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Heat treatment on heme iron and iron-containing proteins in meat: Iron absorption in humans from diets containing cooked meat fractions

Maria N. Garcia, Carlos Martinez-Torres, Irene Leets, Eleonora Tropper, Jose Ramirez, and Miguel Layrisse

Instituto Venezolano de Investigaciones Cientificas, Laboratorio de Fisiopatologia, Caracas, Venezuela

The present study was undertaken to characterize the effect of heat on iron compounds and iron-containing proteins of rabbit meat. We also studied human iron absorption from beef meat precipitates and investigated changes in the cysteine content in beef and rabbit meat caused by cooking processes. Supernatant and precipitate fractions were obtained by an extraction procedure that included homogenization and repeated centrifugations. A 50% decrease of soluble iron was produced by cooking the meat. Cooking also reduced the heme iron content of the meat by 62%. Chromatographic separation of soluble meat extracts showed changes in ferritin, hemoglobin, and myoglobin elution profiles in cooked meat compared with raw meat. Determinations of the cysteine content in raw or cooked meat samples showed a statistically significant reduction ($P < 0.0001$) in the cysteine content in cooked samples compared with raw counterparts. Iron absorption studies in humans feeding the subjects with a typical beef-containing diet, which contained 60% of meat iron as heme iron, showed a 13.5% absorption from heme iron and a 6.3% from nonheme iron. The subjects fed with a diet containing beef precipitate in which only 30% of meat iron was heme iron showed a 7.6% absorption from heme iron and 7.5% from nonheme iron. These results show that although there are important changes in iron-containing proteins, heme iron, cysteine content, and iron absorption by cooking procedures, the factor present in meat responsible for enhancing nonheme iron absorption is not affected by heat and is still present in insoluble meat precipitates. (J. Nutr. Biochem. 7:49–54, 1996.)

Keywords: meat protein; heme iron; cysteine; humans; iron absorption

Introduction

It has been reported that meat promotes iron absorption in two ways. First iron from hemoglobin and myoglobin is more readily available because it is not affected by inhibitors or enhancers present in food that affect nonheme iron absorption and is absorbed by a different mechanism involving specific receptors. Second it promotes absorption because meat contains a factor or factors that enhance heme and nonheme iron absorption.[1–5] However, this effect is not observed with all animal tissues; while beef, pork, lamb, liver, chicken, and fish have a promoting role on nonheme iron absorption, milk and egg proteins inhibit or have no effect on iron absorption from vegetable foods.[2,6–8] Moreover, a study reports that the same protein source could have a "differential" effect on iron absorption: beef meat proteins enhance nonheme iron absorption from a maize gruel but has no effect on iron absorption from a bread meal.[9]

Two mechanisms have been suggested to explain the enhancing effect of animal proteins on iron absorption. First, globin digestion products formed during the digestive process prevent heme and nonheme iron polymerization, maintaining iron in a more soluble form.[10,11] Second, free amino acids such as cysteine alone or glutathione as a

Address correspondence to Dr. Maria N. Garcia at the Laboratorio de Fisiopatologia, Centro de Medicina Experimental, Instituto Venezolano de Investigaciones Cientificas (IVIC), Carretera Panamericana, Km 11, Apartado Postal 21827, Caracas 1020-A, Venezuela.
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tripeptide in humans and serine, tyrosine, etc. in rats chelate the iron in the intestinal lumen and thereby facilitate absorption.[12–14] The enhancing effect of cysteine is observed when the amino acid is administered as a peptide or in gelatine capsules and not when it is premixed with food due to the alkaline buffers of many vegetable foods that promote oxidation of cysteine to cystine.[14,15] The heme iron in meat is partly converted to nonheme iron by heat. Heme iron reduction ranges from 10 to 100%, and this degradation is proportional to the time and type of cooking.[5,16] This article deals with the modifications of heme iron and iron-containing proteins as well as the cysteine content of meat by heat. The effect of cooked meat precipitates on dietary iron absorption in humans is also investigated.

Methods and Materials

To produce radioactive meat, a 2-month-old rabbit was injected in the marginal vein of the ear with 7.4×10^7 Bq of ^{55}Fe. Three months later, the rabbit was exsanguinated by cardiac puncture, and the muscle and blood were removed and kept at –40°C. Twenty-four grams of lean meat was minced in a kitchen blender from low to full speed until completely blended. The minced meat was divided into two equal portions. One portion was cooked in an iron-free receptacle stirring all the time with medium heat until the meat was completely cooked, i.e., when the characteristic red color of raw meat disappeared. The other portion was left raw.

The cooked meat was divided into four portions weighing approximately 3 g each. Each portion was homogenized manually with 10 mL of distilled water at 4°C and centrifuged at 3,500 g for 30 min at 4°C. Supernatants of the cooked portions were pooled, and the precipitates were combined, rehomogenized, and recentrifuged at 3,500 g for 30 min at 4°C. The precipitates were rewashed and recentrifuged until the supernatants were pale yellow in color, usually three or four times. This was necessary to obtain all soluble iron compounds. The same steps were done to the raw meat.

Pooled supernatants of each group, raw or cooked, were filtered, lyophilized, and resuspended in 6 mL of distilled water and this volume was passed through a 100 × 5 cm column (Pharmacia Fine Chemicals, Upsala, Sweden) packed with Sephadex G-100 (Pharmacia Fine Chemicals). In each chromatographic separation, 600 fractions of 5 mL were collected since a protein could be eluted after the elution profile of the column. Commercial standards of ferritin, hemoglobin, and myoglobin were first passed through the column to establish the elution profiles. Chromatographic separation was done at 4°C. The optical density of each fraction was read in a spectrophotometer (Varian Techtron M634, Australia) at 540 nm (hemoglobin and myoglobin) and 400 nm (ferritin).

Total,[17] nonheme,[18] radioactive iron,[19] and protein[20] were measured in minced meat samples, supernatants, and precipitates, and peaks were obtained by gel filtration chromatography of raw or cooked meat. The heme iron content was calculated by substraction of the nonheme iron from the total iron. Each experiment was performed eight times.

Cysteine determinations

Sample preparation for cysteine quantification was based on the method described by Taylor et al.[21] Twenty grams of sample (beef and rabbit meat, raw and cooked) were minced in a kitchen blender for 5 min at fullspeed. The pH was adjusted to 2 with 10 N hydrochloric acid. Pepsin (Hog stomach mucosa, Sigma Chemical Company, St. Louis, MO USA) in an amount equivalent to 0.5% of the protein content of the sample. Samples were incubated in a shaking water bath at 37°C, and the pH was checked every 30 min and maintained between 2 and 2.5 adding 10 N hydrochloric acid when necessary. The digestion was completed after 2 to 2.5 hr indicated by no increases in the pH.

The digest was centrifuged at 2500*g* for 30 min at 0°C. The supernatant, without the upper layer of fat, was filtered and divided into to two equal parts. To one part (cys–), the pH was raised to 8.5 with sodium hydroxide and bubbled with oxygen for 2 hr to oxidize cysteine. The other part, containing cysteine in the reduced form (cys+), was kept intact. The cysteine content of raw or cooked samples from beef or rabbit meat was determined using Ellman's reagent.[22]

Iron absorption studies

Twenty subjects, 14 women and 6 men aged 13 to 40 years, consented to participate in this study. The protocol was approved by the Committee for the Protection of Human Subjects of Scientific Research of this institute. All the volunteers were in good health, and for each one the hemoglobin concentration,[23] serum iron,[24] transferrin saturation,[24] and ferritin[25] was determined.

Eleven subjects received a typical Latin American meal containing beef meat, black beans, rice, and a bread called "arepa" made from precooked maize flour (meal 1). Meat was mixed with 7.4×10^4 Bq of ^{55}Fe hemoglobin and cooked for 10 min. The maize flour was labeled with 2.59×10^4 Bq of ^{59}Fe at the time of dough preparation. The same meal was given to the 9 individuals in the second group but instead of meat, beef meat precipitate prepared as described above was administered (meal 2). This meat was not intrinsically labeled, and after the cooking and extraction procedures, 2.59×10^4 Bq of ^{59}Fe hemoglobin were mixed with the precipitate, and 7.4×10^4 Bq of ^{55}Fe were added to the water used to cook the rice.

For both groups, the food was administered after an overnight fast and no food or drink was allowed for 3 hr after the meal. Fifteen days after the meal, blood was drawn from each subject to measure incorporated red cell radioactivity, and a reference dose of ^{55}Fe-ascorbate was administered. The preparation of the reference dose is described elsewhere.[26] On day 30, blood was drawn to measure iron absorption from this source correcting for the remaining activity of ^{55}Fe in the blood.

Each subject received (raw weight) 20 g of black beans, 25 g of precooked maize flour, and 25 g of rice. Meal 1 contained 50 g of cooked meat with (equivalent to 75 g of raw meat) 5.36 mg of iron, (3.76 mg of nonheme iron and 1.6 mg of heme iron). The iron content of the cooked meat in this diet was, per 100 g, 5.3 mg of total iron, 2.1 mg of nonheme iron, and 3.2 mg of heme iron, which represented 60% of the total iron in the meat. Meal 2 contained 86 g of meat precipitate (equivalent to 174 g of raw meat) 4.69 mg of total iron, 4.09 mg of nonheme iron, and 0.6 mg of heme iron. One hundred grams of precipitate contained 2.3 mg of total iron, 1.6 mg nonheme iron and 0.7 mg heme iron; this value represented only 30% of the total iron content of meat.

Statistical analysis

The mean absorption and standard error were calculated from the logarithm of the percentage of absorption, and the result was retransformed as an antilogarithm to recover the original units. Statistical comparison of two absorption tests as well as cysteine or iron contents was performed according to the Student's *t*-test for paired samples.

Results

The total, nonheme, and heme iron and protein contents of ground rabbit meat samples, cooked and raw, are shown in

Table 1. The total iron content was similar in both the raw and cooked meat; however, there was a significant reduction (62.2%) in heme iron after the meat was cooked. A marked decrease was observed in heme iron content of the supernatants and precipitates of cooked meat. Cooking reduced the heme iron content by 87.4% in the supernatant (2.63 to 0.33 μg/mL) and by 21.8% in the precipitate (1.6 to 1.25 μg/g). Radioactive measurements and protein determinations demonstrated a similar reduction.

The total iron determinations to supernatant and precipitate fractions, obtained as described, demonstrated that the iron distribution in cooked and raw meat was different; the total iron concentration in the raw meat supernatant was greater than in the cooked meat (56 vs. 28%), and proportionally the amount of iron recovered in the precipitate was higher for cooked meat (44% in raw meat vs. 72% in cooked meat), showing that heat changed the iron solubility and increased the proportion of iron recovered in the precipitate.

Chromatographic separation of iron-containing proteins in raw meat by gel filtration showed four well-defined peaks at both 400 and 500 nm (*Figure 1A*). The first three peaks were ferritin (M_r 500,000 Daltons), hemoglobin (M_r 64,000 Daltons) and myoglobin (M_r 16,000 Daltons). The fourth peak eluted after the column elution volume making it difficult to obtain molecular weight estimations because the protein could be interacting with the gel matrix thus delaying its elution. Chromatographic separation of cooked meat supernatants resulted in two peaks at each wavelength (*Figure 1B*). Since these proteins may not be globular, it was not possible to make molecular weight estimations. However, the first peak eluted in a position with a molecular weight between hemoglobin and myoglobin, and the second peak eluted after the column elution volume.

The iron, radioactivity, and protein contents of the peaks obtained from the chromatographic separation of cooked or raw meat are shown in *Table 2*. For raw meat separation, the first peak had the highest iron concentration (15.06 μg Fe/peak) with 76% as nonheme iron and was identified as ferritin. Peaks 2 and 3 contained similar quantities of iron (9.52 and 9.17 μg Fe/peak, respectively) with 65% as heme iron. These two peaks were identified as hemoglobin and myoglobin. Because of unexpected heme iron in peak 1 and nonheme iron in peaks 2 and 3, commercial standards of ferritin, hemoglobin, and myoglobin were used for iron determinations. A 25% heme iron concentration in ferritin and a 35% nonheme iron concentration in hemoglobin and myoglobin were detected validating our results with the fractions studied. The lowest iron concentration (4.55 μg of Fe/peak) was found in the fourth peak, and 86% of it was nonheme iron. The peaks obtained by the chromatographic separation of cooked meat supernatants showed similar iron concentrations (6.01 and 6.48 μg of Fe/peak for peaks 1 and 2, respectively), and 89 to 94% of it was nonheme iron.

Meat cysteine content

The cysteine content was determined to raw or cooked supernatants of beef or rabbit meat after a 2 hr pepsin digestion to release peptides and expose cysteine residues. Raw meat (beef or rabbit) had a higher content of cysteine that cooked counterparts (*Table 3*). Cysteine reduction in beef samples was 13.6%, and for rabbit samples the reduction was 28.5%. Due to the low dispersion of the results, both differences were highly significant with $P < 0.0001$.

Human iron absorption studies

Table 4 shows the heme and nonheme iron absorption from meals 1 and 2 and the hematologic profiles of the groups studied. Iron absorption was normalized according to iron ascorbate absorption by multiplying the observed absorption by the ratio of the composed reference dose mean absorption for all individuals tested to the mean of the reference dose absorption for a given meal.[27] This was done because of the difference in iron status of the subjects tested in meal 1 compared with the iron status of the subjects tested in meal 2.

The heme iron absorption from meal 1 was 13.5%, significantly higher ($P = 0.0031$) than the absorption obtained

Table 1 Total, nonheme, and heme iron, heme iron reduction, radioactivity, and protein content of raw and cooked rabbit meat fractions labeled with ^{55}Fe ($n = 8$)

Meat Preparation	Protein content*	Total iron†	Non-heme iron†	Heme iron†	Heme iron reduction by cooking (%)	Radioactivity‡
Raw meat	210	8.92 ± 0.84	5.11 ± 0.67§[5]	3.81§[7] (42.7%)¶		5348 ± 119
Cooked meat	210	9.87 ± 1.06	8.43 ± 0.78§[5]	1.44[7] (14.5%)	62.2	5431 ± 115
Raw meat supernatant	72.07 ± 1.86§[0]	6.47 ± 0.96§[2]	3.84 ± 0.96	2.63§[8] (40.6%)		2794 ± 81§[9]
Cooked meat supernatant	14.20 ± 0.65§[0]	3.38 ± 1.89§[2,4]	3.05 ± 0.19	0.33§[8] (9.8%)	87.4	631 ± 10§[9]
Raw meat precipitate	146 ± 12.32§[1]	5.16 ± 1.59§[3]	3.56 ± 1.98§[6]	1.60 (31.0%)		1663 ± 324§[2']
Cooked meat precipitate	245 ± 19.2§[1]	8.64 ± 2.89§[3,4]	7.39 ± 1.91§[6]	1.25 (14.5%)	21.8	3892 ± 331§[2']

*Mg/g for the total meat and precipitates, mg/mL for the supernatants.
†μg/g for the total meat and precipitates, μg/mL for the supernatants.
‡Cpm/g for the total meat and precipitates, cpm/mL for the supernatants.
§$P < 0.05$ between the respective superscripts.
¶Percent of heme iron in total iron.

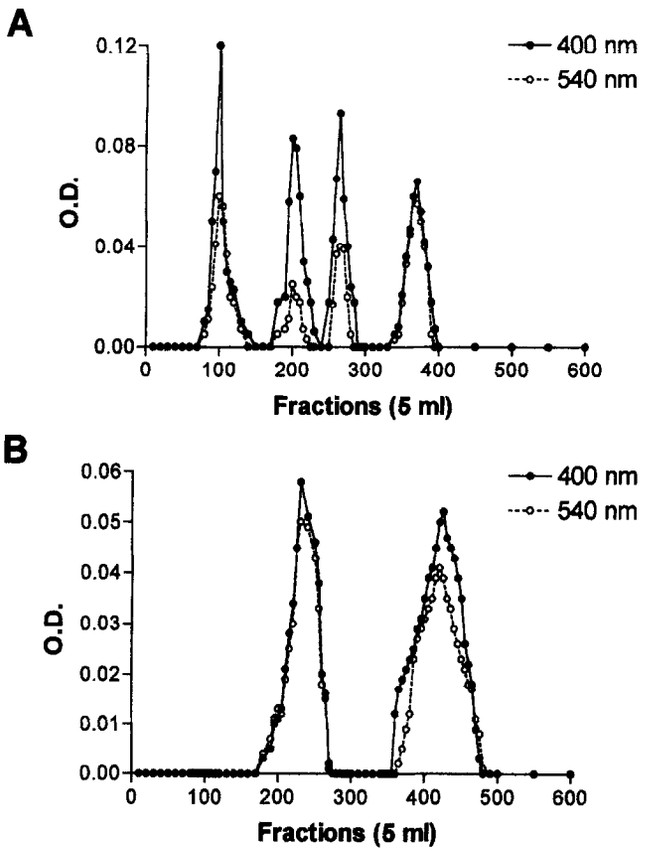

Figure 1 Elution pattern by molecular exclusion chromatography from raw (A) and cooked (B) meat supernatants.

from cooked meat precipitate (meal 2) which was only 7.6%. This difference in iron absorption of almost 50% is in concordance with the reduction in heme iron content found in both meat preparations; meal 1 contained 60% of the total iron as heme iron and meal 2 contained only 30%.

In relation to the nonheme iron absorption, there was no statistically significant difference between the two meals (6.3% from meal 1 and 5.5% from meal 2), indicating that the factor in meat responsible for enhancing nonheme iron absorption was still present in the cooked meat precipitate. In previous studies,[9,15,28] it has been demonstrated that iron absorption from the same meal but without meat is approximately 2.3%, which is significantly lower than the nonheme iron absorption values obtained in this study.

Discussion

Iron absorption varies widely among subjects with normal iron status. In the same subject, daily iron absorption variations are important. However, a number of investigations suggest that an important element in iron absorption is the proportion of calories derived from animal food for two reasons. First, animal proteins or its degradation products enhance nonheme iron absorption, and second, heme iron absorption is more efficient than nonheme iron absorption.

In this study, the results show that 43% of the rabbit raw meat total iron content is heme iron. This percentage is in concordance with those reported by Igene et al. in 1979,[29] Martinez-Torres et al. in 1986,[5] and by Schricker and Miller in 1983[16] who found that 30 to 40% of the beef liver, pork, fish, and chicken meat iron content and 50 to 60% of the beef and veal meat iron content is heme iron. In cooked meat, the nonheme iron content increases resulting in a 62% heme iron decrease. They reported that the reduction in heme iron content may vary from 10 to 100% depending on the time and kind of cooking. The increase in nonheme iron content is derived from hemoglobin and myoglobin iron. Heat treatment possibly causes porphyrin ring oxidation, breakdown, iron liberation, and choleglobin formation. Iron, free from the porphyrin ring, becomes part of the nonheme iron pool.

Iron determinations to supernatants and precipitates show that 56% of the raw meat total iron content remains in the supernatant after the extraction procedure. In 1983, Bogunjoko and coworkers[30] using a similar extraction procedure but with chicken meat, found that 50% of the total iron content is in the supernatant. Chen and colleagues[31] and Sato and Hegarty[32] reported that 86 to 89% of the total iron content is in the supernatant. The differences between these studies are probably related to the extraction procedure. However, this behavior is not observed with cooked meat

Table 2 Total, nonheme, and heme iron, percent heme iron, protein content, and radioactivity obtained by chromatographic separation of raw and cooked rabbit meat labeled with ^{55}Fe ($n = 8$)

Elution Profile	Protein content*	Total Iron†	Non-heme Iron†	Heme-Iron†	Percent Heme Iron‡	Radioactivity§
Raw meat supernatant						
Peak 1	24.6 ± 3.51	15.06 ± 2.37	11.42 ± 2.18	3.64	24	1484 ± 313
Peak 2	14.15 ± 2.98	9.52 ± 2.18	3.31 ± 0.91	6.21	65	1025 ± 141
Peak 3	6.33 ± 0.76	9.17 ± 3.91	3.18 ± 0.66	5.99	65	867 ± 112
Peak 4	5.5 ± 0.99	4.55 ± 0.61	3.93 ± 0.85	0.62	14	429 ± 172
Cooked meat supernatant						
Peak 1	5.3 ± 1.12	6.01 ± 1.87	4.52 ± 1.19	0.39	6.5	410 ± 218
Peak 2	3.97 ± 2.1	6.48 ± 0.86	5.73 ± 2.35	0.75	11.5	270 ± 68

*Mg/g for the total meat and precipitates, mg/mL for the supernatants.
†μg/g for the total meat and precipitates, μg/mL for the supernatants.
‡Percent of heme iron in total iron.
§Cpm/peak for the total meat and precipitates, cpm/mL for the supernatants.

Table 3 Cysteine content of beef and rabbit meat before and after cooking procedures

Meat	Cysteine (mmol/100 g of raw meat)		Percent of reduction
	Raw	Cooked	
Beef	1.213 ± 0.093	1.048 ± 0.072	13.6‡
Rabbit	1.627 ± 0.168	1.164 ± 0.162	28.45‡

*$n = 13$.
†$n = 14$.
‡$P < 0.0001$ paired t-test.

where 72% of the total iron content is in the precipitate fractions, which indicates that during the cooking processes iron precipitates with proteins rendering an insoluble form of iron; this means that only 28% of the total iron content in cooked meat is in soluble form.

Chromatographic separation of rabbit raw meat results in peaks corresponding to ferritin, hemoglobin, myoglobin, and a fourth peak which is probably composed of peptides and contains almost all its iron as nonheme iron. Bogunjoko and coworkers[30] working with chicken meat, reported a four protein elution profile: ferritin, hemoglobin, myoglobin, and a fourth peak, which is not a heme protein and is possibly composed of peptides of low molecular weight. However, using veal muscle, Martinez-Torres and Layrisse[13] found three peaks corresponding to ferritin plus hemosiderin, hemoglobin, and myoglobin. The differences in elution profiles are related to collection times because the fourth peak eluted after the column elution volume. Pollack and Campana in 1980,[33] using guinea pig reticulocytes, reported a five peak elution profile identified as ferritin, hemosiderin, hemoglobin, myoglobin, and a fifth peak composed of low molecular weight peptides that contained iron and could be concentrated in Amicon UM05 membranes (exclusion limit >500) and was not filtered in Biogel P2 exclusion volume (exclusion limit >2,000).

Chromatographic separation of cooked meat-soluble proteins produce two peaks, and it is remarkable that the ferritin peak is not detected. Although it is necessary to realize that it is not possible to make molecular weight estimations because proteins are denatured by heat and the second peak is eluted after column elution volume, it is possible to get information about these proteins. There is an important difference in molecular weight between both proteins. The first one elutes in a molecular range between hemoglobin and myoglobin. The second protein elutes after the column elution volume and is possibly formed by degraded proteins which contain iron or perhaps iron is bound in a nonspecific form. Both peaks contain iron in similar quantities with almost all of it as nonheme iron. The second peak could have peptides that bind iron released from hemoproteins during the cooking of the meat. These low molecular weight peptides have been reported previously,[1,3,9,30,34] and it is believed that they are responsible for the enhancing effect of meat on nonheme iron absorption.

In human iron absorption studies, the low heme iron absorption from cooked meat precipitate (meal 2) compared with the absorption from cooked meat (meal 1) is in accordance with the differences in heme iron content between cooked meat and cooked meat precipitate (60 and 30%, respectively) showing that the reduction of heme iron content in meat significantly reduces the heme iron absorption. The low absorption from cooked meat precipitate is in agreement with the reduction of the percentage of heme iron content which has been previously demonstrated.[2,9,10,26] However, this study also indicates that the factor responsible for enhancing nonheme iron absorption is still present in the precipitate and is not susceptible to heat modifications. One possible explanation is the presence of cysteine-containing peptides which could favor nonheme iron absorption. In this work we demonstrated that although there is a significant reduction in cysteine content it is only approximately 14% and the remaining cysteine could explain, at least in part, the enhancing effect of meat or meat precipitates on nonheme iron absorption. These results support one of the theories to explain the effect of meat on nonheme iron absorption in which the cysteine-containing peptides of the proteins in meat are mainly responsible for such enhancement.[1,2,12,14,15,21]

This study shows that an important amount of heme iron is modified to nonheme iron by heat, which implies that this iron will be susceptible to inhibitors and enhancers present in food. The data presented here also demonstrate that the factor in meat responsible for enhancing nonheme iron absorption is not affected by cooking procedures. With the evidence reported by other authors and in this work, it is tempting to speculate that the meat factor could be a peptide or amino acid released during cooking that is capable of

Table 4 Hematological characteristics and percent of iron absorption from typical Latin American diets of the two groups studied

Meal	Sex	Age	Hemoglobin (mg/dL)	Transferrin saturation %	Ferritin (μg/L)	Iron absorption (%)		
						Heme iron	Nonheme iron	Ascorbate
Meal 1: Meat	10 F/1 M	13–40	11.7 ± 0.4	25.6 ± 2.8	22.1 ± 1.3	13.5*	6.3†	27.2
Meal 2: Cooked meat Precipitates	4 F/5 M	16–40	13.0 ± 0.3	27.3 ± 2.9	44.3 ± 1.2	7.6*	5.5†	

*$P = 0.003$.
†NS.

keeping iron in a more soluble form to be absorbed and/or that the iron is absorbed bound to this factor.

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