Progressive Increases in Dietary Iron Are Associated with the Emergence of Pathologic Disturbances of Copper Homeostasis in Growing Rats

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Abstract

Background: Consumption of a high-iron diet causes copper deficiency in weanling rodents; however, the minimum amount of dietary iron that disrupts copper homeostasis has not been established.

Objective: We tested the hypothesis that dietary iron at only several-fold above physiologic requirements would cause copper depletion.

Methods: Weanling male Sprague-Dawley rats (n = 6/group) were fed AIN-93G-based diets with adequate (88 µg Fe/g = 1×), or excessive (4×, 9.5×, 18.5×, 38×, or 110×) iron content for 7 wk (110× group, due to notable morbidity) or 8 wk (all other groups). Copper-related physiologic parameters were then assessed.

Results: A hierarchy of copper-related, pathologic symptoms was noted as dietary iron concentrations increased. All statistical comparisons reported here refer to differences from the 1× (i.e., control) group. The highest iron concentration (110×) impaired growth (final body weights decreased ∼40%; $P < 0.0001$), and caused anemia (blood hemoglobin and hematocrit decreased ∼65%; $P < 0.0001$) and hepatic copper depletion (>85% reduction; $P < 0.01$). Cardiac hypertrophy occurred in the 110× (∼130% increase in mass; $P < 0.0001$) and 38× (∼25% increase; $P < 0.05$) groups, whereas cardiac copper content was lower in the 110× ($P < 0.01$), 38× ($P < 0.01$), and 18.5× ($P < 0.05$) groups (∼70% reductions). Splenic copper was also depleted in the 110× (>90% reduction; $P < 0.0001$), and in the 38× ($P < 0.001$) and 18.5× ($P < 0.01$) groups (∼70% reductions). Moreover, serum ceruloplasmin activity was decreased in the 110× and 38× (>90% reductions; $P < 0.0001$), and 18.5× ($P < 0.001$) and 9.5× ($P < 0.05$) (∼50% reductions) groups, typifying moderate to severe copper deficiency.

Conclusions: Increasing dietary iron intakes to ∼9.5-fold above dietary recommendations caused copper deficiency. Importantly, human iron supplementation is common, and recommended intakes for at-risk individuals may be ≤10-fold above the RDA. Whether these iron intakes perturb copper metabolism is worth considering, especially since copper deficiency can impair iron utilization (e.g., by decreasing the ferroxidase activity of ceruloplasmin). *J Nutr* 2018;148:373–378.

Keywords: anemia, cardiac hypertrophy, copper deficiency, ceruloplasmin, iron supplementation

Introduction

Iron and copper are essential dietary components for humans and other mammals. Excess or deficient intakes of either of these trace minerals can result in significant morbidity and mortality. Moreover, physiologically relevant interactions between iron and copper have been frequently observed (1). In previous dietary iron-overload experiments, we fed growing rats and mice diets with high iron (∼110-fold in excess of requirements) in combination with adequate or high copper (∼20-fold excess). Unexpectedly, rats and mice consuming the high-iron diet with adequate copper developed severe physiologic perturbations, including premature mortality, growth impairment, severe anemia, cardiac hypertrophy, tissue copper depletion, and

decreased serum ceruloplasmin (Cp) activity. Many of these pathological symptoms typify severe copper deficiency (2, 3). Notably, these abnormalities did not occur in animals consuming the high-iron diet with excess copper (4, 5), essentially proving that these pathologies were caused by copper depletion. The threshold of dietary iron necessary to disrupt copper metabolism has not, however, been experimentally determined. The purpose of the current study was thus to determine the lowest amount of dietary iron that disrupts copper homeostasis.

Defining the minimum dietary iron concentration that antagonizes copper is important, given that many humans consume supplemental iron (6, 7). Individuals most likely to require iron supplements include infants and children, adolescents, and menstruating and pregnant women, particularly amongst those of modest economic means (8–12). Moreover, some pathologic situations may impair intestinal iron absorption, thus increasing the risk for iron depletion and the likelihood that iron supplementation may be recommended. These include gastritis and achlorhydria (13), gastric bypass surgery (14), chronic acid suppression therapy (15), and malabsorptive disorders (16). Dosing varies, but in some cases, supplemental iron at 4- to 10-fold in excess of the RDA may be necessary to correct the iron deficiency (10–12). Given this background, the current investigation was undertaken to test the hypothesis that supplemental iron, at concentrations similar to what humans may consume, will disrupt copper homeostasis. To test this postulate, we fed rapidly growing rats diets with adequate or excessive iron concentrations (with adequate copper) and then assessed the impact on copper homeostasis. Notably, disturbances of copper metabolism were observed in rats consuming iron at only 9.5-fold in excess of requirements.

Methods

Animal experiments

All animal studies were approved by the University of Florida Institutional Animal Care and Use Committee. Three-week-old, weanling, male Sprague-Dawley rats (Envigo) were housed in stainless steel overhanging, wire mesh-bottom cages to minimize coprophagia for 7–8 wk until they were killed. Weanling rats were utilized for this investigation since iron demand is higher during periods of rapid growth, and we sought to model situations in which iron supplementation in humans is more likely. The rats were randomly assigned to the different dietary groups ($n = 6$/group) and had ad libitum access to diet and purified water. A single, large batch of basal AIN-93G diet (17) was first produced, containing all ingredients except for iron (Dyets Inc.). The background iron concentration was then measured by inductively-coupled plasma MS (ICP-MS), followed by adjusting the iron content up to 80 µg/g with ferric citrate. This basal diet was then split into 6 equal parts and carbonyl iron was added to 5 of these to achieve the desired final iron concentrations, which were 80, 320, 760, 1520, 3040, and 8800 µg/g, respectively. All diets contained adequate copper (6–7 µg/g), and were otherwise almost identical (**Supplemental Table 1**). At the low end, 80 µg/g was considered adequate for rapidly growing rats, and at the high end, 8880 µg/g was used since this was the iron concentration used in our previous dietary iron-overload studies (4, 5). Carbonyl iron was used to increase iron content since it is more stable and less prone to cause lipid peroxidation compared to iron salts (such as ferric citrate). Given the differing amounts of carbonyl iron added to each diet, extra corn starch was added to diets with less iron (except the 80 µg Fe/g diet); nevertheless, the energy content of the diets varied by <1% (Supplemental Table 1). Rats were weighed weekly and food consumption was estimated by weighing the amount of food provided daily to each cage of rats. After the dietary treatments, animals were killed by thoracotomy after CO_2 narcosis.

Hematologic parameters, tissue iron concentrations, and Cp activity

Blood hemoglobin (Hb) and hematocrit (Hct) concentrations were assessed using standard assays, as described previously (18). Serum and tissue nonheme iron concentrations, and total iron-binding capacity (TIBC), were determined using standard colorimetric assays (4). Percent transferrin saturation (TSAT) was calculated as: (serum nonheme iron concentration/TIBC) × 100. Serum Cp activity was quantified using a well-established amine oxidase (*para*-phenylenediamine) assay (19–22).

Quantification of mineral concentrations in diets and tissues

Ten pellets of each diet were randomly selected and ground with an acid-washed mortar and pestle. Diet samples and tissues were digested with HNO_3/H_2O_2 on a hot block. Digested samples were filtered (0.45 µm) and analyzed by ICP-MS (NexIon 300, Perkin-Elmer Corp.). Iron concentrations in diets, and iron and copper concentrations in tissues, were normalized by premeasured weights.

qRT-PCR

Total RNA was isolated from kidney [for erythropoietin (*Epo*) expression] and liver [for hepcidin (*Hepc*) expression] with RNAzol RT reagent (Molecular Research Center, Inc.) and relative mRNA expression was determined with a well-established SYBR Green qRT-PCR technique. To minimize amplification of genomic DNA, oligonucleotide primers were designed to span large introns. Expression of each experimental gene was normalized to the expression of cyclophilin, which did not vary significantly among groups. Average fold changes in mRNA expression were calculated by the $2^{-\Delta\Delta Ct}$ analysis method. The following primers were utilized (sequences are 5′ to 3′): cyclophilin, forward CTTGCTGCAATGGTCAACC, reverse TGCTGTCTTTGGAACTTTGTCTGC; *Epo*, forward AGTCGCGTTCTGGAGAGGTA, reverse ACTTTGGTATCTGGGACGGTAA; *Hepc*, forward GGCAGAAAGCAAGACTGATGAC, reverse ACAGGAATAAATAATGGGGCG.

Statistical analysis

The methods used to analyze data were selected after consultation with a biostatistician in the Institute of Food and Agricultural Sciences at the Unversity of Florida. To evaluate the physiologic and pathophysiologic effects of consuming diets with varying iron concentrations, data were first tested for equal variance using Levene's test. Since data sets were not equally distributed, data were log_{10} transformed, and subsequently analyzed by the nonparametric Kruskal-Wallis rank-sum test (Jmp Pro, version 12.2.0). When statistically significant differences were noted with the use of this test, the Wilcoxon post-hoc test was utilized to make comparisons between individual data sets. Some results are depicted as box-and-whisker plots, displaying the minimum, the lower (25th percentile), the median (50th percentile), the upper (75th percentile), and the maximum ranked sample. The mean value is indicated by a "+" sign. Note that for ease of interpretation, the nontransformed data are presented in the figures. The Pearson product-moment correlation coefficient (r) was calculated to determine selected relations between 2 variables (GraphPad Prism, version 7.0).

Results

Diet analysis

ICP-MS analysis of the diets revealed the following iron concentrations: 88, 320, 756, 1475, 3021, and 8834 µg/g. For ease of interpretation, the adequate-iron diet (with 88 µg Fe/g) is designated as 1×, the diet with 320 µg Fe/g as 4× (indicating 4-fold above requirements), and so forth.

Deviations from the experimental plan

The feeding trial was planned to end after 8 wk; however, rats consuming the highest-iron (i.e., the 110×) diet were
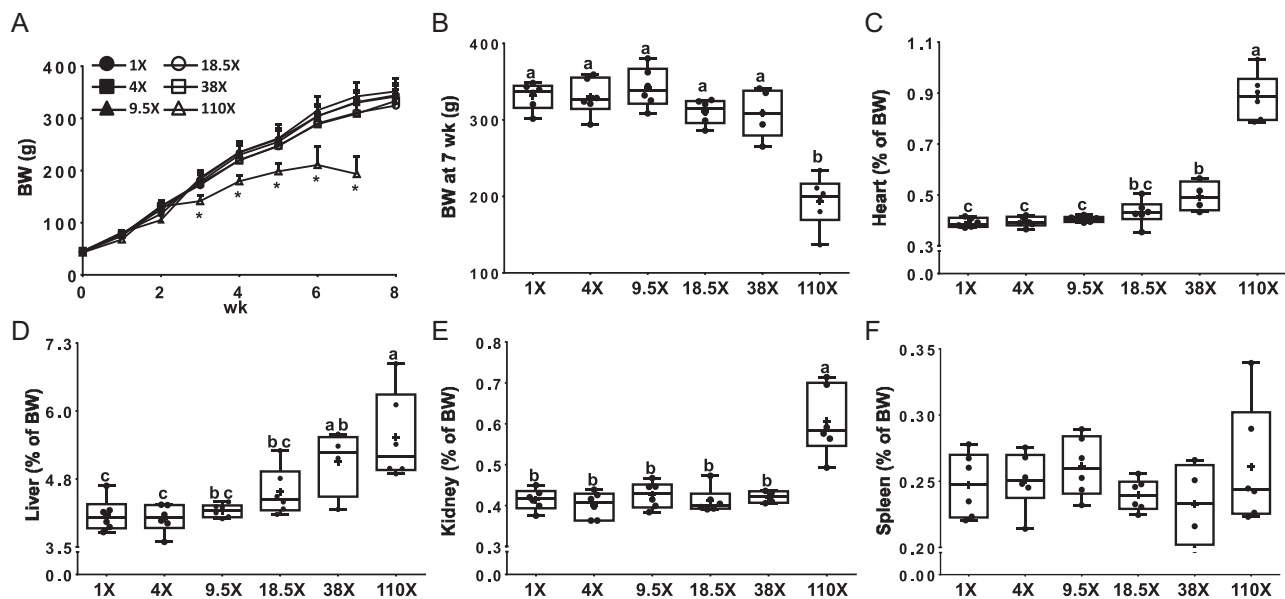
FIGURE 1 Growth curves (A), BW at 7 wk (B), and relative heart (C), liver (D), kidney (E), and spleen (F) weights of male rats fed diets varying in iron concentration for 7–8 wk. All data points are shown, $n = 4$–6. *Different from the other dietary groups at that age, $P < 0.05$ (A). Labeled means without a common letter differ, $P < 0.05$ (B–F). BW, body weight.

hypophagic by 6 wk of age (data not shown) and by 7 wk of age were in obvious distress, and were therefore euthanized a week early (note that blood and tissues were harvested normally from these animals). As a result, data from these animals were compared to rats in the other groups after 8 wk on the diets. Moreover, 2 rats in the $38\times$ group died 1–2 d before the trial was scheduled to end; nonetheless, body weights were recorded, and tissues (but not blood) were successfully harvested from these 2 animals.

Growth rates and organ weights

The growth rate was slower after 3 wk (**Figure 1**A) and final body weights were decreased ($\sim$40%) at 7 wk (**Figure 1**B) in rats consuming the $110\times$ diet (in comparison to all other groups). Cardiac hypertrophy was noted in the $110\times$ (heart mass increased $\sim$130%) and $38\times$ ($\sim$25% increase) groups (**Figure 1**C). Hepatomegaly was also noted in the $110\times$ (increase in mass $\sim$35%) and $38\times$ ($\sim$25% increase) groups (**Figure 1**D). Enlargement of the kidneys was observed in the $110\times$ group ($\sim$45% increase in mass) compared to all other groups (**Figure 1**E). Spleen weights were not different between any dietary groups (**Figure 1**F).

Hematologic and iron-related parameters, and *Epo* mRNA expression

Blood Hb (**Figure 2**A) and Hct (**Figure 2**B) concentrations were reduced by $\sim$65% in the $110\times$ group, compared to all others. Renal *Epo* expression, an established biomarker of erythroid demand (23–25), increased dramatically ($\sim$28-fold) (**Figure 2**C) in rats consuming the $110\times$ diet. Dietary iron consumption >38-fold above requirements is thus necessary to induce anemia in growing rats. Moroever, serum nonheme iron concentrations increased by $\sim$80–90% in the $110\times$ group (**Figure 2**D) compared to the $38\times$, $4\times$ and $1\times$ groups. TIBC was similar in all dietary groups (**Figure 2**E). TSAT increased (by $\sim$75%) in the $110\times$ group (compared to the $38\times$ and $1\times$ groups) (**Figure 2**F).

Tissue iron concentrations and hepatic *Hepc* mRNA expression

Total iron in the liver was higher in rats consuming the $110\times$ (9.7-fold), and $38\times$ and $18.5\times$ ($\sim$3-fold) diets (**Figure 3**A). Hepatic nonheme iron concentrations also increased in the $110\times$ ($\sim$8.5-fold), $38\times$ ($\sim$5-fold), $18.5\times$ ($\sim$4-fold), and $9.5\times$ ($\sim$2-fold) groups (compared to the $1\times$ group) (**Figure 3**B). Hepatic *Hepc* mRNA levels were significantly elevated only in the $110\times$ group ($\sim$4-fold) compared to the $4\times$ and $1\times$ groups (**Figure 3**C). Splenic nonheme iron concentrations increased (by $\sim$2.5-fold) in the $110\times$ group compared to the $4\times$ and $1\times$ groups (**Figure 3**D). Total iron concentrations in the spleen also showed a significant increase (by $\sim$3-fold), but only in the $110\times$ and $18.5\times$ groups (compared to all others) (**Table 1**). Renal nonheme iron concentrations increased in the $110\times$ group (by $\sim$75%), compared to the $4\times$ and $1\times$ groups (**Figure 3**E). Total iron concentrations in the kidney, however, were not different between dietary groups (**Table 1**). Total iron concentrations in bone progressively increased as dietary iron concentrations went up ($\sim$27-fold in the $110\times$ group; 16-fold in the $38\times$ and $18.5\times$ groups; $\sim$2-fold in the $9.5\times$ and $4\times$ groups), compared to the $1\times$ group (**Figure 3**F). Total iron in the heart was not affected by changes in dietary iron (**Table 1**). Overall, these data demonstrate that progressive increases in dietary iron are associated with increases in tissue iron concentrations, which is particularly striking in liver and bone.

Tissue copper concentrations and serum Cp activity

Decrements in copper concentrations in cardiac tissue were noted in the $110\times$ and $38\times$ ($\sim$80% reductions), and $18.5\times$ ($\sim$60% reduction) groups, compared to the $4\times$ and $1\times$ groups (**Figure 4**A). Cardiac copper concentrations inversely correlated with heart weights (**Figure 4**B). Heart enlargement (i.e., hypertrophy) is a well-established, pathophysiologic effect of severe copper deficiency in rodents (3, 26, 27). Hepatic copper concentrations were significantly decreased (<90%) in the $110\times$ group (**Figure 4**C). Serum Cp activity was lower in the $110\times$ and $38\times$ (<90% reductions), and $18.5\times$ ($\sim$60%) and $9.5\times$ ($\sim$35%) groups, in comparison
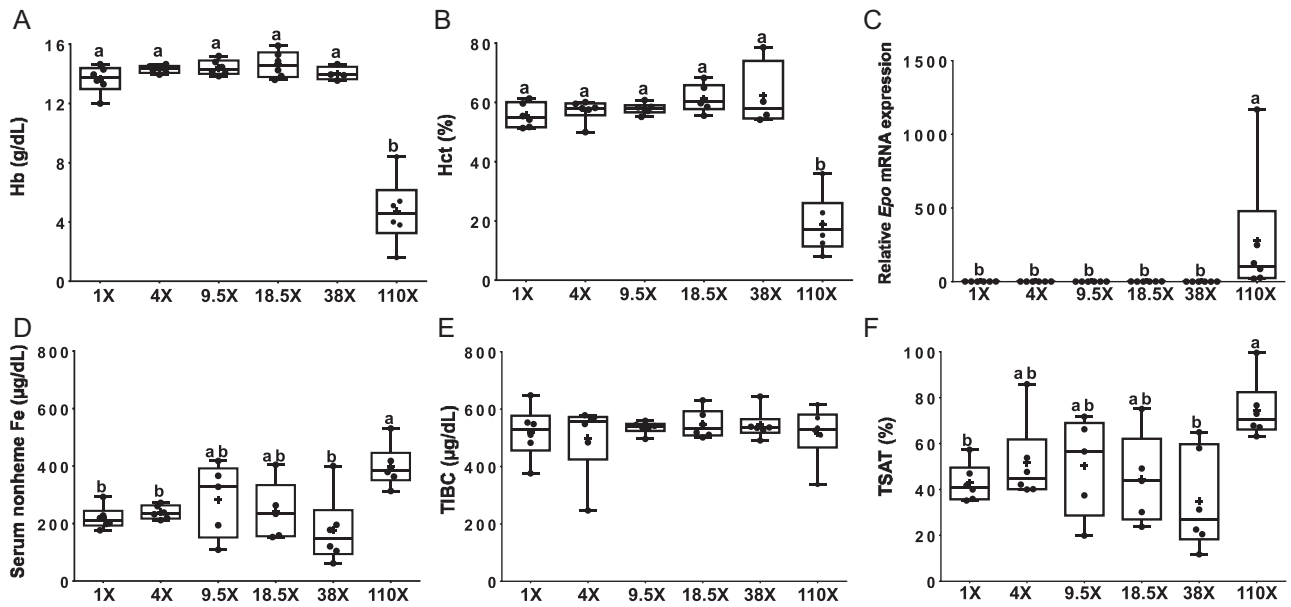
FIGURE 2 Hb (A), Hct (B), renal *Epo* mRNA expression (C), serum nonheme iron (D), total-iron-binding capacity (E) and transferrin saturation (F) of male rats fed diets varying in iron concentration for 7–8 wk. All data points are shown, $n = 4$–6. Labelled means without a common letter differ, $P < 0.05$. *Epo*, erythropoietin; Hb, hemoglobin; Hct, hematocrit; TIBC, total-iron-binding capacity; TSAT, transferrin saturation.

to the 1× group (Figure 4D). Decrements in serum Cp activity are diagnostic for moderate to severe copper deficiency (1, 2, 28, 29). Cp activity correlated with hepatic copper concentrations (Figure 4E) (19). Cp activity also inversely correlated with $\log_{10}$ hepatic nonheme iron concentrations (data not shown) ($r = -0.88$; $P < 0.0001$); that is, as hepatic nonheme iron concentrations increased, serum Cp activity decreased. Furthermore, splenic copper concentrations were lower in the 110× (>90% reduction), 38×, and 18.5× (~65% reductions for both) groups, compared to all others (Figure 4F) In sum, these data confirm earlier studies showing that high-iron intake leads to copper deficiency. Importantly, the current studies extend previous investigations and establish that copper

metabolism was disrupted in growing rats when dietary iron intakes were <10-fold above requirements.

Discussion

Dietary iron loading of rodents is a commonly used intervention to model iron-overload disorders in humans (30, 31). We previously noted that mice and rats fed high-iron diets developed severe copper deficiency (4, 5). These experimental diets contained iron at concentrations far above adequate intake levels (110-fold in excess), but we hypothesized that lower, closer to physiologic amounts of dietary iron would also antagonize
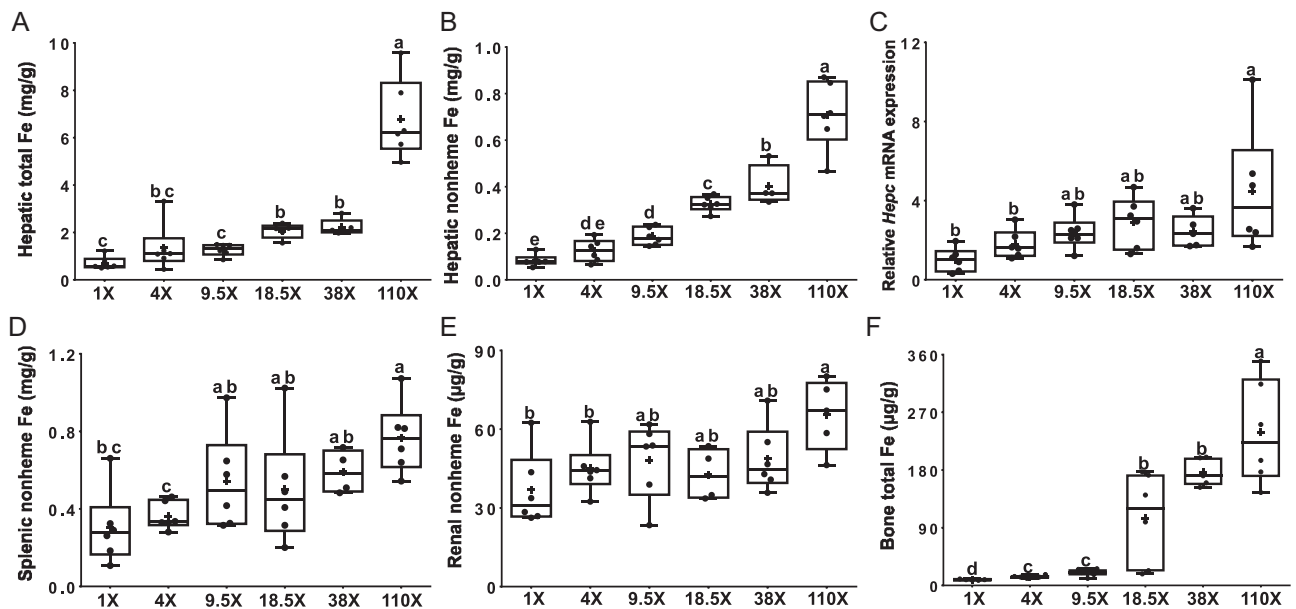

FIGURE 3 Hepatic total iron (A), hepatic nonheme iron (B), hepatic *Hepc* mRNA expression (C), splenic nonheme iron (D), renal nonheme iron (E), and bone total iron (F) of male rats fed diets varying in iron concentration for 7–8 wk. All data points are shown, $n = 4$–6. Labelled means without a common letter differ, $P < 0.05$ *Hepc*, hepcidin.

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TABLE 1 Bone and kidney copper concentrations, and heart, kidney and spleen total iron concentrations of male rats fed diets varying in iron concentration for 7–8 wk[1]

Organ	1 × Fe	4 × Fe	9.5 × Fe	18.5 × Fe	38 × Fe	110 × Fe
Bone Cu, μg/g	0.44 ± 0.05	0.53 ± 0.20	0.65 ± 0.27	0.70 ± 0.35	0.52 ± 0.29	0.52 ± 0.15
Kidney Cu, μg/g	78 ± 16[ac]	84 ± 34[a]	56 ± 11[bc]	53 ± 22[ac]	70 ± 26[ac]	41 ± 45[c]
Heart Fe, μg/g	573 ± 203	720 ± 470	642 ± 394	703 ± 416	463 ± 228	471 ± 186
Kidney Fe, μg/g	880 ± 146	1120 ± 512	1080 ± 340	1480 ± 1160	2390 ± 1350	3650 ± 660
Spleen Fe, μg/g	2680 ± 1130[bc]	2790 ± 446[c]	5510 ± 2290[a]	2920 ± 766[bc]	3560 ± 610[b]	10,200 ± 5520[a]

[1] Tissue mineral concentrations are shown. Values are means ± SDs, n = 5–6. Labeled means without a common superscript letter differ, $P < 0.05$.

copper homeostasis. This is an important consideration since many humans consume supplemental iron (in addition to dietary iron), and it is conceivable that this could lead to copper depletion (32). Notably, some of the experimental diets used here, containing iron at 4- and 9.5-fold above requirements, are within the range of iron intake that humans could achieve when consuming iron supplements (6–8, 13–16).

Consumption of excess dietary iron led to accumulation of nonheme iron in serum, liver, spleen, and bone, as expected. These increases in tissue iron occurred simultaneously with decrements of tissue copper concentrations. In some tissues, however, copper was depleted even though iron concentrations did not increase. For example, cardiac iron concentrations were unaltered by the dietary treatments, yet copper was progressively depleted from the heart as dietary iron increased. Consumption of the diet with ~110-fold excess iron impaired growth and caused anemia and enlargement of the heart, liver, and kidneys, as previously noted (4, 5). Cardiac hypertrophy and hepatomegaly were also observed when dietary iron concentrations were ~38-fold in excess. Tissue copper depletion and depression of serum Cp activity occurred when dietary iron was at even lower concentrations (~18.5- and ~9.5-fold above requirements, respectively). Growth retardation, anemia in the setting of normal serum iron concentrations and TSAT, cardiac hypertrophy, hepatomegaly, tissue copper depletion, and

decrements in serum Cp activity are indicative of copper deficiency in rodents (33).

The current observations are consistent with previous reports demonstrating that high dietary iron can antagonize copper homeostasis (34). For example, high iron intake perturbed copper absorption in infants and adults (32). Also, iron overload was postulated to interfere with copper utilization in humans with aceruloplasminemia (caused by mutation of the *Cp* gene) (35), and in those with acquired copper-deficiency myelopathy (36). Cp, an established biomarker of copper status, was also decreased in individuals with the genetic iron-loading disorder hereditary hemochromatosis (37), supporting the postulate that high iron impairs copper metabolism. Based upon these observations, it was previously suggested that iron supplements should contain copper (32, 34).

In conclusion, this investigation has demonstrated that dietary iron intakes at <10-fold above the adequate level caused moderate to severe copper deficiency in growing rodents, as indicated by suppression of serum Cp activity. Since Cp ferroxidase activity facilitates iron release from stores in the liver (i.e., hepatocytes) and reticuloendothelial macrophages of the spleen, bone marrow, and liver (to support erythropoiesis), suppression of Cp activity could at least partially counteract any positive outcomes from consuming supplemental iron. Since the iron intakes that depressed serum Cp activity are within the range that
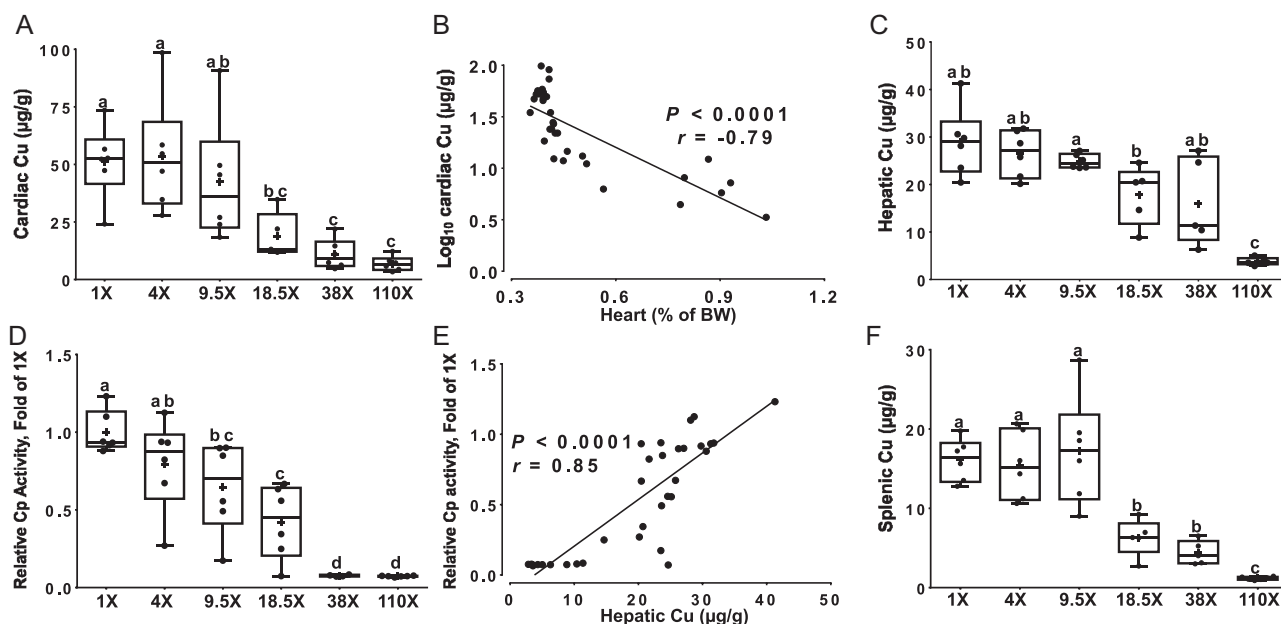

FIGURE 4 Cardiac copper concentrations (A), correlation between cardiac copper concentrations and heart mass (B), hepatic copper concentrations (C), serum Cp activity (D), correlation between serum Cp activity and hepatic copper concentrations (E), and splenic copper concentrations (F) of male rats fed diets varying in iron concentration for 7–8 wk. All data points are shown, n = 4–6. For correlation analyses, the line of best fit is shown along with the correlation coefficient (r), $P < 0.0001$ (B and E). Labelled means without a common letter differ, $P < 0.05$ (A, C, D, F). BW, body weight; Cp, ceruloplasmin.

may be consumed by individuals at risk for iron deficiency, this investigation could have important implications for iron supplementation in humans.

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