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# The effect of proteins from animal source foods on heme iron bioavailability in humans

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ABSTRACT

Forty-five women (35–45 year) were randomly assigned to three iron (Fe) absorption sub-studies, which measured the effects of dietary animal proteins on the absorption of heme Fe. Study 1 was focused on heme, red blood cell concentrate (RBCC), hemoglobin (Hb), RBCC + beef meat; study 2 on heme, heme + fish, chicken, and beef; and study 3 on heme and heme + purified animal protein (casein, collagen, albumin). Study 1: the bioavailability of heme Fe from Hb was similar to heme only (~13.0%). RBCC (25.0%) and RBCC + beef (21.3%) were found to be increased 2- and 1.6-fold, respectively, when compared with heme alone ($p < 0.05$). Study 2: the bioavailability from heme alone (10.3%) was reduced ($p < 0.05$) when it was blended with fish (7.1%) and chicken (4.9%), however it was unaffected by beef. Study 3: casein, collagen, and albumin did not affect the bioavailability of Fe. Proteins from animal source foods and their digestion products did not enhance heme Fe absorption.



## 1. Introduction

There are two kinds of Fe in the human diet: non-heminic Fe (non-heme Fe), present in plant and dairy-based foods, and heminic Fe (heme Fe), present in foods derived from animal tissue (Sharp & Srai, 2007). The mechanisms of non-heme Fe absorption are widely described and are, generally well understood (Andrews, 1999; Fuqua, Vulpe, & Anderson, 2012). However, research on the mechanisms of heme Fe absorption is not fully understood. Some studies suggest that heme Fe released in the enterocyte is internalized by a mechanism of receptor-mediated pinocytosis (Muller-Eberhard & Fraig, 1993). This mechanism has been demonstrated through studies conducted on Heme Carrier Protein 1 Transports (HCP1) (Beard & Han, 2009; Le Blanc, Garrick, & Arredondo, 2012), and its absorption as a saturable process (Pizarro, Olivares, Hertrampf, Mazariegos, & Arredondo, 2003; West & Oates, 2008). Based on studies performed between 1960 and 1980, heme Fe is poorly absorbed when ingested alone (Conrad, Cortell, Williams, & Foy, 1966), but its absorption increases when ingested as Hb (Conrad et al., 1966; Layrisse & Martínez-Torres, 1972). Absorption also increases when heme Fe is ingested in the presence of foods with high levels of meat proteins (Martínez-Torres & Layrisse, 1971; Martínez-Torres, Romano, & Layrisse, 1981). As a result, it has been postulated that proteins from animal source foods and/or their digestion products maintain heme solubility, favoring heme Fe absorption (Conrad et al., 1966; Martínez-Torres & Layrisse, 1971) through: (a) digestion products from meat proteins, which stimulate heme transfer across the enterocyte and/or, (b) meat proteins that enhance the passage of heme Fe through mucin (Hallberg, Bjorn-Rasmussen, Howard, & Rossander, 1979). Data obtained in Caco-2 cell models supports the theory that globin promotes apical uptake of heme (Follett, Suzuki, & Lonnerdal, 2002) and the possible existence of a protein located in the apical region of enterocytes that negatively regulates the absorption of heme and/or polypeptides that may help in the absorption of heme Fe (Uc, Stokes, & Britigan, 2004). However, in Caco-2 cell studies, reduced heme Fe uptake associated with animal proteins in general has been described, whilst purified animal proteins increased heme Fe uptake (Villarroel, Flores, Pizarro, de Romaña, & Arredondo, 2011). Therefore, the

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involvement of proteins from animal source foods and their digestion products in the absorption of heme is not clear. In this article, we evaluate the effect of dietary proteins from animal source foods on the absorption of heme Fe in humans.

## 2. Subjects and methods

### 2.1. Subjects

Forty-five apparently healthy women aged 35–45 years were randomly assigned to three Fe absorption sub-studies (15 in each one). The participants were not taking any medication or vitamins or mineral supplements two months prior to or during the study. None of the participants were blood donors, pregnant or lactating, and all of them were using intrauterine devices or other contraceptive methods at the time of the study. Pregnancy was ruled out using a test for human chorionic gonadotropin in urine. Subjects were characterized by age, weight, height, body mass index and Fe status (biomarkers are described in Section 2.6).

### 2.2. Ethics

Written, informed consent was obtained from all the volunteers before the studies began. The protocol was approved by the Ethics Committee of the Institute of Nutrition and Food Technology, University of Chile and the doses of radioactive isotopes used were approved by the Chilean Commission on Nuclear Energy. Radioisotope labeling of heme Fe protocols in rabbits and calves were approved by the Bioethics Committee of the Institute of Nutrition and Food Technology, University of Chile and by the Bioethics Advisory Committee of the National Fund for Science and Technology (FONDECYT) of Chile.

### 2.3. Heme Fe labeled with radioactive isotopes

Fe isotopes of high specific activity ($^{59}$Fe and $^{55}$Fe) were used as intrinsic markers of heme Fe (NEN Life Science Products, Inc., Boston), which were injected into the marginal ear vein of five male New Zealand rabbits aged 5 mo and ~3 kg of weight (37 MBq $^{59}$Fe diluted in 0.1 mL of a solution of 9 g NaCl/L), and into the jugular vein of two male Holstein Friesian calves aged 4 mo and ~130 kg of weight (740 MBq $^{55}$Fe diluted in 3 mL of a solution of 9 g NaCl/L). Fifteen days after the injection of the isotopes, the rabbits and calves received an overdose of anesthetic (10% thiopental at 25 mg/kg I.V); followed by exsanguinations via the jugular route (Hubrecht & Kirkwood, 2010). The blood of the rabbits and calves was received in containers with 0.11 M sodium citrate in a ratio of 9:1 (v/v) citrate:blood and transferred immediately to the laboratory for processing. Heme Fe compounds were prepared using rabbit and calf blood.

### 2.4. Heme Fe compound preparation

The collected blood was centrifuged at 3207×*g* for 10 min at 10 °C in a refrigerated centrifuge (RC3B Sorvall, Thermo Fisher Scientific, Waltham, MA, USA). Plasma and leukocytes were discarded and red blood cells were washed three times with 9 g/L NaCl. From the red blood cells (RBC), the following was obtained: (a) red blood cell concentrates (RBCC). The RBC of rabbits were frozen in glass balls over 36–48 h and were lyophilized in a freeze dryer (Eyela FD1, Tokyo, Japan) for about 24 h depending on volume. Labeled RBCC with specific activity of 756 kBq $^{59}$Fe/mg of Fe was obtained. The labeled RBCC were mixed in dry form with untagged bovine RBC obtained from a calf that was not treated with radioisotopes, resulting in a dose of 37 kBq/5 mg elemental Fe. (b) Hemoglobin (Hb). Bovine RBC were hemolyzed by adding one volume of deionized water, then stroma proteins were precipitated by adding a 20% solution of ammonium sulfate. The final mixture was ultracentrifuged at 20,000×*g* for one hour (Sorvall RC2B, Thermo Fisher Scientific, Waltham, MA, USA). The supernatant was then collected and dialyzed (cutoff point 8000 D) against deionized water to eliminate ammonium sulfate traces. The labeled Hb was mixed in dry form with untagged bovine Hb resulting in a dose of 111 kBq $^{55}$Fe/5 mg elemental Fe. (c) Heme. Heme was extracted with the technique described by Labbe and Nishida (1957). RBC from bovine and rabbits were treated with strontium 2% chloride in an acetic acid and acetone solution (1:3), and were heated 10 min to boiling point to separate hemin from globin and other proteins. The final solution was then filtered (Whatman paper filter 1) to eliminate protein residues, and heated again for about 1–2 h under an extraction hood to evaporate acetone and part of the water present in the mixture. The heme started to precipitate when the solution was at room temperature. The final product was washed with an acetic acid water solution (1:1), ethanol, and then diethylether, afterward dried at 37 °C overnight. Labeled purified heme with a specific activity of 1,913 kBq $^{59}$Fe and 274 kBq $^{55}$Fe/mg of Fe was obtained. The labeled heme was mixed in dry form with untagged bovine heme such that the result was a dose of 37 kBq $^{59}$Fe or 111 kBq $^{55}$Fe/5 mg elemental Fe.

### 2.5. Study design (Fig. 1)

Three Fe absorption studies were performed. The doses were administered after 8 h of nocturnal fast, and subjects were not allowed to eat again until 4 h after ingestion of the doses. A sample of 15 subjects/group was calculated in order to detect a 5% difference in the absorption of heme Fe. An alpha equal to 0.05, and 80% power, allowed for an estimated 25% loss to follow-up.

#### 2.5.1. Study 1

This study was conducted to determine the absorption of heme Fe from heme only, RBCC, Hb and RBCC plus beef. The subjects received the same dose of 5 mg of Fe as heme, from the different sources, in gelatin capsules (Reutter Co, Santiago, Chile). On day 1, subjects received $^{55}$heme; on day 2, $^{59}$RBCC; on day 14, $^{55}$Hb; and on day 15, $^{59}$RBCC plus 150 g of cow beef (0.79 and 1.39 mg of heme and total Fe/100 g, respectively).

#### 2.5.2. Study 2

This study was designed to measure the effect of heme alone, and heme with fish (*Cilus gilberti*), chicken (*Gallus gallus*) and beef (*Bos taurus*) on heme Fe absorption. The subjects received the same dose of 5 mg of Fe as heme in gelatin capsules (Reutter Co, Santiago, Chile), plus the different meats. On day 1, subjects received

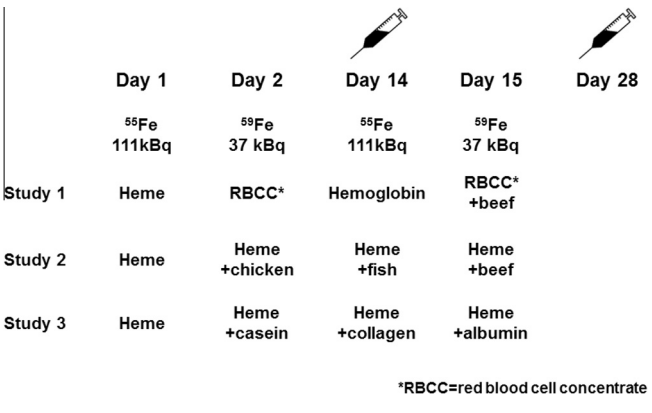


**Fig. 1.** Experimental design.

$^{55}$heme; on day 2, $^{59}$heme plus 150 g chicken (0.81 mg total Fe/100 g); on day 14 $^{55}$heme plus 150 g of fish (1.06 mg total Fe/100 g); and day 15 $^{59}$heme plus 150 g of beef (1,39 mg total Fe/100 g). The meat was steamed at 100 °C for 15 min without other foods or additive, except salt (0.3 g). The iron content of meals were determined by spectrometry atomic absorption, previous digestion of the sample by using nitric, perchloric, and sulfuric acids.

#### 2.5.3. Study 3

Study 3 aimed to measure the effect of collagen, albumin and casein on heme Fe absorption. The subjects received the same dose of 5 mg of Fe as heme with 1.7 g of one of the three protein sources, in gelatin capsules (Reutter Co, Santiago, Chile). On day 1, subjects received $^{55}$heme alone; on day 2, $^{59}$heme plus casein (casein bovine milk, Sigma–Aldrich C3400, Mo, USA); on day 14, $^{55}$heme plus collagen (Collagen from bovine, Sigma–Aldrich 9879, USA); and on day 15, $^{59}$heme plus albumin (albumin bovine serum, Sigma–Aldrich A9418, Mo USA). The amount of protein administered was calculated from the ratio of Fe:globin in Hb (3 mg:1 g; therefore, 5 mg of heme Fe corresponds to 1.7 g of protein.

### 2.6. Blood samples

After 8 h of fasting, 30 mL of venous blood samples were obtained on days 14 and 30 ml on day 28 to measure circulating radioactivity and to determine the Fe status of the subjects using the following Fe status biomarkers: Hb and mean corpuscular volume (VCM) by automated hematology analyzer (Coulter Model ZBI, Hialeah, Fla., and CELL-DYN 3200, ABBOTT Diagnostics, Abbott Park, IL), free erythrocyte protoporphyrin (FEP) (Hematofluorimeter model 206D, AVIV, Lakewood, NJ, USA) (Blumberg, Eisinger, Lamola, & Zuckerman, 1977), serum Fe (SFe) and total Fe binding capacity (TIBC) (Fischer & Price, 1964), and serum ferritin (SF) by ELISA (International Anemia Consultative Group, 1985). Hb, VCM and FEP were measured in fresh blood. SFe, TIBC and SF were measured in serum obtained by centrifugation and stored at −20 °C. The percentage of transferrin saturation (TS) was calculated from the formula TS = SFe/TIBC * 100. The classification of Fe status was defined in accordance with the range/cut-off points established by the Centers for Disease Control (1998) for each of the above biomarkers. Depleted Fe stores were defined as SF < 12 μg/L, Fe deficiency without anemia was defined as normal Hb and two or more altered biomarkers (MCV, FEP, TIBC, SFe, TS or SF), and Fe deficiency anemia was defined as below-normal Hb and two or more of any of the previously mentioned altered biomarkers.

For the calculation of total radioactivity ingested, the radioactivity of 6 aliquots of each compound was counted and these values were used as standards. Measurement of blood radioactivity was performed in duplicate venous samples according to Eakins and Brown (1966). The samples were counted in sufficient time to obtain a counting error of <3% in a liquid scintillation counter (Packard Canberra Company TRI-CAB 1600 TR). The percentages of absorption were calculated on the basis of blood volumes estimated for height and weight (Nadler, Hidalgo, & Bloch, 1962), assuming 80% incorporation of the radioisotope into the erythrocyte (Bothwell, Charlton, Cook, & Finch, 1979). This method is reproducible in our laboratory with a variation coefficient of 5%. Percent of iron bioavailability was calculate by formula: ((cpm/ml blood * blood volume)/(cpm/g meal * meal intake) * 100)/0.8.

### 2.7. Statistics

Because the percentages of Fe absorption and serum ferritin concentrations were not normally distributed according to Shapiro–Wilk test, these values were log-transformed before calculating means, SD, and performing statistical analyses. Results were retransformed to recover original units and were expressed as geometric mean and range ± 1 SD in the Tables and as geometric means ± SEM in a Fig. 1. Fe absorption differences in each study were determined by repeated measured ANOVA and Dunnett's post hoc test ($p < 0.05$). The Pearson correlation coefficient was used to assess these associations. The software package PRISM version 5 (GraphPad Software, Inc, San Diego, Ca, USA) was used for statistical analysis.

## 3. Results

### 3.1. Subject characterization

Two women from studies 1 and 3 were excluded due to protocol violations (they did not attend). No significant differences in the anthropometric and Fe status values were found between groups. Of the 43 women, 6 presented Fe deficiency anemia, 3 Fe deficiency without anemia, 3 Fe depletion and 31 normal Fe status. Serum ferritin values ranged from 1 to 50 μg/L, indicating high variation in Fe stores as it was expected (Table 1). At the beginning of the study, 14 and 29 of the women were classified as normal and overweight, respectively.

### 3.2. Heme Fe absorption studies

The bioavailability of heme Fe from heme did not show significant differences across groups being geometric means and range ±1 SD 13.0% (8.7–19.4), 10.3% (6.8–15.8) and 9.8% (4.8–19.9) for groups 1, 2 and 3, respectively ($p > 0.05$). Correlating this variable with serum ferritin results in $r = -0.62$, $r = -0.55$ and $r = -0.58$ for groups 1, 2 and 3, respectively (all significant $p < 0.025$).

In study 1, the bioavailability of heme Fe from Hb was similar to the bioavailability of heme only. However, when heme Fe was ingested as RBCC or RBCC plus 150 g of beef, heme Fe bioavailability significantly increased nearly 2- and 1.6-fold, respectively ($p < 0.05$). Heme Fe bioavailability between these provisions did not differ statistically (Table 2).

In study 2, heme Fe bioavailability from heme was dramatically reduced when ingested with fish and particularly chicken ($p < 0.05$). Beef did not change heme absorption (Table 3).

In study 3, the presence of purified protein as collagen, casein and albumin did not significantly affect the bioavailability of heme Fe ($p > 0.05$) (Table 4).

Fig. 2 shows the ratios of Fe absorption from Hb, RBCC, RBCC plus beef, heme plus animal meat and animal purified proteins

**Table 1**
Characteristics of the participants.

| Characteristics | Study 1 ($n = 14$) | Study 2 ($n = 15$) | Study 3 ($n = 14$) |
|---|---|---|---|
| Age (y) | 39 ± 6 | 40 ± 5 | 40 ± 5 |
| Weight (kg) | 62.6 ± 6.1 | 61.4 ± 6.7 | 65.2 ± 8.9 |
| Height (m) | 1.55 ± 0.04 | 1.56 ± 0.06 | 1.55 ± 0.08 |
| BMI (kg/m$^2$) | 25.9 ± 2.0 | 25.4 ± 1.9 | 26.9 ± 2.0 |
| Hemoglobin (g/L) | 130 ± 12 | 122 ± 14 | 134 ± 11 |
| MCV (fL) | 88 ± 8 | 85 ± 7 | 88 ± 7 |
| FEP (μmol/L) | 1.17 ± 0.44 | 1.39 ± 0.63 | 1.07 ± 0.40 |
| Serum iron (μg/dl) | 70 ± 33 | 74 ± 35 | 72 ± 33 |
| TIBC (μg/dL) | 346 ± 54 | 318 ± 67 | 327 ± 51 |
| Transferrin saturation (%) | 21.3 ± 11.5 | 24.2 ± 12.8 | 22.9 ± 10.7 |
| Serum ferritin (μg/L)* | 12 (4–34) | 17 (8–38) | 23 (11–45) |
| Heme bioavailability (%)* | 13.0 (8.7–19.4) | 10.3 (6.8–15.8) | 9.8 (4.8–19.1) |

Body mass index (BMI), mean corpuscular volume (MCV), free erythrocyte protoporphyrim (FEP), total iron binding-capacity (TIBC). Mean ± SD.
* Geometric mean and range ± 1 SD in parentheses.

**Table 2**
Heme iron bioavailability of heme, red blood cell concentrate (RBCC), hemoglobin (Hb), and RBCC plus beef.

| Subjects | Heme iron bioavailability (%) | | | | Ratios | | |
|---|---|---|---|---|---|---|---|
| | $^{55}$Heme (H) | $^{59}$RBCC (A) | $^{55}$Hb (B) | $^{59}$RBCC + beef (C) | A/H | B/H | C/H |
| 1 | 8.7 | 6.5 | 5.0 | 10.8 | 0.74 | 0.57 | 1.24 |
| 2 | 4.1 | 22.6 | 2.5 | 2.8 | 5.49 | 0.60 | 0.67 |
| 3 | 10.2 | 24.2 | 13.1 | 31.2 | 2.38 | 1.29 | 3.07 |
| 4 | 12.3 | 16.0 | 14.0 | 23.3 | 1.30 | 1.14 | 1.90 |
| 5 | 15.2 | 22.8 | 24.2 | 21.6 | 1.50 | 1.59 | 1.42 |
| 6 | 20.9 | 46.4 | 27.6 | 46.4 | 2.22 | 1.32 | 2.22 |
| 7 | 19.2 | 25.1 | 39.5 | 48.4 | 1.31 | 2.06 | 2.52 |
| 8 | 15.3 | 34.2 | 16.2 | 25.3 | 2.23 | 1.06 | 1.65 |
| 9 | 15.3 | 33.7 | 28.1 | 36.2 | 2.20 | 1.83 | 2.36 |
| 10 | 14.1 | 36.3 | 8.9 | 14.5 | 2.58 | 0.63 | 1.03 |
| 11 | 14.4 | 13.6 | 5.8 | 10.0 | 0.94 | 0.40 | 0.69 |
| 12 | 14.8 | 24.8 | 17.2 | 24.7 | 1.68 | 1.16 | 1.67 |
| 13 | 15.2 | 89.2 | 31.1 | 53.6 | 5.85 | 2.04 | 3.52 |
| 14 | 13.7 | 19.5 | 10.7 | 21.9 | 1.42 | 0.78 | 1.60 |
| Mean[1] | 13.0 | 25.0 | 13.7 | 21.3 | 1.92 | 1.05 | 1.64 |
| SD[1] | 8.7–19.4 | 13.6–45.9 | 6.2–30.2 | 9.7–46.7 | 1.1–3.4 | 0.6–1.8 | 1.0–2.7 |
| SS[2] | | $p < 0.05$ | N.S | $p < 0.05$ | | | |

[1] Geometric mean and range ± 1 SD.
[2] Statistical significance calculated with respect to heme absorption.

**Table 3**
Heme iron bioavailability of heme only and plus animal meats (fish, chicken, beef).

| Subjects | Heme iron bioavailability (%) | | | | Ratios | | |
|---|---|---|---|---|---|---|---|
| | $^{55}$Heme (H) | $^{59}$Heme + fish (A) | $^{55}$Heme + chicken (B) | $^{59}$Heme + beef (C) | A/H | B/H | C/H |
| 1 | 6.9 | 4.6 | 4.9 | 4.8 | 0.67 | 0.72 | 0.70 |
| 2 | 8.3 | 3.8 | 3.7 | 6.6 | 0.45 | 0.44 | 0.80 |
| 3 | 13.8 | 8.7 | 4.9 | 14.3 | 0.63 | 0.36 | 1.04 |
| 4 | 5.9 | 6.4 | 4.7 | 8.3 | 1.09 | 0.80 | 1.41 |
| 5 | 8.7 | 5.0 | 2.0 | 9.1 | 0.58 | 0.23 | 1.04 |
| 6 | 7.1 | 5.6 | 4.7 | 7.5 | 0.80 | 0.66 | 1.06 |
| 7 | 8.5 | 3.8 | 1.6 | 7.6 | 0.45 | 0.19 | 0.89 |
| 8 | 13.5 | 9.3 | 6.2 | 11.9 | 0.69 | 0.46 | 0.88 |
| 9 | 12.0 | 8.8 | 8.0 | 13.6 | 0.73 | 0.66 | 1.13 |
| 10 | 17.9 | 6.7 | 9.3 | 23.5 | 0.37 | 0.52 | 1.31 |
| 11 | 17.7 | 12.5 | 10.4 | 24.2 | 0.71 | 0.59 | 1.37 |
| 12 | 11.3 | 7.6 | 5.2 | 16.1 | 0.67 | 0.46 | 1.42 |
| 13 | 6.9 | 6.6 | 4.4 | 7.8 | 0.96 | 0.65 | 1.14 |
| 14 | 23.5 | 12.1 | 6.4 | 18.2 | 0.51 | 0.27 | 0.77 |
| 15 | 7.1 | 12.2 | 4.7 | 14.3 | 1.73 | 0.67 | 2.02 |
| Mean[1] | 10.3 | 7.1 | 4.9 | 11.3 | 0.68 | 0.47 | 1.09 |
| SD[1] | 6.8–15.8 | 4.7–10.5 | 3.0–8.0 | 7.0–18.2 | 0.5–1.0 | 0.3–0.7 | 0.8–1.4 |
| SS[2] | | $p < 0.05$ | $p < 0.05$ | N.S | | | |

[1] Geometric mean and range ± 1 SD.
[2] Statistical significance calculated with respect to heme absorption.

against heme Fe bioavailability. According to the present study and in our experimental conditions, purified animal proteins did not favor heme Fe absorption in humans; chicken and fish dramatically reduced absorption, and displayed absorption ratios below 1.0. The presence of 150 g of chicken or fish decreased the bioavailability of heme Fe by 53% and 32%, respectively. However, the red cell stroma was a clear factor that promoted heme Fe absorption with an absorption ratio of 1.92.

## 4. Discussion

Fe deficiency anemia is one of the world's most common nutrition related disorders that occurs, among other causes, when dietary intake of bioavailable Fe is low (World Health Organization, 2001). Heme Fe is absorbed from meat or meat products and represents a relatively small part of total dietary Fe intake but possesses higher bioavailability than non-heme Fe (Hallberg et al., 1979). Fe deficiency is less prevalent in countries in which meat constitutes a significant part of the diet (Uzel & Conrad, 1998). Both Fe forms are absorbed from the diet by different mechanisms (Conrad & Umbreit, 2000), however, the mechanisms by which heme is absorbed are still being studied and the effects of animal proteins on heme Fe bioavailability are unclear. For decades it has been postulated that the proteolytic digestion of animal proteins results in the release of heme, which is maintained in a soluble form causing intraluminal factors that either diminish or enhance the absorption of inorganic Fe to have no effect on heme Fe absorption (Conrad & Umbreit, 2000). However, recent data obtained in Caco-2 cell models supports the hypothesis that trypsin and mucin may enhance the absorption of heme Fe (Jin, Welch, & Glahn, 2006; Uc et al., 2004), and data obtained in our laboratory,

**Table 4**
Heme iron bioavailability of heme only and plus animal purified proteins.

| Subjects | Heme iron bioavailability (%) | | | | Ratios | | |
|---|---|---|---|---|---|---|---|
| | $^{55}$Heme (H) | $^{59}$Heme + casein (A) | $^{55}$Heme + collagen (B) | $^{59}$Heme + albumin (C) | A/H | B/H | C/H |
| 1 | 7.1 | 15.4 | 13.5 | 13.6 | 2.17 | 1.90 | 1.92 |
| 2 | 4.9 | 7.0 | 8.0 | 8.3 | 1.42 | 1.64 | 1.69 |
| 3 | 6.8 | 11.9 | 9.8 | 10.3 | 1.75 | 1.45 | 1.52 |
| 4 | 7.6 | 8.3 | 11.2 | 6.0 | 1.10 | 1.47 | 0.79 |
| 5 | 11.0 | 15.5 | 14.1 | 13.3 | 1.41 | 1.29 | 1.21 |
| 6 | 8.7 | 24.8 | 20.3 | 23.6 | 2.86 | 2.34 | 2.72 |
| 7 | 2.6 | 9.5 | 4.6 | 7.6 | 3.59 | 1.75 | 2.86 |
| 8 | 30.6 | 28.0 | 24.6 | 27.7 | 0.91 | 0.81 | 0.90 |
| 9 | 12.6 | 26.0 | 28.0 | 12.7 | 2.07 | 2.22 | 1.01 |
| 10 | 4.3 | 4.5 | 4.0 | 3.1 | 1.05 | 0.94 | 0.73 |
| 11 | 13.4 | 6.8 | 9.9 | 7.8 | 0.51 | 0.74 | 0.58 |
| 12 | 22.4 | 23.1 | 25.0 | 22.9 | 1.03 | 1.12 | 1.02 |
| 13 | 28.6 | 19.4 | 14.4 | 19.4 | 0.68 | 0.50 | 0.68 |
| 14 | 11.8 | 8.9 | 6.2 | 6.8 | 0.75 | 0.53 | 0.58 |
| Mean[1] | 9.8 | 12.9 | 11.8 | 11.1 | 1.31 | 1.20 | 1.13 |
| SD[1] | 4.8–19.9 | 7.2–23.1 | 6.4–21.7 | 6.0–20.5 | 0.8–2.3 | 0.7–2.0 | 0.7–1.9 |
| SS[2] | | N.S | N.S | N.S | | | |

[1] Geometric mean and range ± 1 SD.
[2] Statistical significance calculated with respect to heme absorption.

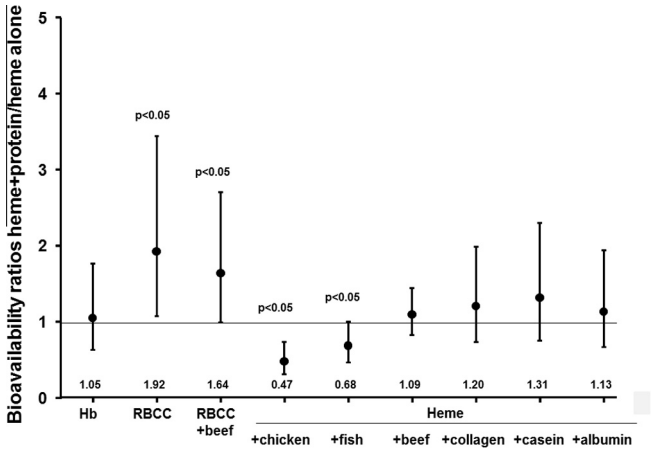


**Fig. 2.** Comparison of ratio: heme Fe absorption from different products as hemoglobin (Hb), red blood cell concentrate (RBCC), RBCC plus beef; heme plus animal meats (fish, chicken, beef); and heme plus animal purified proteins (collagen, casein, albumin)/heme iron bioavailability of heme alone.

using human subjects, suggests that trypsin is the only human gastrointestinal protein than increases heme absorption when Hb plus trypsin is ingested (Cediel et al., 2012).

Studies conducted since the early 1960s provide information on the effect of dietary protein on heme Fe absorption. It is known that heme Fe is poorly absorbed when consumed as a prosthetic group (Conrad et al., 1966), but its absorption increases when ingested as Hb (heme bound to four globin chains) (Conrad et al., 1966; Layrisse & Martínez-Torres, 1972), presumably because heme Fe absorption is facilitated by degraded protein substances from Hb: globin protein prevents the polymerization of heme (Conrad et al., 1966). In our work, a significant increase in heme Fe absorption from Hb was not observed compared to heme alone (study 1, Table 2). We also found that heme Fe bioavailability from RBCC (composed of heme + globin + erythrocyte stroma), was twice that of heme alone and Hb, indicating that the erythrocyte stroma contributes to the promotion of heme Fe absorption. The discrepancies between past findings and the results reported in this present study could be explained as follows: Conrad et al. (1966) induced anemia by phlebotomy in guinea pigs that received intraperitoneal injections with a dose of $^{59}$Fe. The $^{59}$heme was obtained from complete RBC lysates and, therefore, these studies were performed with Hb plus erythrocyte stroma. The Fe absorption studies conducted in humans by Layrisse and Martínez-Torres (1972) also used $^{59}$heme obtained from rabbit RBC lysates. All the studies available to date used complete erythrocyte blended with meals. None of these studies used purified hemoglobin, which was used in the present study (study 1, Table 2).

On the other hand, it has been reported that heme Fe bioavailability is increased two to three times when Hb is ingested with meat (Heinrich, Gabbe, & Kugler, 1971; Layrisse & Martínez-Torres, 1972) or typical amino acids contained in meats such as cysteine (Martínez-Torres et al., 1981). In the present article, contrary to the data reported by these authors, we observed that 150 g of beef did not increase heme Fe absorption compared to heme alone, which was confirmed in study 2 (Table 3). Heme Fe absorption from RBCC (study 1, Table 2) was not increased either, again suggesting that the erythrocyte stroma is the only factor that increases heme Fe bioavailability. With the other meats, 150 g of chicken and fish decreased heme Fe absorption significantly. These results are similar to those reported previously by our group working with Caco-2 cells, wherein it was observed that animal proteins, in general, decrease heme Fe uptake, determined according to animal protein discoloration (Villarroel et al., 2011). The aforementioned observations may be related to (a) the lower content of blood trapped in the muscles compared to beef, on the basis that erythrocyte stroma is the only factor that generates an increase in the absorption of heme Fe, (b) lower concentration of myoglobin (≈15 mg/g beef muscle vs. ⩽5 mg/g of white meats such as chicken or fish) (Livingston & Brown, 1981), and/or (c) the low heme Fe content of these meats (30% equivalent to 0.26 mg heme Fe/100 g of raw breast chicken meat and 37% equivalent to 0.47 mg heme Fe/100 g of tilapia raw meat) (Kongkachuichai, Napatthalung, & Charoensiri, 2002) compared with beef (65% equivalent to 1.30 mg heme Fe/100 g of raw meat) (Valenzuela, de Romaña, Olivares, Morales, & Pizarro, 2009). Recently, other authors have also reported large variability in the heme Fe content values of different meats such as: beef (46% to 78%), chicken (23 to 40%), and fish (around 26%) (Schönfeldt & Hall, 2011). The percentage of heme bioavailability from heme plus fish and chicken observed in this study (study 2, Table 3) is similar to the 7.6% reported by Garcia et al. (1996), wherein subjects were fed with a diet

containing beef precipitate in which only 30% of meat Fe was heme. Regarding the effect of animal source foods and/or animal protein on heme Fe absorption, we did not observe the enhanced effect on absorption that has been reported in other studies conducted in animals, humans (Hallberg et al., 1979; Layrisse & Martínez-Torres, 1972), and Caco-2 cell models with purified animal proteins (collagen and casein) (Villarroel et al., 2011). These results suggest the existence of one or more compounds in the erythrocyte stroma that increase heme Fe bioavailability from the human diet.

On the other hand, at the public health level, interventions such as Fe fortification/supplementation have been promoted in order to mitigate the high prevalence of Fe deficiency anemia around the world (World Health Organization, 2001). Heme Fe–rich blood products have been used for this purpose (Hoppe, Brün, Larsson, Moraeus, & Hulthén, 2013; Seligman, Moore, & Schleicher, 2000; Walter et al., 1993), which demonstrated improvement the Fe status of human subjects, with a low chance of causing gastrointestinal side effects (Frykman, Bystrom, Jansson, Edberg, & Hansen, 1994). It is, therefore, important to continue research in this area in order to determine the erythrocyte stroma factors responsible for the increase in heme Fe absorption.

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## References

Andrews, N. (1999). The iron transporter DMT1. *The International Journal of Biochemistry & Cell Biology, 31*, 991–994.
Beard, J., & Han, O. (2009). Systemic iron status. *Biochimica et Biophysica Acta, 1790*, 584–588.
Blumberg, W. E., Eisinger, J., Lamola, A. A., & Zuckerman, D. M. (1977). The hematofluorometer. *Clinical Chemistry, 23*, 270–274.
Bothwell, T., Charlton, R., Cook, J., & Finch, C. (1979). *Iron metabolism in man*. Oxford: Blackwell Scientific Publications.
Centers for Disease Control and Prevention (1998). Recommendations to prevent and control iron deficiency in the United States. *Morbility and Mortality Weekly Report, 47*, 1–36.
Cediel, G., Olivares, M., Gaitán, D., Flores, S., Brito, A., & Pizarro, F. (2012). Effect of trypsin and mucin on heme iron bioavailability in humans. *Biological Trace Element Research, 150*, 37–41.
Conrad, M., Cortell, S., Williams, H., & Foy, A. (1966). Polymerization and intraluminal factors in the absorption of hemoglobin-iron. *Journal of Laboratory and Clinical Medicine, 68*, 659–668.
Conrad, M., & Umbreit, J. (2000). Iron absorption and transport-an update. *American Journal of Hematology, 64*, 287–298.
Eakins, I., & Brown, D. (1966). An improved method for the simultaneous determinations of 55-iron and 59-iron in blood by liquid scintillation counting. *The International Journal of Applied Radiation and Isotopes, 17*, 391–397.
Fischer, D. S., & Price, D. C. (1964). A simple serum iron method using the new sensitive chromogen tripyridyl-S-triazine. *Clinical Chemistry, 10*, 21–31.
Follett, J., Suzuki, Y., & Lonnerdal, B. (2002). High specific activity heme-Fe and its application for studying heme-Fe metabolism in Caco-2 cell monolayers. *American Journal of Physiology – Gastrointestinal and Liver Physiology, 283*, 1125–1131.
Frykman, E., Bystrom, M., Jansson, U., Edberg, A., & Hansen, T. (1994). Side effects of iron supplements in blood donors: superior tolerance of heme iron. *Journal of Laboratory and Clinical Medicine, 123*, 561–564.
Fuqua, B., Vulpe, C., & Anderson, G. (2012). Intestinal iron absorption. *Journal of Trace Elements in Medicine and Biology, 26*, 115–119.
Garcia, M., Martínez-Torres, C., Leets, I., Tropper, E., Ramirez, J., & Layrisse, M. (1996). Heat treatment on heme iron and iron-containing proteins in meat: Iron absorption in humans from diets containing cooked meat fractions. *The Journal of Nutritional Biochemistry, 7*, 49–54.
Hallberg, L., Bjorn-Rasmussen, E., Howard, L., & Rossander, L. (1979). Dietary heme iron absorption. A discussion of possible mechanisms for the absorption-promoting effect of meat and for the regulation of iron absorption. *Scandinavian Journal of Gastroenterology, 14*, 769–779.
Heinrich, H., Gabbe, E., & Kugler, G. (1971). Comparative absorption of ferri-haemoglobin- $^{59}$Fe-Ferro-haemoglobin- $^{59}$Fe and $^{59}Fe^{3+}$ – $^{59}Fe^{2+}$ in humans with normal and depleted iron stores. *European Journal of Clinical Investigation, 1*, 321–327.
Hoppe, M., Brün, B., Larsson, M., Moraeus, L., & Hulthén, L. (2013). Heme iron-based dietary intervention for improvement of iron status in young women. *Nutrition, 29*, 89–95.
Hubrecht, R., & Kirkwood, J. (2010). *The UFAW Handbook on the care and management of laboratory and other research animals* (4th ed.). Hoboken: John Wiley & Sons Inc.
International Anemia Consultative Group (1985). *Measurement of iron status. A report of the International Anemia Consultative Group (INACG)*. Washington DC: Nutrition Foundation.
Jin, F., Welch, R., & Glahn, R. (2006). Moving toward a more physiological model: Application of mucin to refine the in vitro digestion/Caco-2 cell culture system. *Journal of Agricultural and Food Chemistry, 54*, 8962–8967.
Kongkachuichai, R., Napatthalung, P., & Charoensiri, R. (2002). Heme and nonheme iron content of animal products commonly consumed in Thailand. *Journal of Food Composition and Analysis, 15*, 389–398.
Labbe, R., & Nishida, G. (1957). A new method of hemin isolation. *Biochimica et Biophysica Acta, 26*, 437.
Layrisse, M., & Martínez-Torres, C. (1972). Model for measuring dietary absorption of heme iron:test with a complete meal. *The American Journal of Clinical Nutrition, 25*, 401–411.
Le Blanc, S., Garrick, M., & Arredondo, M. (2012). Heme carrier protein 1 transports heme and is involved in heme-Fe metabolism. *The American Journal of Physiology – Cell Physiology, 302*, 1780–1785.
Livingston, D., & Brown, W. (1981). The chemistry of myoglobin and its reactions. *Food Technology, 25*. 244-222.
Martínez-Torres, C., & Layrisse, M. (1971). Iron absorption from veal muscle. *The American Journal of Clinical Nutrition, 24*, 531–540.
Martínez-Torres, C., Romano, E., & Layrisse, M. (1981). Effect of cysteine on iron absorption in man. *The American Journal of Clinical Nutrition, 3*, 322–327.
Muller-Eberhard, U., & Fraig, M. (1993). Bioactivity of heme and its containment. *American Journal of Hematology, 42*, 59–62.
Nadler, S., Hidalgo, J., & Bloch, T. (1962). Prediction of blood volume in normal human adults. *Surgery, 51*, 224–232.
Pizarro, F., Olivares, M., Hertrampf, E., Mazariegos, D., & Arredondo, M. (2003). Heme-iron absorption pathway is saturable by heme-iron dose in humans. *Journal of Nutrition, 133*, 2214–2217.
Schönfeldt, H., & Hall, N. (2011). Determining iron bio-availability with a constant heme iron value. *Journal of Food Composition and Analysis, 24*, 738–740.
Seligman, P., Moore, G., & Schleicher, R. (2000). Clinical studies of HIP: An oral heme-iron product. *Nutrition Research, 20*, 1279–1286.
Sharp, P., & Srai, S. (2007). Molecular mechanisms involved in intestinal iron absorption. *World Journal of Gastroenterology, 13*, 4716–4724.
Uc, A., Stokes, J., & Britigan, B. (2004). Heme transport exhibits polarity in Caco-2 cells: Evidence for an active and membrane protein-mediated process. *American Journal of Physiology – Gastrointestinal and Liver Physiology, 287*, 1150–1157.
Uzel, C., & Conrad, M. (1998). Absorption of heme iron. *Seminars in Hematology, 35*, 27–34.
Valenzuela, C., de Romaña, D., Olivares, M., Morales, S., & Pizarro, F. (2009). Total iron and heme iron content and their distribution in beef meat and viscera. *Biological Trace Element Research, 132*, 103–111.
Villarroel, P., Flores, S., Pizarro, F., de Romaña, D., & Arredondo, M. (2011). Effect of dietary protein on heme iron uptake by Caco-2 cells. *European Journal of Nutrition, 50*, 637–643.
Walter, T., Hertrampf, E., Pizarro, F., Olivares, M., Letelier, A., Vega, V., & Stekel, A. (1993). Effect of bovine-hemoglobin-fortified cookies on iron status of schoolchildren: A nationwide program in Chile. *The American Journal of Clinical Nutrition, 57*, 190–194.
West, A., & Oates, P. (2008). Mechanisms of heme iron absorption: current questions and controversies. *World Journal of Gastroenterology, 14*, 4101–4110.
World Health Organization (2001). *Iron deficiency anemia: assessment, prevention and control. A guide for programme managers*. Geneva: WHO.