Assessment of Acute Serum Iron, Non-Transferrin-Bound Iron, and Gastrointestinal Symptoms with 3-Week Consumption of Iron-Enriched *Aspergillus oryzae* Compared with Ferrous Sulfate

Amanda E Bries,[1] Chong Wang,[2] Isaac Agbemafle,[1] Brian Wels,[3] and Manju B Reddy[1]

[1]Department of Food Sciences and Human Nutrition, Iowa State University, Ames, IA, USA; [2]Department of Statistics, Iowa State University, Ames, IA, USA; and [3]State Hygienic Laboratory, University of Iowa, Ankeny, IA, USA

ABSTRACT

Background: Iron deficiency anemia (IDA) is a widespread nutritional deficiency, and iron supplementation, especially with ferrous sulfate ($FeSO_4$), is the most common strategy to treat IDA; however, compliance is often poor with daily $FeSO_4$ owing to negative side effects. In a previous study, iron from iron-enriched *Aspergillus oryzae* [Ultimine® Koji Iron (ULT)] was absorbed similarly to $FeSO_4$.

Objectives: The main objective of this study was to assess the safety of consuming ULT in terms of increasing non-transferrin-bound iron (NTBI) and gastrointestinal distress.

Methods: Young female participants ($n = 16$) with serum ferritin <40 μg/L were randomly assigned to a double-blind, 9-wk crossover study with a 3-wk placebo/washout period between treatments. Oral $FeSO_4$ and ULT supplements containing 65 mg Fe were administered daily for 21 consecutive days. On day 1, serum iron (SI), percentage transferrin saturation (%TS), and NTBI were measured for 8 h on the first day of iron consumption. Changes in biochemical indicators were evaluated after 3 wk consumption. Side effects questionnaires were completed weekly on 2 randomly selected weekdays and 1 weekend day for the entire study.

Results: SI, %TS, and NTBI were all markedly higher during hours 2–8 ($P < 0.001$) with $FeSO_4$ than with ULT. Oxidative stress, inflammatory, and kidney and liver function markers remained unchanged with both supplementations compared with placebo. Changes in iron status markers were not significantly different among the 3 treatments. Individual or global side effects were not significantly different among all treatments. Even when common side effects of nausea, constipation, and diarrhea were combined, $FeSO_4$ treatment had a significantly higher effect than ULT ($P = 0.04$) and placebo ($P = 0.004$) only at week 3, but the difference was not significant between ULT and placebo.

Conclusions: Low NTBI production and fewer common gastrointestinal side effects with ULT suggest that it is a safe oral iron supplement to treat IDA. This trial was registered at clinicaltrials.gov as NCT04018300. *Curr Dev Nutr* 2019;3:nzz127.

Introduction

An estimated 12.5% of the global population has iron deficiency anemia (IDA) (1) and it is the most common nutritional deficiency in the world, especially among women and children in developing countries. Negative consequences of IDA include reduced cognitive and physical development and increased mortality of children (2, 3). The WHO guidelines are aimed toward using food fortification, home fortification, or sup-

plementation strategies in treatment of IDA (4). Food iron fortification is one of the most economical strategies to address anemia; however, iron supplementation is more effective in short-term treatment. Ferrous sulfate ($FeSO_4$), the most commonly used oral iron supplement, is highly absorbed and improves iron status, but causes adverse effects such as constipation, diarrhea, and nausea (5). Owing to the quick absorption of $FeSO_4$, iron influx into blood is rapid, saturating transferrin transiently and producing non-transferrin-bound iron (NTBI) (6).

Under normal iron status, transferrin is capable of binding iron present in circulation. It is well known that in chronic iron overload conditions, the capacity of transferrin to bind iron decreases, causing high transferrin saturation and production of NTBI, a highly reactive iron, which induces oxidative stress owing to its involvement in free radical production, as well as potentially damaging DNA, protein, and lipids (7). Research has also demonstrated that circulating NTBI is likely to appear despite the presence of available binding sites on transferrin if the rate of iron influx into plasma exceeds the rate of iron acquisition by transferrin (8). Further consequences of circulating NTBI constitute increased bacterial-pathogenic infections, due to the free iron being utilized by the parasite, causing increased infections and even death in malaria–endemic areas (9). Therefore, it is important to maintain low iron saturation levels to minimize the production of NTBI and thereby reduce systemic inflammation and bacterial infections (10). Furthermore, research indicates that maintaining percentage transferrin saturation (%TS) <35% delays biological aging and lessens the risk of age-associated diseases induced by oxidative stress (11).

$FeSO_4$ is the gold-standard treatment of anemia, especially in pregnant women, but concerns about high soluble iron supplements during pregnancy continue to emerge owing to high amounts of unabsorbed reactive iron in the gut, causing diarrhea, inflammation, and constipation, resulting in low patient compliance (5). There is also a need for a low-risk and safe iron supplement targeted to vulnerable populations with increased physiological need, who may be susceptible to infection.

Ultimine® Koji Iron (ULT) is a source of natural iron produced by fermentation with *Aspergillus oryzae*, also known as *koji* culture. Most of the iron is stored within the mycelia of the *koji* culture. Our recent publication showed that the iron from ULT is as bioavailable as $FeSO_4$ in humans (12). The main objective of this study was to compare the acute effect of consuming 65 mg Fe from $FeSO_4$ and ULT with food, in young female subjects, on serum iron (SI) and NTBI production as a function of time. In addition, we evaluated the effectiveness in improving iron status and safety of 65 mg Fe/d from these supplements by assessing changes in gastrointestinal-related side effects, oxidative stress, and biochemical indicators after 3 wk oral intake.

Methods

Subjects and study design

Women 18–40 y of age were recruited via an Iowa State University (ISU)-wide email. Consented subjects ($n = 126$) completed a prescreening online health questionnaire including demographics (age, gender, education, and ethnicity) and questions pertaining to the initial inclusion criteria: a BMI (in kg/m^2) of 18.5–30; no medication use (except noniron combination oral contraceptives); no blood donation within 2 mo; nonsmoking; nonpregnant or lactating; no history of chronic diseases; no gastrointestinal-associated conditions or dietary intolerances; and no intake of vitamin, mineral, or herbal supplements 1 wk before and during the study period. Subjects were excluded based on the following criteria: hemoglobin (Hb) < 12 g/dL, serum ferritin (SF) $\geq$ 40 μg/L, or abnormal kidney, liver, and basic metabolic panel indicators. A total of 91 consented subjects were screened, of whom only 17 were eligible based on the set inclusion criteria and were randomly assigned to their respective treatment groups. One subject dropped out during

placebo treatment because of reported side effects of gastrointestinal discomfort. A total of 16 subjects completed the 3 arms of the study. We estimated a sample size of 15 subjects for each group was needed to provide a power of 80% ($\beta = 0.20$) to detect an intrasubject difference of 30% in NTBI with $\alpha = 0.05$. Written informed consent was obtained from each participant and the study was approved by the Institutional Review Board (IRB) at ISU: IRB# 17-365.

This 9-wk intervention was conducted at the Nutrition and Wellness Research Center at ISU and was aimed at assessing the acute influx of iron into serum and NTBI as a function of time over 8 h after oral Fe supplementation and change in iron status, safety, and gastrointestinal distress with 3-wk consumption of iron. Seventeen female subjects were enrolled in a double-blind crossover study. They were randomly assigned to receive daily capsules containing 65 mg Fe as either $FeSO_4$ or ULT for 3-wk periods with a 3-wk placebo/washout before treatment crossover (**Figure 1**). A gastrointestinal side effects questionnaire (GISQ) was distributed electronically to participants over 2 randomly chosen weekdays and 1 weekend day during each intervention period. The SI response and NTBI determination procedures are described below. Subjects acted as their own controls and side effects from iron supplementation were monitored throughout the study. General compliance was recorded by documenting the remaining capsules from the returned containers. Safety of supplementation was evaluated via kidney function [blood urea nitrogen (BUN), creatinine, and estimated glomerular filtration rate (eGFR)]; liver function [aspartate aminotransferase (AST) and alanine aminotransferase (ALT)]; oxidative stress [protein carbonyls (PCOs) and thiobarbituric acid reactive substances (TBARS)]; and inflammatory indicators [C-reactive protein (CRP) and hepcidin].

Iron supplements

Each iron supplement contained 65 mg Fe as $FeSO_4$ (Nature Made®) or as ULT (iron-enriched *A. oryzae* containing 8.7% Fe) and placebo capsules were prepared with dextrose monohydrate. Similarly to our previous study (12), a commercial sample of ULT (13) was supplied by Cura Global Health, Inc. All pills were prepared in opaque-colored, pharmaceutical-grade gelatin capsules (Capsuline). New pill containers with 21 capsules (a 3-wk supply) were given to subjects on day 1 of each treatment period to prevent cross-contamination. Subjects were explicitly instructed to only take 1 capsule daily with food, even if they forgot to take it on prior days.

Biochemical assessment

Biochemical indicators were assessed at baseline (day 1) and end (day 21) of treatment period 1 and baseline (day 42)) and end (day 63) of treatment period 2. The effect of the washout period (placebo) was evaluated using the week 3 and week 6 time points. Whole blood and serum were collected and sent to a certified diagnostic laboratory (Quest Diagnostics) for Hb, SI, total iron-binding capacity, %TS, ALT, AST, BUN, eGFR, and creatinine analyses. The SF concentration was determined using an S-22 Spectro Ferritin Kit (Ramco Laboratories, Inc.). Serum aliquots were collected at all 4 visits and stored at $-80°C$ until oxidative indicators were measured within 3 mo of time of collection. Circulating hepcidin, CRP, and soluble transferrin receptor concentrations were measured using commercial ELISA kits (DRG International, Inc.; American Laboratory Products Company; and Ramco Laborato-
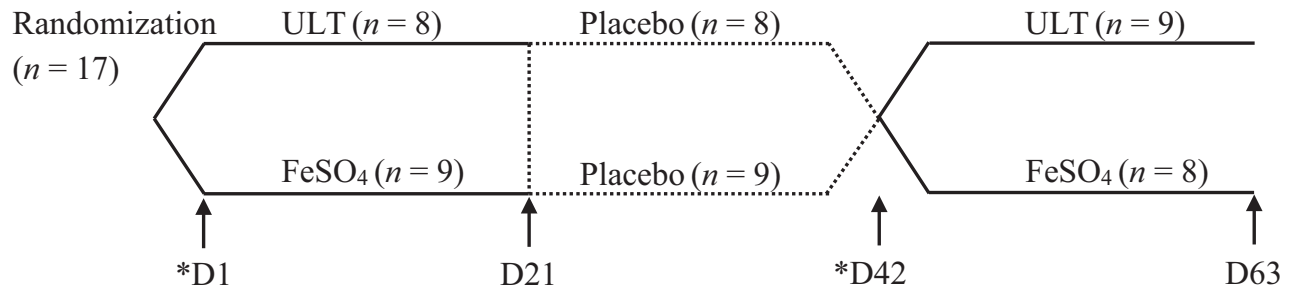
FIGURE 1 Study design for the double-blind crossover study with young female subjects. $n = 16$. *8 h non-transferrin-bound iron and serum iron curve analyses; arrows indicate testing of biochemical parameters. FeSO$_4$, ferrous sulfate; ULT, Ultimine® Koji Iron.

ries, Inc., respectively). Lipid peroxidation (TBARS) was measured as a malondialdehyde colorimetric assay (Cayman Chemical). Serum PCOs were measured based on a modified assay (14).

Acute SI response and NTBI production

To determine NTBI and SI concentrations after iron supplements (FeSO$_4$ and ULT) consumed with a semipurified meal (egg albumin, maltodextrose, and corn oil) after a 10-h fast on days 1 and 42, serum was collected at time points 0 (time of supplementation), 1, 2, 3, 4, 6, and 8 h after supplementation. The ingredients and procedure used in preparing the meals were as previously described (12). During the 8-h period, the subjects consumed unfortified white bread with cheese and butter at 3 h and an apple at 6 h. The NTBI was determined as previously described (15, 16) with modifications. In brief, serum aliquots were rapidly thawed at 37°C for 10 min and incubated with resin-treated 400 mM nitriloacetic acid (NTA) at pH 7.0 for 30 min at room temperature. The serum–NTA complex was then centrifuged in a 30 kD microcon ultracel-30 column (Millipore Sigma) at 7437 × g for 90 min. Sample ultrafiltrates were diluted to a final concentration of 10 mM NTA. To ensure negligible concentrations of NTBI, pooled serum ultrafiltrate obtained from the screening serum of the subjects with SF < 15 µg/L was used to prepare blanks and standards. A pooled ultrafiltrate (10 mM NTA) was used as blank and spiked with 2 and 5 µg/L of iron as quality controls.

Serum NTBI from the Fe–NTA filtered complex was measured using graphite furnace atomic absorption spectrometry (Perkin Elmer AAnalyst600). The lower limit and upper limit of detection were 0.1 and 60 µg/L, respectively. Linearity was established from 0.1 to 60 µg/L ($r = 0.99$) with the iron containing the pooled filtrate. The percentage recovery was 96% with a known 60 µg/L standard, ensuring the accuracy of the measurement.

Side effects questionnaire

We used a modified GISQ assessment tool that was based on a previously reported oral iron supplement questionnaire (17). The GISQ covers gastrointestinal-related side effects commonly reported with oral FeSO$_4$ supplementation. We asked subjects to report the following common side effects due to the iron supplement intake: nausea, heartburn, abdominal discomfort, fatigue, diarrhea, and constipation. The severity of the side effects was recorded on a 7-point Likert scale (0 = absent, 1 = somewhat mild, 2 = mild, 3 = somewhat moderate, 4 = moder-

ate, 5 = somewhat severe, 6 = severe) (see **Supplement 1** for the full questionnaire). Frequency of weekly side effects was the number of reported side effects for 2 randomly selected weekdays and 1 weekend day over the 9-wk study period. From the 6 side effects reported, the most common ones related to iron were nausea, diarrhea, and constipation, which are likely to cause abdominal discomfort (5); these were combined to test the effect of the supplements.

Statistical analysis

Analysis was performed by intention to treat, consistent with CONSORT guidelines (18). All analyses were performed using SAS version 9.4 (2018; SAS Institute Inc.). Changes in SI, TS, and NTBI from baseline to 8 h after administration of 65 mg FeSO$_4$ or ULT were analyzed using repeated-measures regression models over the 8 h time. The biochemical variable values (mean ± SEM) refer to the change from baseline to end for their respective time points within the crossover design. Normality for the biochemical data was tested using the Shapiro–Wilk test and geometric means (95% CIs) were reported for non-normally distributed data. Effects of the treatments on the change were compared using SAS PROC GLIMMIX for repeated-measures ANOVAs with Tukey multiple comparisons to test the difference between least-square means. A total of 16 subjects were included in all biochemical and questionnaire analyses, whereas 15 subjects were included in SI, NTBI, and TS analyses, because 1 subject had difficulty with multiple blood draws.

Data for the side effects were obtained from the online survey of the GISQ exported from Qualtrics™ into Microsoft Excel. The severity of the side effects was recoded from the 7-point Likert scale into 4 levels: 0 = absent, 1 = mild (somewhat mild and mild), 2 = moderate (somewhat moderate and moderate), 3 = severe (somewhat severe and severe). To record the frequency of side effects, we created a dichotomous variable from the 7-point Likert scale as follows: 0 = absent and 1 = present (somewhat mild, mild, somewhat moderate, moderate, somewhat severe, and severe). After the 3-wk supplementation, the frequency of weekly side effects was aggregated to total reported side effects over the 3-wk supplemental period. The models included fixed effects for treatment, period, and sequence; they also included random effects for subjects nested within sequence. Descriptive statistics were presented as frequencies for the side effects. Differences between treatments in the frequency of reported side effects were specified using SAS

TABLE 1 Age, BMI, and biochemical indicators of subjects at baseline[1]

Variable	Values
Age, y	20.6 ± 1.4
Anthropometric measures	
BMI, kg/m^{2}	22.9 ± 2.8
Laboratory measures	
Hemoglobin, g/dL	13.3 ± 0.8
Hematocrit, %	39.2 ± 2.3
Serum ferritin, μg/L	19.3 (15.1, 24.7)
Serum iron, μg/dL	90.5 ± 35.7
Transferrin saturation, %	23.8 ± 8.9
Soluble transferrin receptor, ng/mL	4.6 (3.9, 5.4)
Hepcidin, ng/mL	4.4 (3.3, 5.8)
C-reactive protein, mg/L	1.0 (0.4, 2.3)
Estimated glomerular filtration rate, mL · min^{-1} · 1.73m^{-2}	100.9 ± 13.0
Creatinine, mg/dL	0.8 ± 0.1
Blood urea nitrogen, mg/dL	11.1 ± 3.1
Aspartate aminotransferase, U/L	16.4 ± 3.5
Alanine aminotransferase, U/L	13.2 ± 5.4

[1]$n = 16$. Values are mean ± SD or geometric mean (95% CI).

PROC GLIMMIX. For all statistical analysis, $P < 0.05$ was considered statistically significant.

Results

Subject characteristics

Age, BMI, and biochemical characteristics of the 16 subjects at baseline are shown in **Table 1**. The mean age and BMI of subjects in the study were 21 y and 22.9, respectively. At screening, all participants had normal Hb concentrations ($\geq$12 g/dL) and suboptimal SF concentrations (19.3 ± 8.4 μg/L). One subject was borderline for the SF cutoff concentration at baseline (40.4 μg/L); however, it was 37.4 μg/L at screening.

Acute response of SI, %TS, and NTBI

Mean changes in both %TS and SI concentrations peaked at 4 h with FeSO$_4$ (39.6% ± 5.2% and 27.8 ± 3.6 μM, respectively) and with ULT (11.7% ± 2.0% and 8.3 ± 1.6 μM, respectively) supplements, but the change was less distinct with ULT. The SI progressively decreased after 4 h for FeSO$_4$, but values did not return to baseline within 8 h with either FeSO$_4$ or ULT supplements (**Figure 2**). TS percent rapidly spiked with a 65-mg dose of FeSO$_4$, but the same effect did not occur with ULT (Figure 2). NTBI concentrations peaked at 4 h (0.35 ± 0.17 μM) with FeSO$_4$ and remained above baseline even at 8 h postdosing, although they were not statistically different from baseline concentrations (**Figure 3**). On the contrary, at all time points, ULT NTBI concentrations were nearly unchanged from baseline. As expected, both SI ($r = 0.52$, $P = 0.0001$) and %TS ($r = 0.54$, $P = 0.0001$) were significantly correlated with NTBI when both treatments were combined (**Supplemental Figure 1**).

Biochemical indicators

There were no significant differences in the change of biochemical indicators among the iron supplements and placebo (**Table 2**). Although,
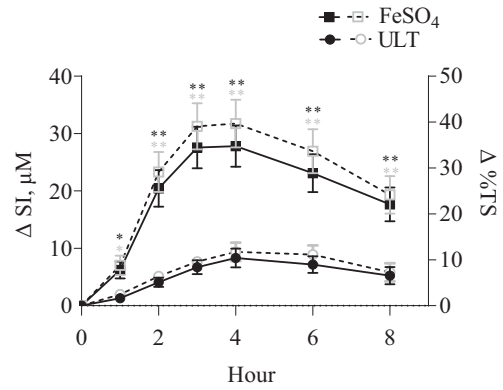

FIGURE 2 Mean ± SEM ($n = 15$) change in SI (solid lines) and %TS (dotted lines) from baseline over 8 h after administration of 65 mg FeSO$_4$ or ULT with a semipurified meal. One subject was removed owing to blood draw complications. Differences between treatments at each time point were analyzed with 2-factor repeated-measures ANOVA. *,**Significant difference between treatments: *$P < 0.01$, **$P < 0.0001$. FeSO$_4$, ferrous sulfate; SI, serum iron; ULT, Ultimine® Koji Iron; %TS, percentage transferrin saturation.

nonsignificantly, SI with ULT was higher than with FeSO$_4$ (mean ± SD: 12.7 ± 11.6 μg/dL and −5.69 ± 10.5 μg/dL, respectively) at the end of the 3-wk supplementation period. Unlike a decline with placebo, improvements in SF were found both with ULT and with FeSO$_4$ supplementation (ULT: 2.03 ± 3.44 μg/L; FeSO$_4$: 9.38 ± 4.91 μg/L; Table 2) but the differences were not statistically significant between the 2 treatments ($P = 0.23$). No other iron indicators were significantly different among the 3 treatments. Nonsignificant changes in inflammatory and oxidative stress markers were observed between treatment groups ($P > 0.05$). Based on kidney and liver function markers, the changes with ULT were not significantly different from those with FeSO$_4$. Compared with FeSO$_4$ and placebo, there were slight improve-
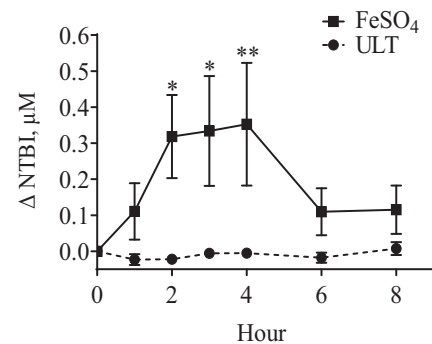
FIGURE 3 Mean ± SEM ($n = 15$) change in NTBI from baseline over 8 h after administration of 65 mg FeSO$_4$ or ULT with a semipurified meal. One subject was removed owing to blood draw complications. Differences between treatments at each time point were analyzed with 2-factor repeated-measures ANOVA. *,**Significant difference between treatments: *$P < 0.01$, **$P < 0.0001$. FeSO$_4$, ferrous sulfate; NTBI, non-transferrin-bound iron; ULT, Ultimine® Koji Iron.

TABLE 2 Change from baseline to 3 wk with supplementation of ULT, FeSO$_4$, and placebo[1]

Biochemical indicators	ULT	FeSO$_4$	Placebo
Iron status			
Hemoglobin, g/dL	0.07 ± 0.12	− 0.04 ± 0.13	0.06 ± 0.16
Hematocrit, %	− 0.07 ± 0.31	− 0.59 ± 0.44	0.12 ± 0.40
Serum ferritin, μg/L	2.03 ± 3.44	9.38 ± 4.91	− 2.61 ± 4.00
Soluble transferrin receptor, ng/mL	− 0.02 ± 0.22	− 0.13 ± 0.21	0.04 ± 0.18
Serum iron, μg/dL	12.7 ± 11.6	− 5.69 ± 10.5	− 5.63 ± 12.5
Transferrin saturation, %	4.63 ± 3.39	0.63 ± 2.72	− 3.44 ± 3.61
Total iron-binding capacity, μg/dL	− 6.06 ± 4.71[a]	− 36.19 ± 9.08[b]	20.19 ± 8.49[c]
Inflammatory markers			
C-reactive protein, mg/L	− 0.41 ± 0.37	− 0.27 ± 0.85	− 0.27 ± 0.52
Hepcidin, ng/mL	0.53 ± 1.00	− 1.47 ± 1.25	− 0.09 ± 0.65
Oxidative stress			
TBARS, μM	0.73 ± 0.97	1.94 ± 0.95	0.90 ± 0.90
Protein carbonyls, nmol/mL	− 0.24 ± 2.00	2.23 ± 3.06	− 6.13 ± 3.91
Kidney and liver function			
Estimated glomerular filtration rate, mL · min^{-1} · 1.73m^{-2}	6.0 ± 2.46[a]	− 0.81 ± 3.42[a,b]	− 1.63 ± 2.29[b]
Creatinine, mg/dL	− 0.04 ± 0.02	0.01 ± 0.02	− 0.69 ± 0.69
Blood urea nitrogen, mg/dL	0.63 ± 0.94	− 0.43 ± 0.76	0.57 ± 1.47
Aspartate aminotransferase, U/L	− 0.94 ± 0.85	0.06 ± 1.15	− 2.19 ± 1.07
Alanine aminotransferase, U/L	0.31 ± 0.63[a]	0.06 ± 0.93[a,b]	3.44 ± 1.02[b]

[1]$n = 16$. Values are mean ± SEM of change from baseline to end for each treatment period. Means without a common letter are significantly different at $\alpha = 0.05$ using a 1-factor ANOVA with Tukey's multiple comparison test. FeSO$_4$, ferrous sulfate; TBARS, thiobarbituric acid reactive substances; ULT, Ultimine® Koji Iron.

ments in eGFR with ULT (ULT: 6.0 ± 2.46; FeSO$_4$: −0.81 ± 3.42; placebo: −1.63 ± 2.29; Table 2) but the differences were nonsignificant ($P = 0.09$). ALT concentrations for placebo were significantly higher from baseline to end than for ULT (Table 2; $P = 0.01$).

Gastrointestinal side effects

Compliance was 97%, 93%, and 95.2% for ULT, FeSO$_4$, and placebo, respectively. Although nonsignificantly, FeSO$_4$ tended to contribute higher incidence of constipation, diarrhea, nausea, and abdominal discomfort than did ULT and placebo (**Table 3**). The differences for global side effects (combined effects) were not significant among the treatments ($P = 0.37$). Even when we combined the most common symptoms (nausea, diarrhea, and constipation) associated with FeSO$_4$, the differences were only significant at week 3 of FeSO$_4$ supplementation compared with both ULT and placebo ($P = 0.04$ and $P = 0.004$, respectively; data not shown), but no differences were found at the preceding weeks.

TABLE 3 Frequency of reported gastrointestinal side effects during the 3-wk supplementation of ULT, FeSO$_4$, and placebo[1]

Symptom	ULT	FeSO$_4$	Placebo
Constipation	1.13 ± 0.42	1.56 ± 0.50	1.06 ± 0.37
Diarrhea	0.63 ± 0.22	1.00 ± 0.33	0.50 ± 0.24
Nausea	0.38 ± 0.18	0.75 ± 0.30	0.44 ± 0.16
Abdominal discomfort	2.50 ± 0.50	2.81 ± 0.56	2.75 ± 0.78
Heartburn	0.13 ± 0.09	0.13 ± 0.09	0 ± 0
Fatigue	1.90 ± 0.55	1.81 ± 0.54	2.00 ± 0.47

[1]$n = 16$. Values are mean ± SEM of frequency of reported gastrointestinal side effects over the 3-wk supplementation period for each treatment period. No significant differences between means for each individual symptom, at $P < 0.05$, using a generalized linear mixed-effects model. FeSO$_4$, ferrous sulfate; ULT, Ultimine® Koji Iron.

Discussion

Despite FeSO$_4$ being the most commonly used supplement for its effectiveness in treating anemia, its rapid absorption is of concern. When a bolus of iron enters the blood quickly, this exceeds the capacity for transferrin to bind the circulating iron, resulting in a transient increase in NTBI concentrations. The catalytically reactive NTBI can promote oxidative stress and inflammatory response in the body (19). Therefore, there is a need for safer alternatives to FeSO$_4$ (20), without compromising iron absorption.

Based on the similar absorption of ULT to FeSO$_4$ in our previous stable isotope study in humans (12), the low SI response with ULT suggested its slow release mechanism, not low absorption. Several studies have demonstrated that the rate in which iron is taken up by the body is dependent on the dose, form of iron, and whether it was taken with or without food (21–23). Both %TS levels and SI concentrations did not return to baseline, even at 8 h postsupplementation, with either iron source. The %TS data are in agreement with a previous study demonstrating that %TS could reach baseline levels only after 24 h of supplementation (24).

Although nonsignificantly, change in SI was higher (after a 10-h overnight fast) with ULT compared with FeSO$_4$ after 3 wk consumption, suggesting that ULT iron may be released beyond 8 h (Table 2). On the contrary, improvement in ferritin was less with ULT, but the change was nonsignificantly different from that with FeSO$_4$. Although we do not know the form of iron in ULT, Perls stain, and DAB/H$_2$O$_2$ iron intensification confirmed that >90% of the iron is inside the *A. oryzae* mycelia (data not shown). We can postulate that the iron from the complex fungal matrix is digested over a longer period of time than FeSO$_4$ and the digested iron may be taken up into enterocytes, processed, and released slowly. Also, it doesn't rule out absorption in the large intestine. Nearly 5 decades ago, a study showed a delayed peak of circulating

iron with Hb iron compared with $FeSO_4$, because of its slow absorption and its alternative heme-absorption pathway [25]. Evidence indicates that heme-iron absorption may be saturable because of the lack of dose-response observed after a 15-mg Fe dose [26]. Therefore, the slow mechanism of release observed in this trial may support a heme-like alternative absorption pathway.

The use of the SI curve as a surrogate for iron absorption is well established [27], and we may interpret that ULT absorption is 3 times lower than that of $FeSO_4$ based on our results. However, caution should be taken when examining different iron sources owing to the differences in digestion rate and mucosal processing time. Our study showed a much lower SI change with ULT than with $FeSO_4$ but based on that we cannot necessarily predict the iron absorption. For example, despite having high bioavailability shown in many studies, plasma iron release in 270 min with NaFeEDTA was much lower than with $FeSO_4$ [23]. The limitation in applying SI curves for predicting iron absorption was clearly discussed by Schümann et al., especially in reference to Hb iron because of its complex digestibility [23]. Therefore, ULT absorption is similar to $FeSO_4$ [12], despite low SI supporting the aforementioned hypothesis.

Under normal physiological conditions, the iron is bound to transferrin in circulation, resulting in negligible amounts of NTBI [20]. When a bolus of iron enters blood with a high dose of iron supplementation, the transferrin becomes quickly saturated, causing a transient increase in NTBI concentrations and a propensity for associated adverse side effects. One study [28] reported that 6.5 mg Fe as $FeSO_4$ resulted in no NTBI production (similar to placebo), but a 65-mg Fe dose induced a 300-fold increase in the AUC of NTBI. Because higher iron doses are given to anemic subjects (200 mg/d) and a 65-mg dose was used in a previous study to assess NTBI [6], this was a reasonable amount for us to use in this study for subjects with an SF < 40 μg/L. The significant association found between SI and both %TS and NTBI suggests the importance iron influx has for %TS and NTBI production. Hence, it is critical for the controlled absorption of iron to thereby reduce the elevation in SI concentrations, minimize the saturation of transferrin, and the subsequent production of NTBI.

NTBI has become a concern because of the involvement of free iron in promoting infection [20]. In a large iron supplementation intervention trial in Pemba, adverse effects, including death, were observed when iron-replete children with malaria were given iron daily [9]. This was primarily attributed to the role of NTBI in promoting the parasitic growth of malaria [29]. More recent evidence from Parkkinen et al. [10] aligns with this observation in a study where they gave hemodialysis patients 100 mg intravenous iron. In their study, they identified significantly higher bacterial growth when cultured in the serum of hemodialysis patients with 80% TS, and the authors directly related it to NTBI availability [10]. Although we cannot directly compare intravenous results and our oral supplementation results, a single 65-mg dose of $FeSO_4$ in healthy subjects in this study reached an absolute mean of 64% TS and $\leq 97\%$ TS in some of the subjects. The mean %TS from ULT was half of that from $FeSO_4$ (34%) and remained at normal concentrations throughout the 8 h. Interestingly, the AUC for NTBI was 19-fold higher for $FeSO_4$ (97.5 ± 61.9) than for ULT (5.5 ± 6.6) (Figure 3, data not shown).

Our findings suggest that %TS $> 60\%$ (as seen with $FeSO_4$) may produce NTBI concentrations at levels that promote systemic inflammation and other adverse effects. On the contrary, iron supplements, like ULT, with no NTBI production may result in less inflammation with long-term administration. Despite our observations of significantly reduced PCOs in rats fed ULT compared with $FeSO_4$ [30], in our short-term human study we found no differences in inflammatory and oxidative stress markers (CRP, PCOs, and TBARS) between ULT and $FeSO_4$. This could be attributed to several confounding variables such as the young age of our subjects, and resilience to acute oxidative stress induction.

One of the goals of this research was also to assess the safety and advantage of ULT supplementation as an alternative supplement to $FeSO_4$, to mitigate the commonly reported negative gastrointestinal side effects and low patient compliance. The higher individual side effects with $FeSO_4$ were not significantly different from those found with ULT. With a larger sample size, we may have detected significant differences; however, the sample size was based on NTBI as the primary outcome. Based on the most common side effects (nausea, diarrhea, and constipation) that were reported in a meta-analysis [5], the combined effects of those 3 gradually increased from week 1 to week 3 for $FeSO_4$ and were significantly different at week 3 compared with ULT and placebo. The increase with time in the reported number of side effects with $FeSO_4$ suggests the body's inability to tolerate its long-term use. On the contrary, side effects with ULT decreased with time. The natural encapsulation of the iron within the fungal matrix may have resulted in slower digestion, potentially reducing the liberation of free reactive iron in the gut. We expect less reactive unbound iron in the distal colon for bacterial growth, creating less oxidative stress, inflammation, and gastrointestinal-related side effects with ULT iron. The inability of the body to tolerate $FeSO_4$ compared with ULT may have accounted for the severe abdominal discomfort and lower compliance with $FeSO_4$ supplementation. Although there was no carryover effect from one iron supplementation to the other, the high frequency of side effects reported in week 1 for the placebo group is in agreement with previous studies [17] and may indicate inflammatory insult to the gut for continued short periods of time after switching to placebo. In a meta-analysis examining the incidence of gastrointestinal symptoms with $FeSO_4$ in 20 trials, the authors reported significant side effects when compared with placebo; however, most of these placebo-controlled trials were not truly double-blind [5]. In Pereira et al.'s [17] double-blind 1-wk intervention study (not crossover), higher side effects were reported in the group supplemented with $FeSO_4$ than in the group on placebo. In their study, symptoms still existed during the washout period after $FeSO_4$ supplementation, suggesting a 7-d washout period is not long enough. The strength in our study was that our treatments were double-blind, with a crossover study of 21 d supplementation and a 21-d washout period between treatments. The limitation of our study was that we were not able to identify significant differences in gastrointestinal side effects between treatment groups. This limitation may have been due to an inadequate sample size, the duration of supplementation, or the duration of the washout period resulting in potential residual side effects. Because our primary objective was to determine the implications of these 2 supplements for NTBI production, we did not account for the power needed for gastrointestinal side effect outcomes. Lastly, although we did see the acute effects of iron supplementation on NTBI production, this was not supported by our inflammatory and oxidative stress measurements. Longer supplementation periods are warranted to potentially see a response in healthy

subjects. Kidney and liver function markers were similarly affected by ULT and FeSO₄, suggesting the safety of ULT consumption.

In conclusion, significantly lower production of NTBI and slightly fewer gastrointestinal side effects (although nonsignificantly so) were found with ULT consumption than with FeSO₄. ULT iron is safe to consume because oxidative stress, inflammatory, and kidney and liver function markers were not elevated. Therefore, ULT may be a safer alternative to oral FeSO₄ in maintaining healthy kidney and liver function, as well as iron status in young women. The results we have to date indicate that ULT has a slow release mechanism, but further studies are needed to identify the form of iron and the mechanism of ULT absorption in humans.

Acknowledgments

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