each blood sample was analyzed by a certified lab (Quest Diagnostics). The serum iron response curves for each treatment were constructed and the AUC was calculated with the change of the serum iron level from baseline. Since iron recovery percentage at maximum (% Fe Recovery at max) showed a significant correlation with percentage of iron absorption measured from erythrocyte incorporation of stable isotope iron in a previous study (28), it was calculated as: [(Δ iron max $\times$ serum volume)/(total iron ingested) $\times$ 100]. The Δ iron max is the difference between the basal serum iron and peak serum iron values. Serum volume was estimated based on a blood volume of 69.6 mL/kg body weight and hematocrit and the detailed calculations are described by Conway et al. (28). The same commercial kits were used to measure CRP, ferritin, and hepcidin as described in the first study. Groups remained blinded until the completion of the study.

Statistical analysis. All statistical analyses were performed using the Graphpad Prism 6 software program. Normally distributed data were presented as the means $\pm$ SDs. Nonnormal data were log transformed before statistical analysis and the geometric means $\pm$ SDs were reported. Paired *t* tests were used to compare iron absorption from ferrous sulfate and ASP in the stable isotope study. Pearson correlation analyses were performed to determine the relations of hepcidin and ferritin concentrations with iron absorption. In the serum iron response study, AUCs, percentage of recoveries at maximum, and change in serum iron at each point for 3 treatments were compared with the use of ANOVA followed by Tukey's multiple comparison test. The differences were considered statistically significant at $P \leq 0.05$.

Results

Stable isotope study

Subject characteristics. General anthropometric and baseline characteristics for hemoglobin, serum CRP, ferritin, and hepcidin

TABLE 1 General baseline characteristics and iron status indicators of all subjects[1]

	Stable isotope study ($n = 16$)	Serum iron study ($n = 17$)
Age, y	23.8 ± 2.8 (20–28)	22.5 ± 3.01 (18–31)
Weight, kg	61.29 ± 9.12 (44.7–80.4)	61.4 ± 6.36 (49.28–71.8)
BMI, kg/m^2	21.8 ± 1.68 (19.1–24.1)	22.3 ± 1.92 (18.7–24.7)
Hemoglobin, g/dL	13.1 ± 0.75 (11.6–14.6)	13.1 ± 0.79 (12.2–14.5)
Hematocrit, %	NA	38.7 ± 2.33 (36.6–44.0)
Serum CRP,[2] mg/L	0.83 ± 2.84 (0.02–8.5)	NA
Serum ferritin,[2] μg/L	14.86 ± 9.79 (5.0–35.4)	16.6 ± 7.18 (6.3–30)
Serum hepcidin,[2] ng/mL	1.92 ± 2.59 (0.11–7.9)	2.57 ± 4.82 (1.0–15.9)

[1]Values are means $\pm$ SDs (range) unless otherwise indicated. CRP, C-reactive protein; NA, not available.
[2]Geometric means $\pm$ SDs (range).

concentrations are shown in **Table 1**. The mean age of the 16 subjects was 24 y, ranging from 20–28 y. The mean BMI of 22 (range 19.1–24.1) was within the normal range. Serum hepcidin concentration averaged 1.92 ng/mL (range 0.11–7.92 ng/mL). Two subjects had hemoglobin concentrations slightly below 12 g/dL (11.6 and 11.8 g/dL), but the mean concentration (13.1 g/dL) was within the normal reference values. The mean serum ferritin concentration was 14.9 µg/L (5–35.4 µg/L). Three subjects had elevated CRP concentrations at baseline (6.67, 7.95, and 8.47 mg/L) and these remained high even after 14 d; however, these subjects had ferritin concentrations in the normal range. Since each subject served as their own control, those subjects were not excluded and, therefore, all participants were used in the analyses.

Iron absorption. The geometric mean fractional iron absorption of ^{57}FeSO$_4$ was 17.1% (range 3.4–51.5%) compared to 15.2% (range 5.5–35.4%) with ^{58}FeASP (**Figure 1**). The 12% difference in iron absorption between the 2 iron supplements was not statistically significant ($P = 0.07$). A strong positive correlation ($R^2 = 0.91$, $P < 0.001$) was found between absorption of both iron compounds (data not shown). In addition, significant inverse relations were found between absorption of both ^{57}FeSO$_4$ ($R^2 = 0.53$; $P = 0.002$) and ^{58}FeASP ($R^2 = 0.58$; $P < 0.001$) and ferritin concentrations as well as hepcidin concentrations (**Figure 2**) ($R^2 = 0.30$; $P = 0.03$ for ^{57}FeSO$_4$ and $R^2 = 0.3$; $P = 0.02$ for ^{58}FeASP).
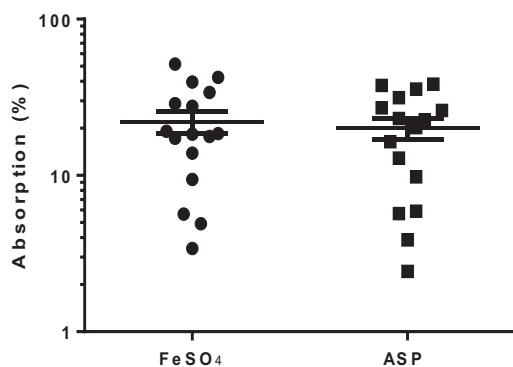

FIGURE 1 Iron absorption of ^{57}FeSO$_4$ and ^{58}FeASP. Values are geometric means $\pm$ SEMs ($n = 16$). The 12% difference between ^{57}FeSO$_4$ and ^{58}FeASP absorption is not significant ($P > 0.05$) based on paired t test. ASP, Aspiron (iron-enriched *Aspergillus oryzae*).

Serum iron study

Subject characteristics. The mean age, weight, BMI, serum ferritin, hemoglobin, hematocrit, and hepcidin of the 17 subjects who completed the study are also listed in Table 1. The 20-mg ASP group had only 16 subjects as 1 subject dropped out after 2 treatment periods. The mean age of the subjects was 23 y (range 18–31 y). The average BMI of 22 (range 18.7–24.7) was also within the normal range. The mean hematocrit, hemoglobin, serum ferritin, and hepcidin concentrations were 38.7%, 13.1 g/dL, 16.6 µg/L, and 2.57 ng/mL, respectively.

Serum iron response. The AUC, % Fe Recovery at max, and CRP concentrations are shown in **Table 2**. The AUCs for FeSO$_4$ 10 mg Fe, 10-mg ASP, and 20-mg ASP were 1674 ± 376, 869 ± 117, and 900 ± 193 (mean $\pm$ SE), respectively. Although the FeSO$_4$ treatment had a 2-fold higher response than both ASP treatments, the differences were not significantly different due to large variation when all 3 treatments were included in the ANOVA analysis. However, when the 10-mg dose was compared only between ASP and FeSO$_4$ by paired t test, the difference was significant. For all the treatments, peak serum iron was reached at 150 min and the % Fe Recovery at max was 11.59 ± 2.10, 6.97 ± 0.89, and 4.08 ± 0.72 (mean $\pm$ SE) for FeSO$_4$, 10-mg ASP, and 20-mg ASP, respectively but the only significant ($P < 0.05$) difference was found between the FeSO$_4$ and 20-mg ASP treatments (Table 2). Serum iron concentration with FeSO$_4$ was significantly $P < 0.05$ higher at 90 min and 120 min than with both doses of ASP (**Figure 3**). The mean concentrations of CRP ranged from 1.64–1.76 mg/L before feeding each iron supplement and were not significantly different among the treatments, suggesting that the differences in AUC among the treatments were not due to CRP status (Table 2).

Discussion

Iron is an essential nutrient for all living organisms, including fungi and yeast. There has been recent interest in iron-enriched yeast for iron fortification (23, 32). Iron-enriched baker's yeast, *Saccharomyces cerevisiae*, has been shown to accumulate around 0.28% iron, 27 times higher than the control (32), but no iron absorption studies in humans with yeast had been reported until recently. A study with mutant *S. cerevisiae* showed promising results in human absorption; however, the iron content of this mutant-yeast biomass was still low (~9 mg/g or 0.9% of dry matter) and has limited utility in nonheat-treated products (23).
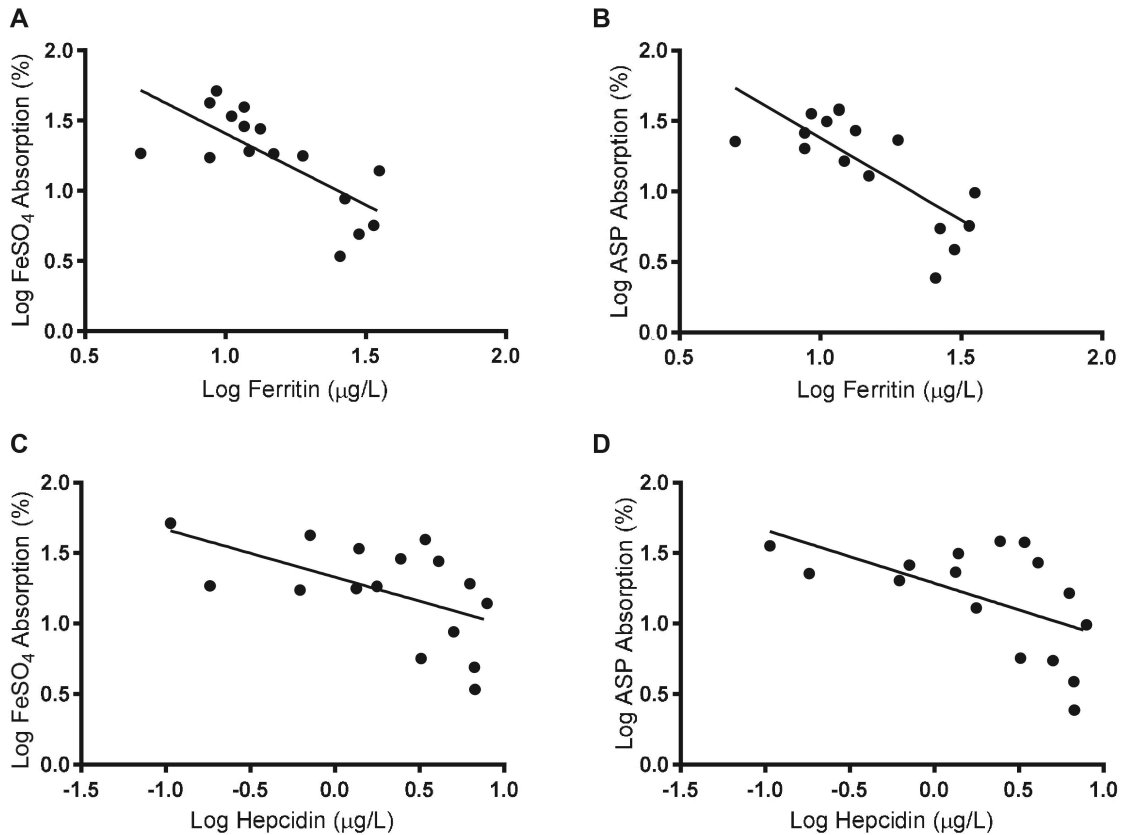
FIGURE 2 Correlation of $^{57}FeSO_4$ and $^{58}FeASP$ absorption with baseline ferritin (A, B) and hepcidin (C, D). An inverse relation was found between serum ferritin and absorption of $^{57}FeSO_4$ (A, $R^2 = 0.53$; $P = 0.0015$) and $^{58}FeASP$ (B, $R^2 = 0.5811$, $P = 0.0006$) and between hepcidin and $^{57}FeSO_4$ (C, $R^2 = 0.3$; $P = 0.028$) and $^{58}FeASP$ (D, $R^2 = 0.31$; $P = 0.02$). ASP, Aspiron (iron-enriched *Aspergillus oryzae*).

Using an innovative fermentation process, fungal biomass of *A. oryzae* was grown in an iron-rich medium to produce ASP with ≤8% iron. When *A. oryzae* is grown in this medium, it has previously been found to take up high amounts of iron and incorporate it in the mycelia (22). The mechanism of iron acquisition is not completely understood with *A. oryzae* compared to other related species like *A. fumigatus* (33). According to the published studies and the known properties of ASP, once the iron enters into the cytosol, it is likely to be transported to the vacuoles and mitochondria. Overall, research in fungi and bacterial systems has shown that iron uptake, storage, and transport are controlled by several mechanisms, ranging from very simple to complex iron uptake systems involving either up- or down-regulation of siderophores, membrane-binding proteins, or transport proteins (33, 34).

Based on the high iron content of ASP, we evaluated iron absorption using a widely accepted stable isotope methodology. Our results showing a nonsignificant ($P > 0.05$) 88% RBV of ASP compared to $FeSO_4$ suggest that the iron associated with ASP is in a highly bioavailable form. The relative absorption (88%) compared to $FeSO_4$ found in our study is higher than the 72% RBV ($P < 0.05$) which was reported in an earlier study with yeast (23). These differences in percentages of absorption between the cheese study and our study may be explained by differences in the meal composition, iron doses used, and the iron status of the subjects. Having similar inverse relations between the fractional iron absorption of $FeSO_4$ and ASP with hepcidin and ferritin, as well as very highly significant correlation between the fractional absorptions of ASP and $FeSO_4$, suggests that the iron from ASP might be regulated similarly through a divalent metal ion transporter 1 (DMT1) or ferroportin (35).

The results from the serum iron study showed that the relative serum iron response based on AUC with ASP was 52–53% of $FeSO_4$, regardless of dose (Figure 3). These differences were not statistically significant when all 3 groups were compared, likely due to the high variation in the serum iron concentrations. However, when 10-mg ASP alone was compared to $FeSO_4$, the AUC and % Fe Recovery at max were significantly different between the 2 groups given the same dose of iron

TABLE 2 Mean of AUC, % Fe recovery at max, and CRP ($n = 17$)[1]

	AUC	% Fe recovery at max	CRP, mg/L
FeSO$_4$ 10 mg	1675 ± 1548	11.59 ± 2.10[a]	1.76 ± 0.44
10-mg ASP	869 ± 481	6.97 ± 0.89[a,b]	1.67 ± 0.45
20-mg ASP[2]	890 ± 771	4.08 ± 0.72[b]	1.64 ± 0.41

[1]Values are means ± SEs. Values in the same column without a common superscript are significantly different ($P < 0.05$). AUC and CRP values were not significantly different among the 3 groups. ASP, Aspiron (iron-enriched *Aspergillus oryzae*); CRP, C-reactive protein; 10-mg ASP, Aspiron providing 10 mg Fe; 20-mg ASP, Aspiron providing 20 mg Fe; % Fe recovery at max, iron recovery percentage at maximum.
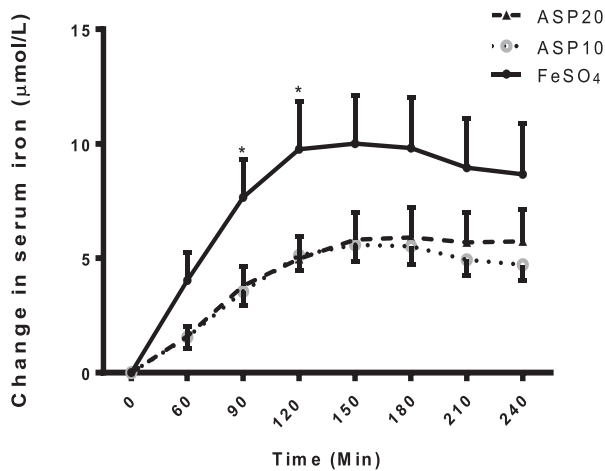[2]$n = 16$.

FIGURE 3 Serum iron curve changes from baseline in female subjects during the 4 h after consuming the same test meal with 10 mg FeSO$_4$, ASP10, and ASP20. Values are means ± SEs, $n = 16$–17. Mean AUCs are not significantly different among the treatments, but change in serum iron with FeSO$_4$ was significantly higher than both ASP doses, only at 90 and 120 min. *$P < 0.05$. ASP, Aspiron (iron-enriched *Aspergillus oryzae*); ASP10, Aspiron providing 10 mg Fe; ASP20, Aspiron providing 20 mg Fe.

(Table 2), suggesting lower absorption of ASP. The differences in RBV in terms of AUC (52% of FeSO$_4$) with 10-mg ASP in study 2 compared to 88% of FeSO$_4$ in study 1 may be attributed to the differences in the meal fed to the subjects. Our results support an earlier study (36) showing that RBV of ferric pyrophosphate (FePP) varied with the food matrix. The RBV of FePP was 15% with rice but 65% with cereal-based meals. Furthermore, adding vitamin C increased FeSO$_4$ absorption 5-fold, compared to only a 3-fold increase with FePP, thus reducing the RBV of FePP to 39% from 62% when vitamin C is in the meal. Therefore, it is possible that ASP absorption was lower than FeSO$_4$ either because of the inhibitory effect of the rice-based meal or because of a potential enhancement in FeSO$_4$ absorption by the orange juice provided with the meal, enlarging the AUC difference between ASP and FeSO$_4$. We would have expected a closer RBV between the 2 studies if we had used the same meal. Nevertheless, the lower RBV observed in the serum iron study does not negate a potential slow-release mechanism associated with ASP since it was shown to be absorbed similarly to FeSO$_4$ in the first study. Serum iron levels started declining for all treatments at 4 h but did not reach baseline values within 4 h, suggesting the need to extend the sampling time further. However, 4 h was shown to be sufficient to measure AUC where iron absorption was correlated with AUC$_{0-4 h}$ (28). In addition, serum iron did not reach baseline even after 6 h in another study (37). These authors suggested that only a fraction of the absorbed iron is cleared in 6 h, therefore extending the sampling time to 6 h in our study would not have been enough to reach baseline values. Furthermore, the serum iron changes for ASP 20 mg remain steady after 150 min, whereas the FeSO$_4$ curve showed a slow downward trend after 180 min. Since CRP concentrations were similar in all treatments, these differences in serum iron response cannot be due to subjects' inflammatory status.

Our results showing similar AUCs with 10 and 20 mg Fe from ASP support the report (38) that there is no linear relation between AUC and iron dose; however, it may also be indicative of a slow-release pattern as already mentioned. It is noteworthy to state that the serum iron concentration or the AUC measured following iron supplementation cannot be used to assess iron utilization but rather for iron release kinetics (39). The kinetics of iron absorption depend on the type of oral iron preparation: compounds that are absorbed slowly inevitably lead to lower maximal plasma iron increases, smaller AUCs, and consequently to possible misinterpretation of the absorption results (36, 39). Therefore, the lack of dose response, similar inflammatory status of the subjects, and slower serum iron reduction over time with the ASP doses may suggest that ASP has a slow release–like mechanism. Further studies are needed to confirm these findings.

Since there is concern over a bolus of iron entering into the serum after high doses of FeSO$_4$ administration which can cause side effects from oxidative damage associated with NTBI, slow-release iron supplements with high absorption are of great interest. As mentioned already, rapid iron absorption with high doses of oral preparations can exceed the rate of transferrin binding and may generate NTBI, which can induce oxidative stress (40). Significantly, lower serum iron with ASP compared to FeSO$_4$ at earlier time points suggests that iron may be released slowly. Contrary to FeSO$_4$, with the slower rate of absorption of ASP, serum iron levels not exceeding transferrin binding capacity are expected with high doses of iron, potentially suppressing the NTBI flux into the plasma, which needs further investigation. This slow rate of absorption pattern may also be associated with the slow digestion of a complex matrix, comprised of protein and carbohydrates, as well as slow release of iron from the vacuoles of the fungi. Iron in fungi cannot be found as free ions but is likely associated with ligands such as polyphosphates and proteins (34). Based on other fungal studies, we can speculate that iron is stored in a ferritin-like molecule or stored as a polyphosphate form in the vacuole (41). Although the pattern of serum iron looks similar, it does not rule out the possibility that the iron uptake mechanism in humans from ASP is mediated by endocytosis as with ferritin, an independent mechanism of DMT1 transport. Our data showing the correlation between hepcidin and iron absorption support the studies showing nanoparticulate Fe(III) can be absorbed by the endocytosis mechanism (42) but can be regulated by ferroportin (43) which is in turn regulated by hepcidin.

As suggested by Conway et al. (28) we used % Fe recovery at max to assess iron absorption. Although the % Fe recovery at max with 20-mg ASP iron was 59% of that of the 10-mg dose, the total amount of iron absorbed (based on the amount of iron ingested and the % Fe recovery at max) was slightly higher with ASP 20 compared to ASP 10 (0.82 compared to 0.70 mg), suggesting no huge advantage of feeding higher doses of ASP iron. The latter is in agreement with reports indicating that only 5 mg Fe can be absorbed at a time and larger amounts exceed the capacity of the active absorption pathway (39).

Overall, with similar iron absorption to ferrous sulfate, a slow rate of iron release into the blood, and potentially causing less side effects, ASP is a promising, innovative natural product for supplementing people with iron deficiency. Furthermore, since ASP contains ∼20–28% of complex carbohydrates, which we suspect to be in indigestible form, further studies are needed to investigate the beneficial effect of ASP on microbiota. As it has been reported that unabsorbed iron in the

colon might induce adverse changes in the beneficial microflora, allowing the growth of pathogenic bacteria (44) and producing free radicals that could cause mucosal cell damage or increase production of carcinogens (8), more studies are needed to explore the iron absorption, slow-release iron mechanism, and side effects including NTBI of high doses of ASP in longer-term supplementation studies.

Acknowledgments

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