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Iron Absorption from an Intrinsically Labeled Lentil Meal Is Low but Upregulated in Women with Poor Iron Status[1,2]

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Abstract

Background: Low iron absorption from important staple foods may contribute to iron deficiency in developing countries. To date, few studies have examined the iron bioavailability of pulse crops as commonly prepared and consumed by humans.

Objective: The objectives were to characterize the iron absorption from a test meal of intrinsically labeled ^{57}Fe lentils prepared as dal, to compare the bioavailability of iron from ^{57}Fe in dal with that observed for a reference dose of ^{58}Fe as ferrous sulfate, and to assess associations between iron absorption and iron status indicators.

Methods: This crossover study included 19 nonpregnant women (n = 6 anemic; hemoglobin: <12.0 g/dL) who consumed 2 test meals on consecutive days in a counter-balanced order, ferrous sulfate (7 mg $FeSO_4$ plus 1 mg ^{58}Fe) and 330 g dal (lentils enriched to 85.1% with ^{57}Fe, 8 mg native ^{57}Fe). Iron absorption was determined by analyzing blood samples taken 14 d after dosing with the use of magnetic sector thermal ionization mass spectrometry.

Results: We found that the mean iron absorption from the dal was 2.20% ± 3.40% and was significantly lower than the 23.6% ± 13.2% observed from the same iron load given as ferrous sulfate (P < 0.001). Absorption of non-heme iron from dal and from ferrous sulfate was inversely associated with serum ferritin (SF; r = −0.50, P = 0.05 and r = −0.81, P < 0.001, respectively) and serum hepcidin (r = −0.45, P = 0.05 and r = −0.60, P = 0.007, respectively). Anemic women absorbed more iron from either source (1.20% from dal, P = 0.10; 18.3% from ferrous sulfate, P = 0.001) compared with women who were iron replete.

Conclusions: Iron absorption from the dal was low overall but upregulated in anemic women. Both SF and hepcidin were inversely associated with iron absorption from both a supplemental and a food-based non-heme iron source in nonanemic and anemic women. *J Nutr* 2015;145:2253–7.

Keywords: pulses, iron, bioavailability, lentils, absorption, non-heme

Introduction

Anemia is the most prevalent nutrition problem worldwide, affecting 1.6 billion people, and increasing data highlight its multiple adverse effects on health outcomes, morbidity, mortality, and quality of life (1, 2). More than 50% of the world's anemia is because of iron deficiency (ID)[6], and, although poor dietary intake remains a main cause, many populations have limited access to bioavailable sources of iron in a predominantly plant-based diet.

Lentils are widely consumed in many areas of the world where anemia and plant-based diets predominate. Depending on the genotype, the iron concentration of conventional lentils can be quite high, ranging from 40 to 92 ppm (3). However, iron absorption from legumes (soybeans, black beans, lentils, mung beans, split peas), measured with the use of extrinsic radioiron tracers, was reported to range from 2% to 4%, and bioavailability from lentils was reported to remain low even in men with serum ferritin (SF) that ranged from 20 to 157 μg/L, likely because of the presence of phytic acid (PA) within the seed and polyphenols in both the seed and seed coat (4).

Biofortification of staple food crops such as lentils may be an effective means of increasing iron status with the use of foods that are readily available and culturally acceptable. Because of

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[6] Abbreviations used: CRP, C-reactive protein; FeEDDHA, iron + chelate complex ethylenediamine di-2-hydroxyphenyl acetic acid, ferric; ID, iron deficiency; PA, phytic acid; SF, serum ferritin; sTfR, soluble transferrin receptor.
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normal genetic variation in PA and polyphenols, biofortified varieties can be selected to enhance the iron content of this food in varieties with lower normal concentrations and known inhibitors of iron absorption (PA and polyphenols).

To address iron absorption from a biofortified plant source, lentils were intrinsically labeled with a stable iron isotope (^{57}Fe). Iron absorption from intrinsically labeled lentils was compared with absorption of iron given as ferrous sulfate (labeled with ^{58}Fe) in a group of healthy young women with a range of iron status. Determinants of iron absorption were evaluated from each test meal. Results from this study will inform larger human efficacy studies that involve biofortified lentils.

Methods

Subjects. Twenty healthy, nonpregnant young women with a range of iron status aged 18–35 y were recruited to participate in this feeding study, beginning June 2013. Volunteers were eligible for the study if they were not taking any vitamin, mineral, or herbal supplements. Study participants agreed to refrain from use of supplements for at least 1 mo before the study and throughout the 2-wk study period. Most women reported consuming a typical diet that included animal tissue, legumes, dark greens, and fortified cereal/grain products. Women were eligible to participate if they did not have a history of intestinal or malabsorption problems, blood disorders, ulcers, joint disease, or did not ingest any medications known to affect iron homeostasis. Informed written consent was obtained from each volunteer, and the study was approved by Cornell University's Institutional Review Board.

Study design. A randomized crossover design with 2 meals was used, with each woman serving as her own control. The 2 meals were extrinsically labeled 8 mg $FeSo_4$ (containing 1 mg ^{58}Fe) and an intrinsically labeled non-heme iron source given as a lentil meal, dal (containing 8 mg iron as ^{57}Fe).

On the first morning of the study, women in a fasted state (≥8 h) were admitted to the Human Metabolic Research Unit at Cornell University. On arrival, height and weight were measured (in street clothing and without shoes) with the use of a stadiometer and a calibrated digital scale, respectively. After completing a survey on health and dietary habits, a baseline sample of 15 mL whole blood was obtained and centrifuged to collect plasma and serum.

Each woman ingested the lentil meal or the ferrous sulfate on alternate days in a random, counterbalanced order so that half of the study cohort was administered the lentil meal on day 1 and the ferrous sulfate on day 2, whereas the other half of the study participants was administered the test meals in the reverse order. For the lentil meal, women were required to consume the entire portion of dal. After the dal was ingested, the bowl was rinsed with deionized water and the rinse was consumed. The ferrous sulfate dose was orally administered to each woman by syringe along with 2 mL raspberry-flavored sugar syrup (Humco). The syringe was weighed before and after administration to obtain the exact amount of tracer ingested. Total iron intake from the ferrous sulfate tracer and the dal meal was set at 8 mg to avoid differences in iron absorption because of iron load alone. The only beverage allowed during consumption of all meals in the laboratory was deionized water. One and one-half hours after consuming each test meal, volunteers consumed a standard lunch that consisted of tomato soup, pretzels, and water. Two weeks after the second tracer dose was ingested, subjects returned to the Human Metabolic Research Unit again in a fasted state, and venous blood samples were collected.

Test meals. The lentil meal consisted of 330 g dal (117 g uncooked lentils) and contained a total of 8 mg ^{57}Fe. The iron content of the ingredients used to prepare the dal are listed in **Table 1** (5). Before cooking, the dehulled lentils were rinsed with deionized water, and all food preparation was done in stainless steel cookware that was cleaned with deionized water to minimize any metal contamination. The intrinsic iron content of the lentil variety used in this study was 0.06 ± 0.001 mg/g, and the dal was 0.03 ± 0.004 mg/g (n = 5 samples). All lentils needed for the feeding study were prepared in 1 large batch, and weighed 330-g aliquots were frozen until consumed.

TABLE 1 Iron content of the lentil meal (dal) ingredients

Ingredient	Weight per serving, g	Fe content,[1] μg/g
Vegetable oil	15	0
Onions, chopped	25	2
Garlic, chopped	3.8	17
Turmeric	0.5	420
Salt	2.4	0
Lentils	117	60
Deionized water, mL	250	0
Serving (before cooking), g	414	499

[1]For ingredients other than lentil, iron content information was obtained from the USDA National Nutrient Database for Standard Reference (5).

Ferrous sulfate (Silarx) was prepared at a concentration of 75 g/L. Stock solution of ^{58}Fe was prepared at a concentration of 0.49 g/L. Each subject ingested the ferrous sulfate/^{58}Fe dose with 2 mL flavored syrup. Total iron content of a duplicate portion of the lentil meal and of the ferrous sulfate solution was measured with atomic absorption spectrophotometry (PerkinElmer Analyst 800; PerkinElmer Inc.). The isotopic composition of the tracers (both in the oral solution and the prepared meal) was validated with the use of a ThermoQuest Triton TI Magnetic Sector Thermal Ionization Mass Spectrometer (ThermoQuest Corporation). The final isotopic abundance of the ^{57}Fe in the prepared lentil meal was 85.1%. The enrichment of the ^{58}Fe ferrous sulfate solution was 97.9%.

Tracer preparation. Iron isotopes (^{57}Fe at 96.3% enrichment and ^{58}Fe at 99.9% enrichment) were purchased as metal from IsoFlex USA. The ^{58}Fe was converted into a sterile, pyrogen-free solution of ferrous sulfate by Anazao Health Corporation.

The intrinsically labeled lentils used in this experiment (Canadian lentil variety CDC Maxim) were hydroponically grown, and all iron provided across the growth cycle was provided as ^{57}Fe. The cultivation conditions and methods used in this process were described elsewhere (6). Briefly, in the hydroponic growth medium, iron + chelate complex ethylenediamine di-2-hydroxyphenyl acetic acid, ferric (FeEDDHA) was used as the source of iron. This is an extremely stable and highly appropriate form of iron chelate for dicots (6). To make this chelate, the ^{57}Fe iron powder was first dissolved in concentrated hydrogen chloride, and then most of the hydrogen chloride and water was evaporated off at 100°C, as a hydrogen chloride-water azeotrope. The result was a concentrated $^{57}FeCl_3$ stock solution with low acidity, which could easily be diluted with high-purity water to a target concentration. This $^{57}FeCl_3$ salt was chelated in a 1:1 ratio with high-purity EDDHA (Aldrich Chemical Co.). The EDDHA was first dissolved in water and 2 equivalents NaOH, and then the $^{57}FeCl_3$ was slowly added to the EDDHA solution, forming a FeEDDHA chelate. The pH was adjusted to 5 with slow addition of 0.1N NaOH. Lentils were harvested in Ithaca, NY, and dehulled before food preparation at the University of Saskatchewan's Crop Development Centre in a Satake TM-05 grain-testing mill (Satake Engineering Co.).

Laboratory analysis. Serum ferritin was measured with the use of a commercially available enzyme immunoassay procedure (Ramco Laboratories Inc.). Serum transferrin receptor (sTfR) was measured with the use of an ELISA (Ramco Laboratories Inc.). Total body iron was calculated with the use of the ratio of sTfR to SF as described by Cook et al. (7). Hemoglobin was analyzed with the use of the HemoCue 201 (HemoCue Inc.). Serum folate, vitamin B-12, and C-reactive protein (CRP) were analyzed with the use of an Immulite 1000 immunoassay system (Immulite). Serum hepcidin was analyzed with the use of Hepcidin-25 (human) ELISA (Bachem).

Whole blood samples (0.5 mL) were digested with 4 mL concentrated Ultrex nitric acid in a polytetrafluoroethylene beaker. Samples were then

dried overnight on a hot plate at 80°C and re-dissolved in 7 mol/L ultrapure hydrogen chloride (Ultrex II; JT Baker). Iron was extracted with the use of an anion exchange chromatography method as previously detailed (8).

Extracted iron samples (8 μL) were loaded onto a rhenium filament (H Cross Co.) along with 4 μL silica gel (Sigma-Aldrich Inc.) and 4 μL phosphoric acid (0.7N). With the use of magnetic sector thermal ionization MS (Triton TI; ThermoQuest Corporation), isotopic ratios of ^{57}Fe to ^{56}Fe ($^{57/56}$Fe) and ^{58}Fe to ^{56}Fe ($^{58/56}$Fe) were measured in blood samples, and the $^{57/56}$Fe and $^{58/56}$Fe ratios obtained were normalized to the ratio of ^{54}Fe to ^{56}Fe ($^{54/56}$Fe). The fractional abundance values used were 0.02317 for ^{57}Fe and 0.00308 for ^{58}Fe. Relative SDs obtained for all blood samples averaged 0.02% and 0.19% for $^{57/56}$Fe and $^{58/56}$Fe, respectively.

Calculations. Iron absorption was calculated with the use of previously described methods (9, 10). The quantity of ^{57}Fe and ^{58}Fe incorporated into erythrocytes was determined with the use of the measured 2-wk enrichment and by estimating total circulating iron with a mean blood volume for women (70.0 mL/kg), the concentration of iron in the hemoglobin (3.47 g/kg), and each subject's hemoglobin (in g/L) and body weight (in kg). The final calculation for iron absorption was determined on the basis of the assumption that 80% of the absorbed isotope was incorporated into erythrocytes. Absorption of ^{58}Fe from the ferrous sulfate dose was adjusted to account for the fractional abundance of ^{58}Fe that was present in the ^{57}Fe-labeled lentils (2.04%). The calculations needed for this correction were previously reported (10).

Data analysis. All statistical analyses were completed with the use of SPSS version 22 (IBM). Paired *t* tests were used to determine relations between the 2 iron sources and iron absorption. Anemic and nonanemic women were compared in secondary analyses with the use of ANOVA. Pearson's correlation and simple linear regression analyses were used to explore relations between biomarkers of iron status and iron absorption. Data distributions were viewed by examining the normal quantile plots and histograms, and normality was assessed with the use of the goodness-of-fit test (Shapiro-Wilk *W* test). Normally distributed data are presented as means ± SDs, and data that were not normally distributed (^{57}Fe and ^{58}Fe absorption, age, BMI, CRP, SF, sTfR, and serum hepcidin) are presented as the geometric means ± SDs. Variables that were not normally distributed were transformed with the use of a natural logarithm before analysis. Results were considered significant at $P < 0.05$.

Results

Subject characteristics. General characteristics of subjects that completed the 2-wk study are shown in **Table 2**. All women had vitamin B-12 and folate status within normal ranges (>200 pg/mL and >5.00 μg/L, respectively) (11). None of the women had elevated CRP concentrations (CRP: >10.0 mg/L) (12). Six of the 19 women were anemic (hemoglobin: <12.0 g/dL) (13), and 5 of those who were anemic had SF <20.0 μg/L (with 3 of those with SF <12.0 μg/L) (14). One of the anemic women and 2 other nonanemic women had sTfR concentrations >8.50 mg/L, indicating tissue iron deficiency. Of the 6 anemic women, 4 had a calculated total body iron concentration [SF relative to tissue iron stores (7)] <1.00 mg/kg, and 3 of these women also had SF <12.0 μg/L.

As expected, SF ($P = 0.002$) and hepcidin ($P = 0.01$) were significantly lower in anemic ($n = 6$) than in nonanemic ($n = 13$) women. Among all women in the study, hemoglobin was significantly correlated with both SF ($r = 0.81$, $P < 0.001$; $n = 19$) and hepcidin ($r = 0.69$, $P = 0.001$; $n = 19$). Serum ferritin was also significantly correlated with hepcidin ($r = 0.86$, $P < 0.001$; $n = 19$).

Iron absorption. Mean percentage of iron absorption from ferrous sulfate (^{58}Fe) was significantly greater than from the intrinsically labeled ^{57}Fe lentil meal (23.6% ± 13.2% vs. 2.20% ± 3.40%, respectively; $P < 0.001$). No significant effect of meal order was found on iron absorption from the oral iron or the lentil meal.

Determinants of iron absorption. Iron absorption from ferrous sulfate was significantly greater in the anemic (34.3% ± 13.7%; $n = 6$) than in the nonanemic (16.0% ± 7.10%; $n = 13$; $P = 0.001$) women. A trend for greater iron absorption from the ^{57}Fe lentil meal was found in the anemic (2.2% ± 5.6%; $n = 6$) than in the nonanemic (0.90% ± 1.40%; $n = 13$; $P = 0.10$) group. In all women, the ln of SF and ln of hepcidin were inversely associated with ln of percentage of iron absorption from lentils ($r = -0.46$, $P = 0.05$; $r = -0.45$, $P = 0.05$, respectively; $n = 19$), and from the ln of ferrous sulfate absorption ($r = -0.81$, $P < 0.001$; $r = -0.60$, $P = 0.007$, respectively; $n = 19$). Serum hepcidin and SF were both inversely related with the ln of iron absorption from ferrous sulfate (serum hepcidin: $P = 0.007$, $y = 3.50–0.25\times$; $R^2 = 0.36$; SF: $P < 0.001$, $y = 4.52–0.42\times$; $R^2 = 0.65$) and lentils (serum hepcidin: $P = 0.05$, $y = 0.89–0.35\times$, $R^2 = 0.21$; SF: $P = 0.05$, $y = 1.77–0.44\times$, $R^2 = 0.21$; **Figure 1**). Linear regression models revealed that SF alone explained 53% of the variance in iron absorption from the supplemental source and 17% from the lentil meal. Serum hepcidin explained 36% of the variance in iron absorption from the supplemental source and 21% from the lentil meal. Together, SF and hepcidin explained 54% of the variance in iron absorption from the supplemental iron source and 22% from the lentil meal.

TABLE 2 Characteristics and iron status indicators of nonpregnant, young women

Variable	Anemic ($n = 6$)	Nonanemic ($n = 13$)	All subjects ($n = 19$)
Age,[1] y	23.4 ± 4.13 (19.0–30.0)	23.9 ± 5.01 (18.0–34.0)	23.7 ± 4.64 (18.0–34.0)
Weight,[2] kg	62.5 ± 12.6 (46.4–84.2)	56.8 ± 5.90 (48.9–67.5)	58.6 ± 8.64 (46.4–84.2)
BMI,[1] kg/m^2	24.4 ± 4.20 (19.0–28.9)	23.8 ± 2.80 (18.4–25.4)	24.2 ± 3.20 (18.4–28.9)
Hemoglobin,[2] g/dL	10.7 ± 0.80** (9.7–11.70)	13.2 ± 0.70 (12.0–14.7)	12.4 ± 1.40 (9.70–14.7)
Serum folate,[2] μg/L	20.1 ± 7.90 (12.4–35.0)	18.4 ± 4.60 (9.20–26.4)	19.0 ± 5.70 (9.20–35.0)
Serum vitamin B-12,[2] pg/mL	610 ± 125 (458–776)	453 ± 201 (161–793)	497 ± 194 (161–793)
Serum C-reactive protein,[1] mg/L	0.60 ± 0.90 (0.22–2.08)	1.10 ± 1.90 (0.24–6.64)	0.90 ± 1.70 (0.22–6.64)
Serum ferritin,[1] μg/L	10.4 ± 16.9* (4.40–48.6)	61.8 ± 33.4 (31.7–123)	35.2 ± 38.5 (4.40–123)
Serum transferrin receptor,[1] mg/L	6.90 ± 12.0 (2.60–34.5)	4.50 ± 3.40 (2.00–14.2)	5.10 ± 7.30 (2.00–34.5)
Total body iron,[2] mg/kg	0.02 ± 5.59** (0.00–9.07)	7.80 ± 2.67 (3.92–12.9)	5.48 ± 5.29 (0.00–12.9)
Serum hepcidin,[1] μg/L	1.90 ± 7.00* (0.33–16.4)	12.6 ± 6.00 (3.78–23.2)	6.90 ± 7.50 (0.33–23.2)

[1] Data are geometric means ± SDs (range). *, **Different from non-anemic women: *$P < 0.05$, **$P < 0.001$.
[2] Data are means ± SDs (range).

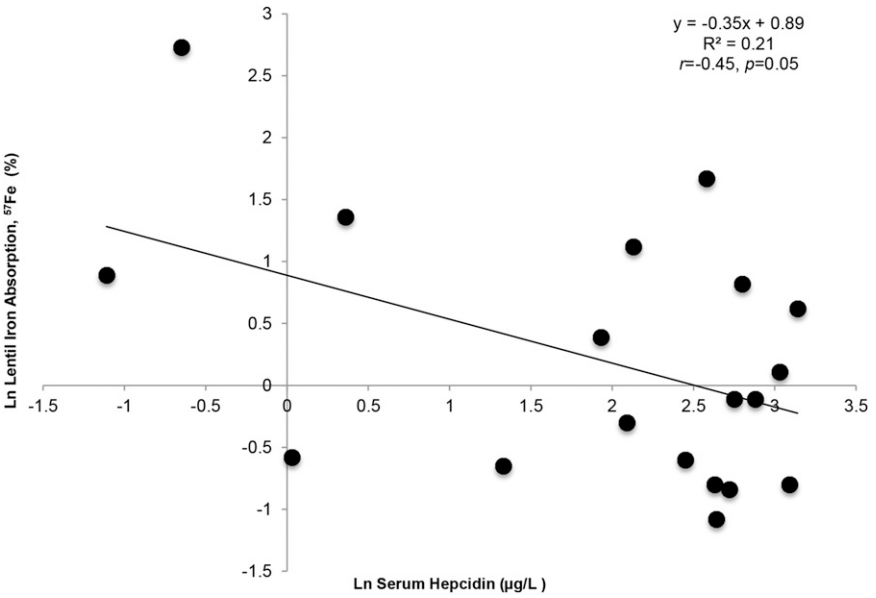

FIGURE 1 Serum hepcidin was significantly inversely related to iron absorption from the ^{57}Fe lentil meal in nonpregnant, young women (n = 19). The ln of serum hepcidin was correlated with the ln of percentage of iron absorption from dal (r = −0.45, P = 0.05) and from the ln of ferrous sulfate absorption (r = −0.60, P = 0.007; not shown).

Discussion

Sufficient iron must be absorbed from the diet to offset daily iron losses and to prevent development of ID and ID anemia. Failure to meet this nutritional necessity is a driving force in the development of ID anemia of persons who depend on nondiverse diets of staple food crops. Thus, the biofortification strategy was developed for crops such as legumes because these crops can be major sources of dietary iron and therefore can help prevent ID. This study adds to the growing body of literature of the bioavailability of non-heme iron sources from a staple food crop, and, to our knowledge, it represents the first direct measurement of iron absorption from intrinsically labeled lentils in women with a range of iron status, which is a useful first step to assess the bioavailability of iron in lentils as we move this crop forward with iron biofortification.

With the use of intrinsically labeled lentils, we observed that the percentage of iron bioavailability from a lentil meal was significantly lower than that observed from a similar iron dose of ferrous sulfate; however, the amount of iron obtained from this commonly ingested food source is similar to previous human studies that evaluated absorption of non-heme iron from other plant-based foods, including sweet potato and the common bean (8, 15–17). This is interesting because the PA concentrations of the lentils used in this study were also on the low side of normal, resulting in a PA-to-iron molar ratio of ~5.5:1. Although this PA content is lower than many other grains and legumes, adding iron absorption enhancers (e.g., vitamin C that would be abundant in potatoes) and/or reduction of inhibitors (polyphenols, PA) may positively alter iron bioavailability of lentil and other crops (15, 18). At present, the exact role of polyphenols in lentils remains unclear. In the present study, the lentils were dehulled, and as such the polyphenol concentrations would be much lower than in whole lentils (19). We suggest, therefore, that the primary inhibitor of iron absorption in the present study is likely PA.

It should be also noted that the meals consumed in this study contained only lentil. Variability in food combinations often consumed along with lentils (e.g., rice, potatoes, vegetable curry, and fish) would certainly influence the iron bioavailability of the lentils, perhaps by diluting out the inhibitory factors (e.g., PA, polyphenols). For example, in a series of experiments conducted with the use of the in vitro digestion/Caco-2 cell culture method, we found that the total amount of iron absorbed from traditional Bangladeshi meal plan models depended on iron concentration and that PA and polyphenols were likely inhibitors of iron uptake (20). The more lentil replaced rice in the meal plan model (lentil being the main contributor of iron), the higher relative iron bioavailability of the meal plan model. In those experiments we found that the addition of small amounts of fish had no significant impact on relative iron bioavailability.

In the present study, a 330-g serving of dal provided, on average, 0.10 mg absorbable Fe, corresponding to 6.60% of the estimated average daily amount of absorbed iron required by nonpregnant women (1.50 mg/d). However, it is important to note that the serving of dal used in this study was much larger than the average serving typically consumed by women in developing countries on a daily basis, such as Bangladesh. Polished rice accounts for at least 66% of total food (in g) consumed in Bangladeshi women, and lentils comprise ~2–3% of total food intake (21). Although the dal used in this study was close to a traditional Bangladeshi preparation, no rice or vegetable curry was served as would be traditional (e.g., dal bat). In addition, if affordable and/or available, local Bangladeshi people may add fish to the meal. We did not add these meal components in this study because they may have affected iron absorption via additional PA, polyphenols, or heme iron. As stated previously, the primary goal of the present study was to provide some baseline information as to what an intrinsically labeled lentil could deliver in terms of absorbable iron.

The iron absorption from lentils alone (not incorporated into dal) was not tested in this human study, because that is not how lentils are commonly consumed. Because of genotypic, environmental, and other factors, iron concentration of lentils varies widely (3). In vitro studies conducted on the whole lentil used in this study displayed similar relative iron bioavailability to other whole and dehulled red lentil varieties of similar iron content (3, 22). The additional ingredients added to the lentil (turmeric, salt, onion, garlic, and vegetable oil) may have added additional polyphenolic compounds to the dehulled lentil, possibly decreasing iron bioavailability in the human study. However, previous work has shown turmeric not to inhibit iron absorption from non-heme iron-based meals in young women, despite its high

polyphenolic content (23). The additions of garlic and onion were actually shown to significantly improve iron bioavailability from non-heme iron sources (pulse- and grain-based meals) in an in vitro model, likely because of their sulfur compounds (24).

As expected, hemoglobin was significantly correlated with both SF and hepcidin, and SF was also significantly correlated with hepcidin in our subjects. Both SF and serum hepcidin were inversely related to the ln of iron absorption from ferrous sulfate and the lentil meal. Serum ferritin alone explained 53% of the variance in iron absorption from the supplemental source and 17% from the lentil meal. Serum hepcidin explained 36% of the variance in iron absorption from the supplemental source and 21% from the lentil meal. This is similar to what was reported by other investigators (8, 25).

In conclusion, this study shows that the total amount of iron absorbed from a traditional Bangladeshi lentil meal is low. Although ID remains the most common nutrient deficiency in the world, biofortification efforts should focus on not only increasing mineral content but also on reduction of known absorption inhibitors such as PA that can be found at relatively high concentrations in foods such as lentils. Additional efficacy studies are needed to assess iron bioavailability not only of staple food crops but also of mixed meals because they are regularly consumed in regions where ID is prevalent.

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