Production of stable-isotope-labeled bovine heme and its use to measure heme-iron absorption in children[1–4]

Paz Etcheverry, Gordon E Carstens, Erin Brown, Keli M Hawthorne, Zhensheng Chen, and Ian J Griffin

ABSTRACT

Background: The use of stable isotopes has provided valuable insights into iron absorption in humans, but the data have been limited to nonheme iron.

Objective: Our objectives were to produce heme iron enriched in ^{58}Fe and to use it to study the absorption of heme iron and the effect of iron and zinc intakes on heme-iron absorption in children.

Design: Labeled bovine heme was produced in a bovine model. Forty-eight children were randomly assigned to consume identical meals containing 1 of 3 doses of labeled heme iron (2, 4, or 8 mg as hemoglobin) and 1 of 2 doses of inorganic zinc (1 or 9 mg); successful measurements of iron absorption, zinc absorption, or both were made in 40 of these subjects. We hypothesized that fractional heme-iron absorption would decrease as heme-iron intake increased and that higher zinc intakes would decrease heme-iron absorption.

Results: ^{58}Fe heme was produced with an enrichment (mass/mass) of 9.5%. Fractional iron absorption in children was significantly affected by the intake of heme iron ($P = 0.0013$) and of zinc ($P = 0.0375$), but, contrary to expectations, heme-iron absorption was higher at higher zinc intakes. Absolute heme-iron absorption was higher in the group with higher zinc intakes, but only for those with the lowest heme-iron intake (2 mg; $P = 0.0147$). Although fractional zinc absorption decreased as zinc intake increased ($P = 0.031$), absolute zinc absorption continued to increase across the intake range studied ($P = 0.018$).

Conclusions: Heme iron intrinsically labeled with ^{58}Fe can be produced at sufficient enrichments for use in human studies. In children, heme iron and zinc absorption decrease as the dose of each mineral increases. Heme iron did not inhibit zinc absorption. At lower heme intakes, zinc intakes may increase heme-iron absorption. *Am J Clin Nutr* 2007;85:452–9.

INTRODUCTION

Iron deficiency is the most common micronutrient deficiency worldwide, affecting up to 2 billion persons, and children and pregnant women bear a disproportionate burden (1). Stable isotopes of iron have been used to study iron absorption in high-risk populations and have provided valuable insights into human iron metabolism (2, 3).

Dietary iron can be either heme iron or nonheme iron. Stable iron isotopes have been widely used as intrinsic or extrinsic labels to measure nonheme-iron absorption. For example, to measure iron absorption from sow milk, one can add the isotopic label either to the milk (extrinsic labeling) or to the sow's feed and subsequently produce milk enriched with the isotope (intrinsic labeling). In many cases the 2 methods produce similar results because the extrinsic nonheme iron label enters a "common pool" with intrinsic nonheme iron present in the food, and absorption of the label reflects the absorption of the intrinsic nonheme iron (4). However, because heme iron is absorbed by a different mechanism than is nonheme iron, it is unlikely that absorption of a nonheme-iron isotope will reflect heme-iron absorption. Therefore, labeled heme is required. It is fairly simple to intrinsically label heme with radioactive iron because relatively low enrichments are required, and this has been done for >50 y (5). Typically, animals are injected with ^{59}Fe or ^{55}Fe, and labeled tissue is collected. Usually, labeled hemoglobin is used, either from rabbits (6) or swine (7, 8); however, veal muscle (9) and rabbit liver (10) have also been used. Although these labeled products may provide important insights into iron metabolism in adults (11), they are not ethically appropriate for use in children or pregnant women—populations that are at high risk of iron deficiency. The study of heme-iron absorption in these populations requires stable-isotope-enriched heme, but to our knowledge no successful attempts to intrinsically label heme iron with a stable iron isotope have been reported.

The objectives of this study were *1*) to produce heme that is sufficiently enriched to allow studies in children consuming physiologically and nutritional meaningful amounts of heme iron, *2*) to study the effect of heme-iron intake on heme-iron absorption, *3*) to examine the effect of zinc intake on heme-iron absorption, and *4*) to examine the effect of heme-iron intake on

zinc absorption. We hypothesized that as the intake of heme iron increases the fractional heme-iron absorption will decrease and absolute heme-iron absorption will increase. We also hypothesized that an increase in heme-iron intake will reduce fractional zinc absorption and an increase in zinc intake will reduce fractional heme-iron absorption.

SUBJECTS AND METHODS

Intrinsic labeling protocol

Animal management and isotope administration

A Holstein calf (birth weight: 41.7 kg) was removed from its dam at 3 d of age and was transported to an outdoor pole barn with 2×3 m pens located at the research center at Texas A&M University. On arrival, ≈ 200 mL blood (an estimated 10% of total blood volume) was collected via jugular venipuncture and discarded. Twice a day, the calf was fed 1.9 L whole cow milk that was warmed to $37\,°C$ and supplemented with 25 mg α-tocopheryl acetate/d and 200 mg Mg/d (as magnesium chloride). This diet was selected because it has been shown to cause iron deficiency anemia in calves (12) and was therefore expected to lead to increased incorporation of the subsequently administered ^{58}Fe into red blood cells.

^{58}Fe (91.55 atom%) was purchased from Trace Sciences International (Ontario, Canada) and converted to aqueous solutions of ferrous sulfate as described previously (3). The resulting solution was diluted to ≈ 1 mg/mL, filtered through a sterile 0.22-μm Millex-GP sterilizing filter unit (Millipore Corp, Bedford, MA), and stored in sterile autoclaved 10-mL glass ampules at $4\,°C$ until required.

On day 28, the calf was gently restrained and a jugular venous catheter was inserted following a normal aseptic technique; ≈ 200–300 mL blood was withdrawn through the catheter and discarded. Labeled ferrous sulfate (produced as described above) was slowly infused through the catheter over 20–25 min. After the infusion, the catheter was flushed with 25 mL normal saline and removed. The calf's heart rate and respiratory rate were monitored continuously during the course of the intravenous infusion. The total amount of intravenously administered iron was 150 mg (equivalent to ≈ 137 mg ^{58}Fe).

Blood was collected from the calf 14 d after the infusion of ^{58}Fe. An intravenous catheter was inserted into the jugular vein, and 250 mL whole blood was withdrawn over 5 min and stored in 50-mL plastic containers, each containing 1000 IU heparin. Normal saline (250 mL) was then infused into the calf over 5 min. The calf was then sedated with 5 mg ketamine/kg and 2 mg xylazine/kg intravenously. Blood was withdrawn through the catheter until the flow of blood from the catheter decreased, the jugular vein and carotid arteries were severed, and blood was collected into sterile bowls before being transferred to preheparinized 50-mL plastic tubes. Postmortem, the liver was dissected free and frozen at $-20\,°C$.

Anticoagulated blood was transferred to 50-mL test tubes and centrifuged at $3800 \times g$ in a centrifuge (model J2-21; Beckman Coulter, Fullerton, CA) for 5 min. The serum was removed and discarded, and the red blood cells were washed 3 times in normal saline before being stored at $-20\,°C$. The Animal Use Committee of Texas A&M University approved the animal protocol.

Determination of iron enrichment and iron content in red blood cells

Red blood cells (≈ 1 mL) were digested overnight in 10 mL concentrated nitric acid in a hot plate. After being dried, each sample was dissolved in 0.6 mL of 6 N HCl and loaded onto a polyethylene column containing 2 mL anion exchange resin (AG 1-8X resin; Bio-Rad Laboratories, Hercules, CA). The columns were washed with 6 mL of 6 N HCl followed by 0.5 mL of 0.5 N HCl. Iron was released from the columns by adding 1 mL of 0.5 N HCl. The eluate was collected in polytetrafluoroethylene vials, heated to dryness on a hot plate, and dissolved in 40 μL of 3% ultrapure HCl. The isotope ratios in the iron solution were then analyzed by thermal-ionization magnetic sector mass spectrometry (MAT 261; Finnigan, Bremen, Germany). The total iron content in each fraction was measured via inductively coupled argon plasma emission spectrometry.

^{58}Fe enrichment of the samples was calculated from the equation

$$\text{Iron enrichment} = {}^{58}\text{Fe content of the sample}/$$
$$\text{total iron content of the sample} \quad (1)$$

Isotope yield was calculated from the equation

$$\text{Yield} = \text{total } {}^{58}\text{Fe recovered from animal}/$$
$$\text{amount of } {}^{58}\text{Fe administered} \quad (2)$$

Human absorption protocol

Study subjects

We recruited 48 children aged 4–8 y from the Houston metropolitan area. Subjects were eligible for the study if they were healthy, did not have any food allergies, and were not taking medications or herbal supplements. Ten of the 48 children (26%) were taking vitamin or mineral supplements that they were asked to discontinue ≥ 2 wk before beginning the study. Informed written consent was obtained from the parents, and verbal assent was obtained from the children. The Institutional Review Board for Baylor College of Medicine and Affiliated Hospitals approved the protocol.

Experimental design

On the first day of the study, the children were admitted to the General Clinical Research Center (GCRC) at Texas Children's Hospital. Their heights and weights were measured with the use of a stadiometer and calibrated scale, and a local anesthetic cream (L-M-X-4; Ferndale Laboratories, Inc, Ferndale, MI) was applied over a subcutaneous vein in the antecubital fossa.

The children were given a study meal (*see* below) that was extrinsically labeled with zinc-67 as aqueous zinc chloride and with ^{58}Fe as ^{58}Fe-labeled bovine hemoglobin. Children were randomly assigned to 1 of 6 groups to receive meals that contained 1 of 3 different amounts of iron as bovine hemoglobin (2, 4, or 8 mg) and 1 of 2 different amounts of zinc (1 or 9 mg) in a 3×2 factorial design (*see* below). After consuming their meals, the children received zinc-70 (0.5 mg) as aqueous zinc chloride intravenously. The parents of the children were given coolers with a container and received oral and written instructions on the methods of collecting a urine sample 72 h later. Two weeks later,

TABLE 1
Iron and zinc contents of the test meals

	Meal					
	A	B	C	D	E	F
Iron content (mg)						
50 g Tomato sauce[1]	0.90	0.90	0.90	0.90	0.90	0.90
8 Frito Scoops corn chips[2]	0.55	0.55	0.55	0.55	0.55	0.55
15 g Cheddar cheese	0.10	0.10	0.10	0.10	0.10	0.10
40 g chicken breast	0.30	0.30	0.30	0.30	0.30	0.30
Hemoglobin iron	2.00	4.00	8.00	2.00	4.00	8.00
Total iron	3.85	5.85	9.85	3.85	5.85	9.85
Total heme iron[3]	2.12–2.24	4.12–4.24	8.12–8.24	2.12–2.24	4.12–4.24	8.12–8.24
Zinc content (mg)						
50 g Tomato sauce[1]	0.17	0.17	0.17	0.17	0.17	0.17
8 Frito Scoops corn chips[2]	0.10	0.10	0.10	0.10	0.10	0.10
15 g Cheddar cheese	0.47	0.47	0.47	0.47	0.47	0.47
40 g chicken breast	0.35	0.35	0.35	0.35	0.35	0.35
^{67}Zn tracer	1.00	1.00	1.00	1.00	1.00	1.00
Unlabeled zinc acetate	0.00	0.00	0.00	8.00	8.00	8.00
Total zinc	2.09	2.09	2.09	10.09	10.09	10.09
Iron:zinc						
Mass ratio (mg:mg)	1.8:1	2.8:1	4.7:1	0.4:1	0.6:1	1.0:1
Molar ratio (mol:mol)	2.2:1	3.3:1	5.5:1	0.4:1	0.7:1	1.1:1
Heme iron:zinc						
Mass ratio (mg:mg)	1.0:1	2.0:1	3.9:1	0.2:1	0.4:1	0.8:1
Molar ratio (mol:mol)	1.2:1	2.3:1	4.6:1	0.3:1	0.5:1	0.9:1

[1] Two 227-g cans tomato sauce, 50 g water, 1 packet (34.8 g) McCormick Mild Chili seasoning (Hunt Valley, MD).

[2] Frito-Lay, Dallas, TX.

[3] Based on the assumption that 40–80% of the iron in chicken is heme iron (as has been shown for beef; 9, 14, 15).

the children returned to the GCRC to provide blood samples for the measurement of hemoglobin, ferritin, transferrin receptors, and iron-isotope enrichment. An alternative study design, in which subjects consumed many of the different meals in random order, was rejected because it would have required many more visits to the GCRC, more venipunctures, and more intravenous infusions and would have been too great a burden for the study participants.

Study meal

The study meal given to the children was made with ground chicken. Because we were studying the effect of heme iron on zinc absorption and vice versa, we chose a type of meat with the least amount of endogenous heme iron (0.3 mg total Fe per serving, 0.12–0.24 mg heme iron per serving; *see* below) and one that was expected to be well accepted by the children.

Because the children were randomly assigned to receive 1 of 3 different intakes of heme iron, the ground raw chicken was divided into 3 equal batches of 800 g each. One batch had 110.3 mL bovine red blood cells added to it (0.363 mg Fe/mL), which corresponded to 2 mg total Fe/40 g serving; another batch had 220.7 mL red blood cells added to it (4 mg total Fe/40 g serving), and the final batch had 441.4 mL red blood cells added to it (8 mg total Fe/40 g serving). Each batch was thoroughly mixed and cooked over a low heat. Such treatment has been shown to not affect the absorption of hemoglobin (13). To avoid losing any isotope, we did not drain the chicken. Once cooled, the chicken was divided into 20 equal-sized serving portions (each equivalent to 40 g raw chicken) and stored in a freezer until the day of the study, when the portions were thawed. Seasoned

tomato sauce (50 g) was stirred into the mixture, which was heated in the microwave for 15 s. The subjects ate the meal with 8 corn chips (Fritos Scoops Corn Chips; Frito-Lay, Dallas, TX) and grated mild cheddar cheese (15 g). The iron and zinc contents of the test meals are shown in **Table 1**. The molar ratio of iron to zinc varied from 0.4:1 to 5.5:1. The molar ratio of heme iron to zinc was estimated based on the assumption that 40–80% of the iron in chicken is heme iron, as has been shown for lean beef (9, 14, 15), and varied from 0.3:1 to 4.6:1. Most of the heme iron in the meals (>85%) was from the labeled hemoglobin. The heme iron present in the chicken meat made a relatively small contribution to the total heme iron content.

Approximately 1 h before the meals were served to the children, either zinc-67 (1 mg) alone or zinc-67 (1 mg) and zinc acetate (8 mg) were added to the meals, depending on whether a child was receiving a total of 1 or 9 mg Zn, respectively. The children were observed as they ate the study meal to monitor their food consumption. The actual intakes were assessed by weighing the bowls of food both before and after consumption.

Isotope preparation

Zinc-67 (88.6% enrichment by mass), zinc-70 (95.6% enrichment by mass), and ^{58}Fe (91.5% enrichment by mass) were purchased as metals from Trace Sciences Inc (Ontario, Canada). Zinc isotopes were converted to aqueous solutions of zinc as previously described (16, 17) and tested for sterility. Zinc-70, the intravenous solution, was also tested for pyrogenicity before use.

Sample preparation and analysis

Zinc was purified from the urine samples using ion-exchange chromatography. Isotope enrichments were measured by magnetic sector thermal ionization mass spectrometry. Isotope ratios were expressed with respect to the nonadministered isotope, zinc-66, and corrected for differences in fractionation by using the ratio of zinc-64 to zinc-66 (17).

Iron absorption was calculated from incorporation of ^{58}Fe into red blood cells. Isotope ratios also were measured by thermal ionization magnetic sector mass spectrometry (MAT 261; Finnegan) (16). Ratios were expressed relative to the nonadministered isotope, ^{56}Fe, and corrected for temperature-specific differences in fractionation by using the ratio of ^{54}Fe to ^{56}Fe and converted to a tracer:tracee ratio.

Serum ferritin concentration was measured with an electrochemiluminescence immunoassay (Elecsys 1010/2010; Roche Diagnostics Corporation, Indianapolis, IN) consisting of a double-antibody sandwich method, which, on application of voltage, emits chemiluminescence that is measured by a photomultiplier. Serum transferrin receptor concentration was measured with an enzyme immunoassay test kit (Ramco TfR test kit; Ramco Laboratories Inc, Stafford, TX).

Zinc and iron-absorption measurements

Zinc and iron absorption were measured as previously described (2). Briefly, zinc absorption was calculated from the fractional excretion of the oral and intravenous isotope in the 72-h urine sample (2, 3) by using the equation

Zinc absorption $=$ (TTR of oral isotope/dose of oral isotope)/

(TTR of intravenous isotope/dose of intravenous isotope)   (3)

where TTR is the tracer:tracee ratio.

Red blood cell incorporation of the oral isotope was calculated by using the following equation (2, 3):

Red blood cell incorporation $=$ TTR of isotope $\times$ Fe$_{Circ}$ (4)

where Fe$_{Circ}$ is the total amount of iron circulating as hemoglobin and was calculated as

Fe$_{Circ}$ $=$ hemoglobin concentration (g/dL)

$\times$ blood volume $\times$ weight (kg)

$\times$ 3.47 mg Fe/g hemoglobin $\times$ 1 dL/100 mL   (5)

Blood volume was assumed to be 75 mL/kg in boys and 66 mL/kg in girls (18). Iron absorption was then calculated by assuming that 90% of the absorbed iron was incorporated into red blood cells (2, 3).

Definitions of anemia and iron deficiency

Anemia was defined as a hemoglobin concentration <11.5 g/dL, as recommended by the Centers for Disease Control and Prevention (19). A serum ferritin concentration ≤ 12 ng/mL was used as a marker of iron deficiency (19).

Statistical analysis

Normally distributed data were expressed as means $\pm$ SDs. Data that were not normally distributed were log transformed and reported as the geometric mean and interquartile range. Ethnicity

was determined by the report of the child's parent and was coded as "Caucasian" or "non-Caucasian" (American American, Asian, or Hispanic).

Differences in continuous variables between the groups were analyzed by using a 2×3 factorial analysis of variance (ANOVA) with iron dose (3 levels) and zinc dose (2 levels) as the independent variables. Differences in nominal variables between the 6 groups were analyzed by using chi-square or Fisher's exact test. Differences in iron and zinc absorption were analyzed by using a 2×3 factorial model, with iron or zinc absorption as the dependent variables and iron intake (3 levels) and zinc intake (2 levels) as the independent variables. Statistical interaction between the 2 independent variables was assessed (ie, the effect of independent variable A different depending on independent variable B). In the case of iron absorption, the data also were analyzed with measures of iron status (serum ferritin and serum transferrin receptor concentrations) as additional covariates. Post hoc testing was carried out by using Scheffe's test. Statistical significance was assumed at $P < 0.05$.

Before the analysis, serum ferritin, fractional iron absorption, and absolute iron absorption were log transformed (to the base 10) to normalize their distributions and are reported in the text as geometric means and interquartile ranges. The statistical analysis was carried out by using SPSS (version 13 for Microsoft Windows; SPSS Inc, Chicago, IL) and STATVIEW version 5.0.1 for MacIntosh (SAS Institute Inc, Cary NC).

The study was powered to detect a clinically meaningful difference of 1 SD in iron absorption between the groups. A sample size of 48 gave an 80% power of detecting such a difference between any of the 3 iron intakes ($n = 16$ children each) at $P < 0.05$. We did not expect a statistical interaction between the 2 independent variables (iron intake and zinc intake), and the study was not powered to detect such an effect. Power calculations were performed with DSTPLAN version 4.2 (University of Texas MD Anderson Cancer Center, Houston, TX).

RESULTS

Intrinsic labeling protocol

The calf tolerated the intravenous infusion of ^{58}Fe well; no changes in vital signs or distress were observed. When killed, 3500 mL whole blood was collected from the calf, which was equivalent to 900 mL concentrated red blood cells. The ^{58}Fe enrichment was 9.5%. The liver (1000 g wet weight) had an average total iron content of 44.6 ± 7.5 mg and enrichment of $12.0 \pm 0.47\%$. A total of 30 mg ^{58}Fe was recovered from red blood cells and 5.35 mg from the liver. Total yield was 26% of the administered dose (22% from red blood cells and 4% from the liver).

Human absorption protocol

Study demographics

Forty-eight subjects were recruited; 8 ate less than half of the labeled meal and were excluded from the analysis. Three subjects were excluded from the analysis of iron absorption because an insufficient amount of blood was drawn for analysis. Four subjects were excluded from the analysis of zinc absorption because the intravenous infusion of zinc either could not be given or infiltrated subcutaneously ($n = 3$) or the urine sample could not

TABLE 2
Demographic characteristics of the participants[1]

	Value
Age (y)	5.8 ± 1.5 (40)
Weight (kg)	23.4 ± 6.4 (40)
Hemoglobin (g/dL)	12.6 ± 0.7 (39)
Ferritin (ng/mL)[2]	26.3 ± 10.9 (39)
log Ferritin	1.4 ± 0.2 (39)
Serum transferrin (μg/mL)	5.3 ± 1.3 (39)

[1] All values are $\bar{x} \pm$ SD; n in parentheses.
[2] The geometric $\bar{x}$ is 24.1 ng/mL, and the interquartile range is 17–31.

be analyzed ($n = 1$). The demographic characteristics of the remaining 40 subjects who had successful measurements of iron absorption, zinc absorption, or both are shown in **Table 2**. The final population included 12 girls and 28 boys, 24 of whom were white and 16 of whom were nonwhite (11 Hispanics, 4 African Americans, and 1 Asian); 37 of these subjects were included in the analysis of iron absorption and 36 in the analysis of zinc absorption.

One subject had a hemoglobin concentration <11.5 g/dL, and one had a ferritin concentration ≤12 ng/mL. The geometric mean serum ferritin concentration was 24.1 ng/mL (interquartile range: 17–31 ng/mL) and ranged from 9 to 64 ng/mL.

The randomized groups did not differ in age ($P = 0.73$), weight ($P = 0.84$), hemoglobin concentration ($P = 0.77$), log serum ferritin concentration ($P = 0.44$), or serum transferrin receptor concentration ($P = 0.98$). The groups had similar sex distributions ($P = 0.54$) and ethnic distributions ($P = 0.22$). Those subjects who completed the study did not differ from those who did not in terms of age ($P = 0.80$), weight ($P = 0.61$), hemoglobin concentration ($P = 0.18$), log serum ferritin ($P = 0.29$), sex ($P = 0.25$), or ethnicity ($P = 0.52$).

Iron absorption

Of the 37 children included in the analysis of iron absorption, 33 completed the entire meal. The remaining 4 children consumed 50%, 53%, 74%, and 77% of the meal. Iron absorption (fractional and absolute) in the 6 groups is shown in **Table 3**. When analyzed by ANOVA, log percentage iron absorption was significantly related to iron ($P = 0.013$) and zinc ($P = 0.0375$) intakes. There was a nonsignificant trend toward a heme iron–zinc interaction ($P = 0.0643$). Unexpectedly, heme-iron absorption was higher in the subjects with a higher intake of zinc. When the effect of zinc intake on heme-iron absorption was analyzed by unpaired t tests separately at the 3 different heme iron doses, a significant effect of zinc on heme-iron absorption was apparent at the 2-mg dose of heme iron ($P = 0.0102$) but not at the 4-mg ($P = 0.52$) or 8-mg ($P = 0.83$) doses.

The effect of iron and zinc intakes on absolute iron absorption was assessed by 2-factor ANOVA (Table 3). Neither iron intake ($P = 0.0504$) nor zinc intake ($P = 0.0551$) had a significant effect on absolute iron absorption, but a significant interaction between iron and zinc was observed ($P = 0.0482$). When the effect of zinc intake on absolute iron absorption was assessed by using an unpaired t test at each iron intake level individually, a significant zinc effect at a heme intake of 2 mg ($P = 0.0147$), but not at a heme intake of 4 mg ($P = 0.56$) or 8 mg ($P = 0.66$), was observed; this finding was consistent with the similar analysis for fractional heme-iron absorption (*see* above).

TABLE 3
Fractional and absolute iron absorption in the study groups

Group	Iron dose	Zinc dose	Percentage iron absorption[1,2]	Iron absorption[1,3]
	mg	*mg*	*%*	*μg*
A ($n = 6$)	2	1	13.0 (11.4–16.9)	260 (228–338)
B ($n = 7$)	4	1	11.1 (6.8–19.1)	405 (270–693)
C ($n = 7$)	8	1	8.6 (5.8–10.3)	691 (462–824)
D ($n = 7$)	2	9	32.6 (22.3–52.3)	625 (410–1045)
E ($n = 5$)	4	9	14.6 (11.2–18.9)	507 (334–712)
F ($n = 5$)	8	9	8.3 (7.2–9.7)	628 (576–679)

[1] All values are geometric $\bar{x}$; interquartile range in parentheses.
[2] Significantly affected by iron intake ($P = 0.0013$) and zinc intake ($P = 0.0375$) without a significant statistical interaction between iron intake and zinc intake ($P = 0.06$); assessed by 2-factor ANOVA.
[3] No significant effect of either iron ($P = 0.0504$) or zinc ($P = 0.0551$) intake alone on absolute iron absorption was found by 2-factor ANOVA, but the iron-zinc interaction was significant ($P = 0.0482$). When the effect of zinc intake on absolute iron absorption was assessed with an unpaired t test at each iron intake separately, a significant effect of zinc intake on absolute iron absorption was observed at the 2-mg ($P = 0.0147$) heme-iron intake but not at the 4-mg ($P = 0.56$) or 8-mg ($P = 0.66$) intake.

These findings were little affected by the addition of log serum ferritin, serum transferrin receptor concentration, or both to the model. Neither log serum ferritin nor serum transferrin receptor concentration was significantly related to log fractional iron absorption, either alone ($P = 0.14$ for log serum ferritin and $P = 0.35$ for serum transferrin receptors) or in combination ($P = 0.18$ for log serum ferritin and $P = 0.35$ for serum transferrin receptors).

Zinc absorption

Thirty-six children were included in the analysis of zinc absorption. Thirty-two completed the entire meal, and the remaining 4 children consumed 50%, 53%, 74%, and 77%. Zinc absorption, both as percentage absorption (%) and as absolute absorption (mg), is shown for the 6 groups in **Table 4**. When fractional zinc absorption was examined with a 2 × 3 ANOVA with zinc intake level and iron intake level as independent variables in the analysis, iron intake was unrelated to fractional zinc absorption ($P = 0.87$), but zinc intake was inversely related to

TABLE 4
Fractional and absolute zinc absorption in the study groups

Group	Iron dose	Zinc dose	Percentage zinc absorption[1,2]	Zinc absorption[1,3]
	mg	*mg*	*%*	*μg*
A ($n = 6$)	2	1	23.0 ± 7.0	0.23 ± 0.07
B ($n = 7$)	4	1	23.3 ± 10.8	0.23 ± 0.11
C ($n = 7$)	8	1	23.2 ± 18.9	0.24 ± 0.19
D ($n = 7$)	2	9	10.5 ± 5.8	0.98 ± 0.55
E ($n = 5$)	4	9	14.9 ± 5.0	1.39 ± 0.45
F ($n = 6$)	8	9	12.2 ± 4.5	1.14 ± 0.41

[1] All values are $\bar{x} \pm$ SD. ANOVA was used for the statistical analysis.
[2] Significantly affected by zinc intake ($P = 0.006$) but not by iron intake ($P = 0.87$); no significant iron-zinc interaction was observed ($P = 0.90$).
[3] Significantly affected by zinc intake ($P < 0.0001$) but not by iron intake ($P = 0.34$); no significant iron-zinc interaction was observed ($P = 0.33$).

fractional zinc absorption ($P = 0.0059$). No significant statistical interaction between the effect of iron intake and zinc intake was observed ($P = 0.90$). Absolute zinc absorption was unaffected by iron intake ($P = 0.34$), but significantly increased as zinc intake increased ($P = 0.0018$); no significant statistical interaction between the effect of iron intake and zinc intake was observed ($P = 0.33$).

DISCUSSION

Intrinsic labeling protocol

Although radiolabeled heme has been used to study iron metabolism in humans, we are unaware of any reports of the successful production of stable-isotope-labeled heme. In the present study we were able to produce bovine hemoglobin and liver enriched with the stable isotope at ≈10% (hemoglobin) to 12% (liver) or between 30–40-fold as enriched as natural samples. The chemical form of iron in the liver is likely to be heterogeneous, and we did not attempt to determine the exact forms. When veal muscle is labeled with ^{59}Fe, however, most is in the form of 2 heme iron proteins—myoglobin (65%) and hemoglobin (30%)—with only small amounts as ferritin and transferrin (9). When ^{59}Fe is used to intrinsically label rabbit liver, much more of the isotope appears to be in the form of ferritin and hemosiderin (10). The liver tissue produced could, therefore, be used to assess the absorption of stable-isotope-labeled ferritin in humans. The yield of [58]Fe in this study was relatively low, despite our attempts to create iron deficiency in the calf. These difficulties did not prevent us from producing hemoglobin iron of sufficient enrichment to be used to measure the absorption of heme iron from physiologically or nutritionally meaningful amounts. However, they greatly increased the cost of producing labeled [58]Fe.

Human protocol

Labeled hemoglobin was used to study iron absorption in children. Before the study began, taste tests of the study foods (by the investigators) had found the meals to be palatable and to have no adverse sensory qualities. However, for whatever reason, a small number of the children ate little or none of the labeled meal. The children who did not consume the meal did not differ in any appreciable way from the remaining children, and we believe the results as presented are representative of the population as a whole. However, the loss of subjects did reduce the power of our study. For the comparison of heme-iron absorption, we planned on effective group sizes of 16 (at each heme-iron intake), which would have been sufficient to detect a between-group difference of 1 SD. In the final analysis, our effective group size was closer to 12–13 at each heme intake level, which was sufficient to detect a between-group difference of 1.2–1.3 SDs.

We showed that heme-iron intake was inversely related to fractional heme-iron absorption across the intake range from 2 mg to 8 mg. This finding is consistent with the data of Pizarro et al (20), who showed that fractional heme-iron absorption in adults declined as heme-iron intake increased from 0.5 to 30 mg.

In our study, the geometric mean iron absorption from 4 mg heme iron was between 13% and 16%. This range is broadly similar to that found by investigators who described mean fractional absorption rates in adults of 10–17% from 5 mg heme as rabbit hemoglobin (21), 22% from 2 to 4 mg heme as veal muscle

(mostly myoglobin) (9), and 16% from 5 mg heme consumed with meat (22). We are aware of only one study in children in which the mean fractional iron absorption in children aged 1–18 mo was 5% from 5 mg heme iron, which increased to 8% in those with low iron stores (7). This lower estimate may in part be due to the unusual meal used in that study—an aqueous solution of hemoglobin with or without 2/3 strength cow milk in the absence of meat, because meat is a known enhancer of heme-iron absorption (9, 22). Our study meal did include calcium (as cheddar cheese), and it is possible that this lowered the heme-iron absorption in our study (14).

Studies in adults have suggested an inverse relation between heme-iron absorption and several measures of iron status (9, 11, 23), although the relation is less than that seen for nonheme iron (9, 11, 23). We saw no such relation between heme-iron absorption and serum ferritin or serum transferrin receptor concentrations. This finding may have been partly due to the narrow range of iron status in our subject population or lack of statistical power, because our study was not designed to detect such a relation. A further measure of iron status is the reference dose of iron absorption, where an aqueous solution of isotope is given in the fasted state with vitamin C. Although some studies have found that this is not a useful measure of iron status in some infants (24), it is possible that it may have shown a relation between iron status and heme-iron absorption in our study.

Increasing intakes of heme iron had no effect on zinc absorption. Previous studies have shown no effect of zinc on heme-iron absorption from a hamburger meal (25) but an inhibitor effect from an aqueous solution of zinc chloride when given with heme iron (26). Our test meal was more similar to the former (25), and confirms the lack of effect of heme iron on zinc from a complex meal.

To our surprise, the higher intake of zinc was associated with higher heme-iron absorption, although only at the lower heme-iron intakes. This is contrary to data we have reported in Indonesian children in whom zinc oxide reduced nonheme-iron absorption (3). Why the findings of the current study suggest the opposite relation for heme iron is unclear, but it is possible that either zinc or some other component of the zinc acetate solution increases heme absorption.

Several mechanisms for this unexpected effect are possible, including the digestion and release of heme from hemoglobin, solubilization of the heme within the gut lumen, mucosal trafficking, or the activity of one of the enzymes involved in heme-iron absorption. Similar mechanisms have been used to explain the significant increases in heme-iron absorption when meat (27) or niacin (28) is consumed or to explain the decrease in heme-iron absorption when meat is coingested with calcium (14, 29). The effect of zinc intake on heme-iron absorption was only seen at lower intakes of heme iron. This is consistent with an effect on the solubility of heme or on the activity of enzymes involved in absorption, where a beneficial effect would be most apparent at lower heme-iron intakes, but less apparent at higher heme-iron intakes, after which the absorptive capacity for heme iron was nearer saturation. It is unlikely that the observed difference is an artifact due to differences in how the meal was prepared or cooked because all of the children consuming a given amount of iron consumed meals made in the same batch and cooked in an identical manner. The differences in zinc content were achieved by adding zinc acetate to the meal after the food had been prepared, because prolonged cooking can denature heme iron and

reduce its absorption (30). An alternative explanation for this finding is that the result is a spurious one. The sample size in our study, limited as it was by cost and availability of labeled hemoglobin, was small, and this finding may simply represent a type I error. Only further study will determine whether this finding is real or not.

Our study was not designed to compare absorption of heme and nonheme iron. However, we previously measured iron absorption in children aged 4–8 y who consumed very similar meals that contained 3.3 mg nonheme iron from either ground beef or low-phytate soy protein (31). Geometric mean iron absorption was 7.6% from the chili made with beef protein and 3.6% from the chili made with low-phytate soy protein. The current study suggests that geometric mean heme-iron absorption from 4 mg heme iron (13–16%) is higher, consistent with the expectation that heme-iron absorption would be greater than nonheme-iron absorption (11).

As expected (32), and as hypothesized, fractional zinc absorption decreased as the zinc load of the meal increased. This finding agrees with recent data from de Romana et al (33), who showed that fractional zinc absorption was inversely related to zinc intake in stunted anemic Peruvian children. Despite the decrease in fractional absorption, absolute zinc absorption continued to increase as zinc intake increased.

Cooked ground beef contains $\approx$2.8 mg Fe/100 g (34). This equates to a heme iron content of 1.1–2.2 mg/100 g, depending on whether one assumes that 40% (14) or 80% (9, 15) of the iron in ground beef is heme. The lowest heme-iron intake we studied was equivalent to between 90 and 180 g cooked ground beef. This is a relatively large amount of beef for a child aged 4–8 y to consume. To study heme-iron absorption from smaller amounts of heme iron, techniques that measure iron isotope ratios with greater precision or lead to greater enrichment of heme iron with stable isotopes will be required.

REFERENCES

1. Cook JD, Skikne BS, Baynes RD. Iron deficiency: the global perspective. Adv Exp Med Biol 1994;356:219–28.
2. Abrams SA. Using stable isotopes to assess mineral absorption and utilization by children. Am J Clin Nutr 1999;70:955–64.
3. Herman S, Griffin IJ, Suwarti S, et al. Co-fortification of iron fortified flour with zinc sulfate, but not zinc oxide, decreases iron absorption in Indonesian children. Am J Clin Nutr 2001;76:813–7.
4. Cook JD, Layrisse M, Martinez-Torres C, Walker R, Monsen ER, Finch CA. Food iron absorption measured by an extrinsic tag. J Clin Invest 1972;805–15.
5. Walsh RF, Kaldor I, Bradling I, Georg EP. The availability of iron in meat: some experiments with radioactive iron. Australas Ann Med 1955; 4:272–6.
6. Hallberg L, Solvell L. Absorption of hemoglobin iron in man. Acta Med Scand 1967;181:335–54.
7. Heinrich HC, Gabbe EE, Whang DH, Bender-Gotze C, Schafer KH. Ferrous and hemoglobin-^{59}Fe absorption from supplemented cow milk in infants with normal and depleted iron stores. Z Kinderheilkd 1975; 120:251–8.
8. Gabbe EE, Heinrich HC, Bruggemann J, Pfau AA. Iron absorption from hemiglobin (stable oxidation product of hemoglobin) in relation to the dose in subjects with normal and depleted iron stores. Nutr Metab 1979; 23:17–25.
9. Martinez-Torres C, Layrisse M. Iron absorption from veal muscle. Am J Clin Nutr 1971;24:531–40.
10. Martinez-Torres C, Renzi M, Layrisse M. Iron absorption by humans from hemosiderin and ferritin, further studies. J Nutr 1976;106:128–35.
11. Roughead ZK, Hunt JR. Adaptation in iron absorption: iron supplementation reduces nonheme-iron but not heme-iron absorption from food. Am J Clin Nutr 2000;72:982–9.
12. Blaxter KL, Sharman GAM, Macdonald AM. Iron-deficiency anaemia in calves. Br J Nutr 1957;11:234–46.
13. Lam-Po-Tang PR. Absorption of haemoglobin iron in man. Med J Aust 1969;1:115–7.
14. Hallberg L, Rossander-Hulthén L, Brune M, Gleerup A. Inhibition of haem-iron absorption in man by calcium. Br J Nutr 1992;69:533–40.
15. Harrington CF, Elahi S, Merson SA, Ponnampalavanar P. A method for the quantitative analysis of iron speciation in meat by using a combination of spectrophotometric methods and high-performance liquid chromatography coupled to sector field inductively coupled plasma mass spectrometry. Anal Chem 2001;73:4422–7.
16. Ames SK, Gorham BM, Abrams SA. Effects of high compared with low calcium intake on calcium absorption and incorporation of iron by red blood cells in small children. Am J Clin Nutr 1999;70:44–8.
17. Griffin IJ, King JC, Abrams SA. Body weight-specific zinc compartmental masses in girls significantly exceed those reported in adults: a stable isotope study using a kinetic model. J Nutr 2000;130:2607–12.
18. Hawkins WW. Iron, copper and cobalt. In: Beaton GH, McHenry EW, eds. Nutrition: a comprehensive treatise. New York, NY: Academic Press, 1964:310–72.
19. Yip R. Recommendations to prevent and control iron deficiency in the United States. MMWR Morb Mortal Wkly Rep 1998;47:1–29.
20. Pizarro F, Olivares M, Hertrampf E, Mazariegos DI, Arredondo M. Heme-iron absorption is saturable by heme-iron dose in women. J Nutr 2003;133:2214–7.
21. Callender ST, Mallett BJ, Smith MD. Absorption of haemoglobin iron. Br J Haematol 1957;3:186–92.
22. Bjorn-Rasmussen E, Hallberg L, Isaksson B, Arvidsson B. Food iron absorption in man. Applications of the two-pool extrinsic tag method to measure heme and nonheme-iron absorption from the whole diet. J Clin Invest 1974;53:247–55.
23. Hallberg L, Hulten L, Gramatkovski E. Iron absorption from the whole diet in men: how effective is the regulation of iron absorption? Am J Clin Nutr 1997;66:347–56.
24. Davidsson L, Ziegler EE, Kastenmayer P, Hurrell RF. Erythrocyte incorporation of iron by infants: iron bioavailability from a low-iron infant formula and an evaluation of the usefulness of correcting erythrocyte incorporation values, using a reference dose or plasma ferritin concentrations. Br J Nutr 2000;84:847–53.
25. Solomons NW, Jacob RA. Studies on the bioavailability of zinc in humans: effects of heme and non-heme iron on the absorption of zinc. Am J Clin Nutr 1981;34:475–82.
26. Valberg LS, Flanagan PR, Chamberlain MJ. Effects of iron, tin, and copper on zinc absorption in humans. Am J Clin Nutr 1984;40:536–41.
27. Hallberg L, Bjorn-Rasmussen E, Howard L, Rossander L. Dietary heme iron absorption. A discussion of possible mechanisms for the absorption-promoting effect of meat and for the regulation of iron absorption. Scand J Gastroenterol 1979;14:769–79.
28. Conrad ME, Benjamin BI, Williams HL, Foy AL. Human absorption of hemoglobin iron. Gastroenterology 1967;53:5–10.

29. Hallberg L, Brune M, Erlandsson M, Sandberg AS, Rossander-Hulthen L. Calcium: effect of different amounts of nonheme- and heme-iron absorption in humans. Am J Clin Nutr 1991;53:112–9.

30. Martinez-Torres C, Leets I, Taylor P, Ramirez J, del Valle Camacho M, Layrisse M. Heme, ferritin and vegetable iron absorption in humans from meals denatured of heme iron during the cooking of beef. J Nutr 1986; 116:1720–5.

31. Etcheverry P, Hawthorne KM, Liang LK, Abrams SA, Griffin IJ. Effect of beef and soy proteins on the absorption of non-heme iron and inorganic zinc in children. J Am Coll Nutr 2006;25:34–40.

32. King JC, Cousins RJ. Zinc. In: Shike M, Ross AC, Caballero B, Cousins RJ, eds. Modern nutrition in health and disease. 10th ed. Philadephia, PA: Lippincott Williams and Wilkins, 2006:271–85.

33. de Romana DL, Salazar M, Hambidge KM, et al. Longitudinal measurements of zinc absorption in Peruvian children consuming wheat products fortified with iron only or iron and 1 of 2 amounts of zinc. Am J Clin Nutr 2005;81:637–47.

34. Schakel SF. Maintaining a nutrient database in a changing marketplace: keeping pace with changing food products—a research perspective. J Food Comp Anal 2001;14:315–22.