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Utilization of Iron from an Animal-Based Iron Source Is Greater Than That of Ferrous Sulfate in Pregnant and Nonpregnant Women[1–3]

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Abstract

Heme iron absorption during pregnancy and the role of hepcidin in regulating dietary heme iron absorption remains largely unexplored. The objective of this research was to examine relative differences in heme (animal based) and nonheme (ferrous sulfate) iron utilization. This study was undertaken in 18 pregnant (ages 16–32 y; wk 32–35 of gestation) and 11 nonpregnant women (ages 18–27 y). Women were randomly assigned to receive both an animal-based heme meal (intrinsically labeled ^{58}Fe pork) and labeled ferrous sulfate (^{57}Fe) fed on alternate days. Blood samples obtained 2 wk postdosing were used to assess iron status indicators and serum hepcidin and iron utilization based on RBC incorporation of iron isotopes. Heme iron utilization was significantly greater than nonheme iron utilization in the pregnant (47.7 ± 14.4 vs. 40.4 ± 13.2%) and nonpregnant women (50.1 ± 14.8 vs. 15.3 ± 9.7%). Among pregnant women, utilization of nonheme iron was associated with iron status, as assessed by the serum transferrin receptor concentration ($P = 0.003$; $r^2 = 0.43$). In contrast, heme iron utilization was not influenced by maternal iron status. In the group as a whole, women with undetectable serum hepcidin had greater nonheme iron utilization compared with women with detectable serum hepcidin ($P = 0.02$; $n = 29$); however, there were no significant differences in heme iron utilization. Our study suggests that iron utilization from an animal-based food provides a highly bioavailable source of dietary iron for pregnant and nonpregnant women that is not as sensitive to hepcidin concentrations or iron stores compared with ferrous sulfate. J. Nutr. 140: 2162–2166, 2010.

Introduction

Iron (Fe) deficiency affects ~1.6 billion people globally, with pregnant women being at increased risk (1). Anemia during pregnancy is associated with labor/delivery complications, preterm delivery, low birth weight, reduced infant Fe status, impaired mother-child interactions, and increased infant and maternal mortality (2–4). Due to these adverse outcomes, an understanding of dietary Fe bioavailability from all dietary iron sources is needed. These data will help inform the development of public health programs and nutritional recommendations.

Dietary Fe is obtained from nonheme (mostly plant-based or supplemental sources) and heme (mostly animal based) sources. Although the cellular process of nonheme Fe absorption is largely known, the proteins involved in heme Fe absorption have yet to be fully characterized (5,6). Previous research in nonpregnant women found heme Fe absorption was ~3 times higher than that of nonheme Fe (7). Absorption of nonheme iron in men has also been found to be more responsive to alterations in body Fe stores compared with absorption of heme Fe (8). The impact of the increased Fe demands of pregnancy on heme compared with nonheme Fe absorption remains unknown and to our knowledge heme Fe absorption has not been assessed during pregnancy in humans.

Hepcidin is a small hormone produced by the liver that is now known to be a key regulator of Fe homeostasis (9). This hormone is inversely associated with absorption of nonheme Fe in nonpregnant women and men (10,11). At present, no human data on the role of this hormone on iron absorption during

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[3] This trial was registered at clinicaltrials.gov as NCT01019096.

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pregnancy are currently available. An improved understanding of possible relationships between serum hepcidin and absorption of dietary Fe from both heme and supplemental Fe sources could potentially provide valuable information and therapeutic options for anemic pregnant women.

To address these issues, we undertook an Fe utilization study using 2 oral stable iron isotopes (^{57}Fe and ^{58}Fe) in a group of pregnant and nonpregnant women. Fe utilization from an intrinsically labeled animal-based heme was compared with absorption of ferrous sulfate. We hypothesized that women would have higher Fe utilization from the heme source and that hepcidin would be associated with both heme and nonheme Fe utilization.

Materials and Methods

Twenty nonsmoking, pregnant women (≥19 y; n = 10) and adolescents (≤18 y; n = 10) were recruited between 2008 and 2009 from the Strong Midwifery Group and the Rochester Adolescent Maternity Program in Rochester, NY. All women had uncomplicated pregnancies at the time of recruitment. Informed written consent was obtained and the study was approved by the Institutional Review Boards of Cornell University and the University of Rochester. Twelve healthy, nonsmoking, nonpregnant women ages 18–27 y were recruited in 2009 from Ithaca, NY. Nonpregnant participants did not consume any supplements for 1 mo prior to the study and during the study. Informed written consent was obtained from each participant and the study was approved by the Institutional Review Board of Cornell University. Exclusion criteria for both groups included underlying malabsorption diseases or diagnosed medical problems known to affect Fe homeostasis.

Stable Fe isotopes were purchased as the metal: ^{57}Fe at 94.69% enrichment and ^{58}Fe at 93.34% enrichment from Trace Sciences International. The nonheme Fe tracer (^{57}Fe) was prepared as a sterile, pyrogen-free solution of ferrous sulfate by Anazao Health using a similar method to that reported by Kastenmayer et al. (12). The Fe tracer (^{58}Fe) used for intrinsic labeling of heme was first converted from the metal into a solution of ferrous citrate in our laboratory and was sent to Analytical Research Labs for sterility testing. Isotopic composition of tracers was validated using magnetic sector thermal ionization mass spectrometry (TIMS)[9] (Thermo Scientific Triton TI, Thermo Fisher Scientific). The heme iron source was prepared by intrinsically labeling (^{58}Fe) porcine muscle and RBC at Baylor College of Medicine following previously published methods (13) as approved by the Baylor College of Medicine Institution Animal Care and Use Committee. In brief, a 3-d-old female piglet was housed in a stainless steel stall and fed a diet to induce Fe deficiency (Fe-free sow replacement milk, Research Diets). Piglets are typically given i.m. Fe at birth (100–200 mg); this standard Fe injection was replaced with two 75-mg doses of ^{58}Fe as ferrous citrate. At 28 d of age, the piglet was killed at a Texas A&M University facility approved by the USDA and the State of Texas. The RBC and muscle were collected and processed (13). Isotopic enrichment of ^{58}Fe in the muscle and RBC was quantified by TIMS and did not significantly differ between these 2 sources (18.0 ± 0.80% and 17.3 ± 0.01%, mean ± SD, respectively).

To accommodate recruitment, 2 batches of the heme Fe meal were prepared. The first batch prepared for the pregnant participants used enriched ground pork (203 g) and enriched RBC (3.9 g) in combination with tomato sauce (Hunts) and chili seasoning (McCormick). Each serving provided 0.46 mg of ^{58}Fe from pork and 0.46 mg of ^{58}Fe from RBC for a total ^{58}Fe dose of 0.92 mg and 7.9 mg total Fe/serving. In the second batch of pork, nonenriched ground pork (198 g) and enriched RBC (3.5 g) were combined with tomato sauce and chili seasoning. The intrinsically labeled pork provided 0.6 mg of labeled ^{58}Fe RBC and a total iron load of 9.9 mg Fe/serving. All labeled meals were well mixed and frozen as individual 320-g servings until use. Aliquots of both test meals were sent to Microbac Laboratories for food safety testing and analyzed for isotope abundance by TIMS and total Fe content by atomic absorption spectrophotometry (PerkinElmer Analyst 800). The nonheme iron source administered was given as, 8.2 mg of ^{57}Fe as ferrous sulfate (total Fe, 8.6 mg) flavored with 2 mL of raspberry syrup containing 0.391% ascorbic acid (Humco).

Clinical studies in the pregnant participants were undertaken at Strong Memorial Hospital's Clinical and Translational Science Institute (CTSI). Women came to the CTSI during the last trimester of pregnancy (32–35 wk gestation) on 2 consecutive days. Women were asked to discontinue prenatal supplementation for 3 d before and 2 d following dosing. On the study morning, each fasting woman's height and weight were recorded with the use of a stadiometer and a calibrated scale. Women received a standard breakfast and 2 h later ingested the test meal (intrinsically labeled ^{58}Fe heme meal or ^{57}Fe as ferrous sulfate) without other food or liquids. The labeled Fe was fed randomly (coin toss for order) so that one-half of the women received the heme meal on the first day and the ferrous sulfate on the second day or these forms of iron in the reverse order. Each participant served as her own control to compare relative differences in iron utilization, which are not affected by possible differences in blood volume and RBC iron incorporation between participants. To further explore relative differences in each of the pregnant and nonpregnant groups, the ratio of ^{58}Fe utilization:^{57}Fe utilization was compared.

After ingesting each test meal, women remained in the CTSI for an additional 2 h before consuming a standard lunch. All women were then given a standard dinner and snacks to take home and consume. The pregnant adolescents remained overnight at the CTSI between test meals to minimize transportation difficulties. All participants consumed the same amounts and types of foods on the second test day as those ingested on the first day. Participants returned to the CTSI 2 wk after the second Fe tracer was ingested for collection of a blood sample. Clinical studies in nonpregnant participants were undertaken at the Human Metabolic Research Unit at Cornell University by using the same protocol as detailed above.

Hemoglobin and hematocrit were analyzed at the Strong Memorial Hospital's clinical laboratory by using the Cell Dyn 4000 system (for pregnant participants) and a Coulter hematology analyzer at the Human Metabolic Research Unit (for nonpregnant participants). The hemoglobin cutoffs used to define anemia were based on CDC guidelines (14). Whole blood was centrifuged and serum was stored at −80°C until analysis. Serum ferritin was measured using an enzyme immunoassay (Ramco Laboratories). Ferritin concentrations < 20 μg/L were indicative of depleted iron stores (15). An indicator of inflammation [C-reactive protein (CRP)] and serum folate, vitamin B-12, and erythropoietin were assessed using the Immulite1000 immunoassay system. Serum soluble transferrin receptor (TfR) was measured using an ELISA (Ramco Laboratories). Total body iron (TBI) was calculated based on a formula developed using serial quantitative phlebotomy data in healthy men and nonpregnant women: {TBI (mg/kg) = −[log (serum transferrin receptor/serum ferritin) − 2.8229]/0.1207} (16,17). Although validation of this equation is not possible in pregnant women, this measure has been utilized in pregnant populations (16,18). Serum leptin was measured by ELISA (Millipore). Serum hepcidin was measured using a competitive serum ELISA (Intrinsic Life Sciences) (19). The lower limit of detection for this assay is 5 μg/L.

To assess iron utilization, blood (0.5 mL) was digested and iron was extracted using anion exchange chromatography (20). Iron isotope ratios were measured using TIMS and corrected for fractionation as previously reported (20,21). Fe utilization was determined as described in previous publications (12,20,22). The enrichment of each isotope in RBC was measured as the degree to which the natural abundance (NA) ratio in RBC was increased as a result of incorporation of the stable Fe tracer. NA ratios utilized were 0.02317 for $^{57/56}$Fe and 0.00307 for $^{58/56}$Fe. The total circulating Fe (mg) was estimated by using the participants' weight (kg), a blood volume estimate (65 mL/kg for nonpregnant and 70 mL/kg for pregnant participants), hemoglobin concentration (kg/L), and the concentration of Fe in hemoglobin (3.47 g/kg) as reported by Fomon et al. (23). Total Fe incorporated was then calculated using the RBC enrichment, total circulating Fe, the Fe dose administered and the NA of isotope given [NA_{57}, 0.0214; NA_{58},

[9] Abbreviations used: CRP, C-reactive protein; CTSI, Clinical and Translational Science Institute; NA, natural abundance; PE, preeclampsia; TBI, total body iron; TIMS, thermal ionization MS; TfR, transferrin receptor.

0.00287 (24)]. Final values for RBC Fe incorporation were determined based on the assumption that 80% of the absorbed Fe would be incorporated into erythrocytes (25,26). An additional correction factor was used to correct for the small amount of ^{58}Fe that was contributed by the ^{57}Fe tracer (12).

Data were analyzed using JMP 8.0 (SAS Institute). Linear regression analyses were used to examine relationships among Fe status, hepcidin, and Fe utilization in each of the nonpregnant and pregnant groups. Paired *t* tests were used to determine the significance of relationships between heme and nonheme Fe utilization in participant groups. Differences between participant groups were compared using a *t* test or the Wilcoxon's rank sum test for nonparametric data. Differences in categorical variables race (black/white) and ethnicity (Hispanic/non-Hispanic) were compared using Fisher's exact test. Serum hepcidin concentrations below the limit of detection were artificially assigned a value of 0 μg/L and for data transformation the raw value zero plus one was utilized as previously described (27). A cohort of 20 pregnant women was selected to provide a power of 80% using an α level of 0.05 to detect a 4.5% difference in iron utilization, allowing for an attrition rate of 20%. Variables were tested for normality using a goodness-of-fit test. Those that were not normally distributed were transformed (natural log) prior to statistical testing. Results were analyzed with and without the 2 participants who developed preeclampsia (PE). The results and *P*-values are given for the complete population unless inclusion of PE participants affected the significance as noted in the results. Differences were considered significant at $P < 0.05$.

TABLE 2 Iron status indicators for pregnant and non-pregnant participants[1,2]

Variable	Pregnant, *n* = 18	Nonpregnant, *n* = 11
Hemoglobin,* *g/L*	114 (101–129)	130 (118–144)
Anemic,[3] *%*	<110 g/L: 39	<120 g/L: 9
Ferritin,* *μg/L*	17.5 (4–77)	40.5 (17–96)
<12 μg/L	22	0
<20 μg/L	61	9
Transferrin receptor,* *mg/L*	6.1 (4–9)	4.2 (4–71)
>8.5 mg/L,%	11	0
TBI,* *mg/kg*	2.3 (-3–9)	6.0 (3–9)
<0 mg/kg, %	22	0
Hepcidin,* *μg/L*	0 (0–207)	16.0 (0–44)
CRP,* *g/L*	3.5 (0–66)	0.66 (0–5)
Folate, *nmol/L*	37.9 (18–100)	38.6 (18–41)
Vitamin B-12, *pmol/L*	214 (151–552)	224 (111–726)
Erythropoietin,* *IU/L*	30.5 (16–80)	12.7 (8–40)
Leptin,* *μg/L*	40.6 (6–106)	9.8 (3–23)

[1] Values are mean (range) of normally distributed data and median [range] for non-normally distributed data or percent. *Groups differed, $P < 0.05$.
[2] To convert mg of iron to mmoL, divide the mg quantity by the atomic weight of iron (55.847).
[3] For pregnant, Hb < 110 g/L; for nonpregnant, <120 g/L.

Results

Nonpregnant participants. General characteristics of the study participants are provided in **Table 1**. Three nonpregnant participants were overweight. One nonpregnant participant did not attend the second day of dosing and was removed from the study. Final data were collected on 11 nonpregnant participants. Iron status indicators of in the study participants were characterized (**Table 2**). Only 1 woman was anemic (Hb = 118 g/L). Three women had undetectable serum hepcidin and they all had serum ferritin values near the value thought to be indicative of depleted iron stores (<20 μg/L).

In women who were not pregnant, Fe utilization from the intrinsically labeled pork meal (50.1 ± 14.8%) was greater than that of ferrous sulfate (15.3 ± 9.7%; $P < 0.001$). Fe utilization was not significantly affected by the order that the 2 forms of Fe were fed. Fe status was not significantly related to either nonheme or heme Fe utilization with the exception of the serum TfR concentration, which was related to heme Fe utilization ($P = 0.04$; $r^2 = 0.39$). Serum hepcidin was related to serum ferritin ($P = 0.03$; $r^2 = 0.42$) and TBI ($P = 0.03$; $r^2 = 0.44$). In this group, serum hepcidin was not significantly associated with iron utilization from either iron source. The ratio of ^{58}Fe utilization:^{57}Fe utilization was 4.8 ± 3.1 in the women who were not pregnant.

TABLE 1 General characteristics of the participants[1]

Variable	Pregnant, *n* = 18	Nonpregnant, *n* = 11
Age at enrollment,* *y*	18.5 (16–32)	21.5 (18–27)
BMI at time of study,* *kg/m*	32.5 (23.3–46.5)	23.0 (18.9–25.8)
Prepregnancy BMI, *kg/m*	25.0 (20.7–43.6)	N/A
Gestational age at study, *wk*	33.4 (32.0–34.6)	N/A
Race,* *%*		
African American	50	0
Caucasian	50	73
Native American	0	9
Asian	0	18
Ethnicity,* *%*		
Hispanic	39	0
Non-Hispanic	61	100

[1] Values are mean (range) of normally distributed data and median [range] for non-normally distributed data or percent. *Groups differed, $P < 0.05$.

Pregnant participants. Among enrolled pregnant women, 50% had a normal BMI, 22% were overweight (BMI > 25 kg/m^2), and 28% were obese (BMI > 30 kg/m^2) as assessed by prepregnancy medical chart weights (29). Both prepregnancy BMI ($P = 0.01$) and racial composition of the pregnant cohort were significantly different from the nonpregnant cohort (Table 1).

Two pregnant participants did not complete the study; one did not finish the heme meal and the other did not return for the 2-wk blood draw. The final study population included 18 pregnant participants. Two pregnant participants (ages 16 and 20 y) developed PE; in these women, blood samples were obtained at delivery which occurred at 10 and 14 d postdosing.

Anemia was prevalent (Table 2). The majority (67%) of these pregnant women had undetectable serum hepcidin and depleted iron stores in the 3rd trimester of pregnancy. Fe utilization from the intrinsically labeled pork meal (47.7 ± 14.4%) was greater than that observed for ferrous sulfate (40.4 ± 13.2%) ($P = 0.04$). Fe utilization was not significantly affected by the order that the 2 forms of Fe were fed. The ^{58}Fe utilization:^{57}Fe utilization ratio was 1.26 ± 0.44 in the pregnant women, a value that differed from that of the nonpregnant cohort ($P = 0.003$).

Nonheme Fe utilization was inversely associated with TBI ($P = 0.02$; $r^2 = 0.29$) and directly associated with TfR ($P = 0.003$; $r^2 = 0.43$). In contrast, relationships between heme Fe utilization and maternal TBI ($P = 0.08$) and TfR ($P = 0.06$) only approached significance. Neither heme nor nonheme iron utilization were related to BMI, leptin, or CRP. Hepcidin was directly associated with serum ferritin ($P < 0.0001$; $r^2 = 0.63$) and TBI ($P = 0.0005$; $r^2 = 0.54$). In the pregnant women, serum hepcidin was not significantly associated with non-heme iron utilization. However, serum hepcidin was correlated with nonheme Fe utilization

(P = 0.03; r^2 = 0.17) when data from the pregnant and nonpregnant participants were combined (n = 29). When the 2 participants with PE were excluded from the analysis, this relationship was further strengthened (P = 0.01; r^2 = 0.24). In the group as a whole, women with undetectable serum hepcidin (<5 μg/L, n = 15) had greater nonheme Fe utilization compared with those with detectable serum hepcidin (n = 14; P = 0.02). This difference tended to occur in the pregnant group alone (P = 0.1; n = 12 and 6, respectively). In contrast, heme Fe utilization was not significantly associated with serum hepcidin in the group as a whole or within each cohort.

Discussion

Iron utilization from the heme animal-based iron source was significantly higher than that observed for ferrous sulfate in both the pregnant and nonpregnant groups. This research supports the existing literature on the enhanced bioavailability of heme iron in men and nonpregnant women (8,29,30) and extends the relevance of these findings to pregnant women.

The role of hepcidin in the regulation of Fe absorption during pregnancy has been largely unexplored. In the current study, serum hepcidin was inversely associated with markers of Fe status in the pregnant and nonpregnant populations. Likewise, previous research has established relationships between urinary hepcidin and maternal iron status in pregnant Bangladeshi women (31). In our study, nonheme Fe utilization was inversely related to serum hepcidin as evidenced by the finding that women with undetectable levels of serum hepcidin had significantly greater nonheme Fe utilization. Serum hepcidin explained ~24% of the variation in nonheme Fe utilization in healthy pregnant and nonpregnant participants, which is similar to our previous research in nonpregnant women (26%) (10). The nonsignificant findings in the pregnant or nonpregnant cohorts alone are likely due to the small sample size and limited range of iron status in each of the individual groups. Even when combining data from both groups, there was only 23% power to detect a significant correlation between serum hepcidin and nonheme iron absorption. Although there appeared to be a trend for greater nonheme iron utilization in pregnant women with undetectable serum hepcidin, to fully explore this association in pregnant women, our data indicated that over 100 participants would be needed to achieve a power of 80%. Using animal models, hepatic hepcidin expression has been found to decrease during pregnancy, resulting in increased Fe absorption (32). Hepcidin regulates nonheme Fe absorption by binding to ferroportin, effectively blocking Fe export (33), and hepcidin may also inhibit apical iron uptake in the enterocyte (34). Although heme iron does not enter the enterocyte through the same pathway as nonheme iron, the exact mechanisms are yet to be fully elucidated and may or may not involve the recently identified heme carrier protein/proton coupled folate transporter (35,36). The process by which heme exits the enterocyte likewise has not been definitively characterized. Absorbed heme may be degraded in the cell into nonheme iron and subsequently exported entirely using nonheme iron transport mechanisms. The presence of heme exporters in the enterocyte may also suggest that some heme is exported intact (6). Potential differences in cellular iron trafficking may explain the lack of association between heme Fe utilization and hepcidin.

Limitations of the current study design include our estimation of RBC Fe incorporation. In nonpregnant women, RBC Fe incorporation of absorbed iron is relatively stable at 80%; however, estimates of RBC Fe incorporation during pregnancy can be highly variable and are affected by Fe supplementation and maternal iron stores (22). Furthermore, incorporation of dietary iron into maternal RBC during pregnancy reflects maternal utilization of iron but does not capture the amount of absorbed iron that was transferred directly to the fetus. In a prior study of nonheme iron absorption, the amount of nonheme dietary Fe transferred to the fetus underestimated maternal Fe absorption by ~5% (37). In the current study, each participant served as her own control, thus the relative difference in heme compared with nonheme iron utilization was not affected by errors introduced by blood volume and RBC incorporation assumptions.

Differences in iron status and composition of the labeled heme meal (labeled meat and RBC vs. labeled RBC alone) among the pregnant and nonpregnant participants preclude us from making conclusions about the impact of pregnancy alone on heme and nonheme iron utilization. As expected, the pregnant participants had significantly higher rates of iron depletion compared with nonpregnant participants. An examination of the ratios of heme:nonheme iron utilization in each group indicated that there appeared to be less difference in iron utilization between the 2 forms of iron among pregnant participants. A similar attenuated relationship between iron stores and heme Fe absorption was reported among blood donors (lower Fe status) in whom a 2-fold difference between heme and nonheme iron absorption was noted compared with the 5-fold difference in Fe-replete nonblood donors (8). The degree to which differences in the composition of the labeled heme meal substantially affected values of iron utilization is unknown and is a limitation of our study design. In an early radiolabeled veal study, 95% of administered isotope was found in hemoglobin and myoglobin (30). We did not assess the relative partitioning of iron into heme compared with nonheme sources in the labeled pork, but prior research has reported this to vary from 31 to 66% (38). The race/ethnicity of the pregnant participants in our study was not comparable to the nonpregnant participants. To our knowledge, no data at present have found cellular iron flux to vary as a function of race, although race/ethnicity differences in iron status previously have been reported (39,40). Additional research is needed to further understand the role of pregnancy, independent of iron status or race, on upregulating iron utilization. Furthermore, although leptin and CRP did not appear to be associated with iron utilization and iron status in this group, larger studies are needed to assess the role of obesity/inflammation and its possible contribution to iron deficiency in this population.

In summary, both pregnant and nonpregnant women had a significantly higher utilization of heme iron relative to utilization of a similar iron load from ferrous sulfate. Heme iron utilization was not significantly influenced by iron stores or serum hepcidin in either the pregnant or nonpregnant women. During pregnancy, compliance with prenatal iron supplementation is a common problem. Thus, emphasis on how to best meet iron requirements from the diet alone is an important concern. The significantly greater bioavailability of heme iron may help to achieve requirements for absorbed dietary iron, which are particularly high during the 3rd trimester of pregnancy. Further research is required to understand the mechanisms by which heme iron utilization is enhanced and to characterize the ability of these 2 forms of iron to meet both maternal and fetal iron demands during pregnancy.

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