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Copper supplementation reverses dietary iron overload-induced pathologies in mice

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Abstract

Dietary iron overload in rodents impairs growth and causes cardiac hypertrophy, serum and tissue copper depletion, depression of serum ceruloplasmin (Cp) activity and anemia. Notably, increasing dietary copper content to ~25-fold above requirements prevents the development of these physiological perturbations. Whether copper supplementation can reverse these high-iron-related abnormalities has, however, not been established. The current investigation was thus undertaken to test the hypothesis that supplemental copper will mitigate negative outcomes associated with dietary iron loading. Weanling mice were thus fed AIN-93G-based diets with high (>100-fold in excess) or adequate (~80 ppm) iron content. To establish the optimal experimental conditions, we first defined the time course of iron loading, and assessed the impact of supplemental copper (provided in drinking water) on the development of high-iron-related pathologies. Copper supplementation (20 mg/L) for the last 3 weeks of a 7-week high-iron feeding period reversed the anemia, normalized serum copper levels and Cp activity, and restored tissue copper concentrations. Growth rates, cardiac copper concentrations and heart size, however, were only partially normalized by copper supplementation. Furthermore, high dietary iron intake reduced intestinal ^{64}Cu absorption (~60%) from a transport solution provided to mice by oral, intragastric gavage. Copper supplementation of iron-loaded mice enhanced intestinal ^{64}Cu transport, thus allowing sufficient assimilation of dietary copper to correct many of the noted high-iron-related physiological perturbations. We therefore conclude that high- iron intake increases the requirement for dietary copper (to overcome the inhibition of intestinal copper absorption).
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Keywords: Anemia; Cardiac hypertrophy; Ceruloplasmin; Intestine; Copper absorption; Dietary iron loading

1. Introduction

Iron and copper are essential dietary components for humans, and as such, inadequate intakes of both trace minerals are detrimental to human health. Additionally, given the propensity of iron and copper to potentiate the formation of damaging oxygen free radicals, excesses of both metals can lead to tissue damage and associated pathological outcomes. Iron and copper metabolism is thus tightly regulated by sophisticated homeostatic mechanisms. Furthermore, given their similar physiochemical properties, it is not surprising that notable iron-copper interactions have been documented in humans and other mammals over the past several decades [1,2].

Iron-overload diseases, including hereditary hemochromatosis, and iron-loading anemias such as β-thalassemia, are common in humans [3–5]. Rodent models of these human genetic diseases exist, but researchers can also model iron-loading disorders by feeding rodents diets with high concentrations of iron, creating so called dietary iron overload [6–8]. We previously utilized this approach to assess the influence of variable copper intakes on the iron-overload phenotype. Unexpectedly, we noted that high-iron fed rats and mice developed distinct pathologies which were characteristic of copper deficiency, including growth impairment, tissue copper depletion, cardiac hypertrophy, hepatomegaly and depression of serum ceruloplasmin (Cp) activity [9,10]. Notably, supplementing the high-iron diets with extra copper (~25-fold in excess of requirements) prevented the development of most of these physiological perturbations, demonstrating that high-iron intake disrupted copper homeostasis. Interestingly, it has previously been suggested that high iron can antagonize copper [11,12], which is consistent with these observations.

The current investigation was designed to expand upon our previous studies to determine whether copper supplementation could reverse these high-iron-related abnormalities once established. We hypothesized that increasing copper intake would be effective at

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mitigating these pathophysiological disturbances. To test this postulate, we first established the time course of iron loading, and then tested different supplemental copper concentrations for different periods of time, to establish the correct experimental parameters. Having determined the most appropriate experimental design, notably, we demonstrate that increasing copper intake to ~3.5-fold above requirements was effective at preventing and reversing many of the negative outcomes associated with high-iron intake.

2. Materials and methods

2.1. Animals and experimental design

Three-week-old, C57BL/6 mice (Jackson Laboratories; Bar Harbor, ME) were housed in shoe-box cages under standard laboratory conditions (23±2°C, humidity 60–70%, 12 h light/dark cycles). Mice were provided AIN-93G-based diets (Dyets Inc.; Bethlehem, PA) with high (HFe) (~8800 ppm) or adequate (AdFe) (~80 ppm) iron content with adequate copper content (7–8 ppm). These diets were identical to those used in our previous studies [9,10], which established that high-iron feeding for 4 or more weeks caused notable copper-related pathologies in weanling mice. To assess the time course of tissue iron loading and development of associated high-iron-related pathologies, weanling, male mice, were fed the AdFe or HFe diet for 2, 3 or 4 weeks. It was determined that 4 weeks of high-iron feeding was required for the development of more severe copper depletion, so this time period was selected for further experimentation. To test the efficacy of supplemental copper to correct high-iron consumption-related pathologies, copper (II) sulfate pentahydrate (Sigma; cat. # C-6283) was dissolved in purified water at 78.1 or 781 mg/L, which equates to 20 or 200 mg/L of copper, respectively. 200 mg/L Cu was chosen to mimic our previous work, which utilized high-copper diets [9,10], while the 20 mg/L dose was chosen since it was previously established that this copper concentration could reverse copper depletion-related cardiac hypertrophy in rodents [13]. Initially, weanling, male mice were fed the experimental diets for 4 weeks and supplemental copper (at two concentrations) was provided in the drinking water for the last 2 weeks. This experimental approach was designed to determine which supplemental copper concentration would prevent the development of the pathologies associated with high dietary iron intake. The most appropriate copper concentration (i.e., 20 mg/L) was then selected for further studies designed to determine whether supplemental copper could reverse the high-iron-related pathologies once already established. Moreover, given notable sex differences in iron and copper metabolism, we also sought to determine which sex was most appropriate for further experimentation. Male and female mice were thus fed the experimental diets for 4 weeks, with supplemental copper added to the drinking water (at 20 mg/L) for the last 2 weeks. Males were selected for additional experimentation since they developed more severe copper-depletion-related pathologies. Finally, to investigate the ability of supplemental Cu to reverse the high-iron-related pathologies once established, weanling, male mice, were fed the experimental diets for 7 weeks, with supplemental copper (20 mg/L) added to the drinking water for the last 3 weeks. The different feeding/supplementation regimens were designed to answer different questions regarding the influence of high dietary iron intake on copper homeostasis. Experimental mice were weighed weekly, and average food and water consumption was estimated by weighing the amount of food and water provided daily to each cage. This allowed us to assess any differences in growth rates or energy intakes, as well as estimate the amount of copper consumed by the experimental animals. Mice were sacrificed by CO_2 narcosis followed by thoracotomy. All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Florida.

2.2. Hematological parameters, and serum and tissue iron and copper quantification

Blood hemoglobin (Hb) and hematocrit (Hct) levels were determined as described previously [14]. Tissue nonheme iron levels were determined using a standard protocol, as previously described [9,14]. Briefly, 10–50 mg (wet weight) tissues samples were digested in 200–1000 μL acid solution (3 mol/L HCl, 10% trichloroacetic acid) and incubated at 65°C for 20 h and then centrifuged. Ten ul of the supernatant was then mixed with 200 μL of chromogen solution (0.01% bathophenanthroline disulphonate, 0.1% thioglycolic acid, 1.5 M sodium acetate in high-purity water). After 10 min incubation at room temperature, absorbance was measured at 535 nm by spectrophotometry. Serum nonheme iron levels were quantified using a standard colorimetric method [10]. To quantify total iron and copper content, tissue and serum samples were digested with HNO_3 in a water bath (initial temperature 95°C) overnight, and then diluted in purified water to keep the final acid concentration between 2–5%. The diluted samples were then filtered (0.45 μm) and analyzed by inductively-coupled plasma mass spectrometry (ICP-MS) (NexIon 300, Perkin-Elmer Corp.; Norwalk, CT). Iron and copper concentrations in tissues were normalized by weight, and in blood, by volume.

2.3. Serum Cp protein levels and activity

Serum Cp protein expression levels were determined by Western blotting. Briefly, serum protein concentrations were determined with the BCA Protein Assay Kit (Thermo Scientific). Equal amounts of serum protein (30 μg) were loaded onto 6% SDS-PAGE gels and immunoblotting was carried out using a standard protocol [15]. The primary anti-Cp antibody, which was used at a 1:1000-fold dilution, was from Sigma (cat. # C0911). We previously established the validity of this reagent [16]. The secondary antibody, which was used at a 1:2000-fold dilution, was horseradish peroxidase (HRP)-conjugated donkey, anti-goat IgG (Santa Cruz Biotechnology; cat. # sc-2020). Ponceau S staining and imaging of the stained blots was used to normalize Cp protein levels (Image-Pro Plus). Serum Cp activity was determined by an amine oxidase (*para*-phenylenediamine [*p*PD]) assay, as described previously [16].

2.4. qRT-PCR

Total RNA was isolated with RNAzol RT reagent (Molecular Research Center Inc.; Cincinnati, OH) and SYBR-Green qRT-PCR was performed as previously described [17,18]. Oligonucleotide primers were designed to span large introns to avoid amplification of genomic DNA. Standard-curve reactions validated each primer pair, and melt curves routinely showed single amplicons. Expression of experimental genes was normalized to expression of *Rps18*. Primer sequences were as follows (5′ to 3′): *Rps18*, forward – TTCCAGCACATTTTGCGAGTA, reverse – CACGCCCTTAATGGCAGTGAT; erythropoietin (*Epo*), forward – ATGAAGACTTGCAGCGTGGA, reverse – AGGCCCAGAG GAATCAGTAG; erythroferrone (*Erfe*), forward- ATGGGGCTGGAGAAC, reverse-TGGCATTGTCCAAGAAGACA.

2.5. Copper absorption experiments

These experiments were done in the dark phase (at night), since mice are nocturnal. Mice were fasted for 3 h prior to a ^{64}Cu transport solution being delivered by oral, intragastric gavage. The transport solution (100 μl) contained 20 μCi ^{64}Cu diluted into PBS buffer containing 0.1 N HCl and 3 μM $CuCl_2$. Immediately after gavage, mice were given *ad libitum* access to the same diet (AdFe or HFe) and water [with or without supplemental copper] that they had been consuming, and were sacrificed 8 h later. Radioactivity was measured using a WIZARD^2 Automatic Gamma Counter (Perkin Elmer; Waltham, MA), and counts were corrected by the half-life of ^{64}Cu. ^{64}Cu absorption was calculated as follows: ([disintegrations per minute (dpm) in the entire carcass plus blood (after the entire GI tract was removed)] divided by [total dpm administered by gavage]) × 100. Radioactivity in blood was expressed as dpm/μl and radioactivity in tissues as dpm/mg wet weight.

2.6. Statistical analysis

The data analysis approach that was utilized was developed upon consultation with a biostatistician. Statistical analyses were performed using the JMP (v 13.2.0) and GraphPad Prism (v 7.0.1) computer programs. Data are presented 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. All data were first tested for homogeneity of variances by Bartlett's test. If the data were not equally distributed, then data were $\log_{10}$ transformed prior to running statistical analyses (as indicated in the figure legends). The trends in data were then analyzed using a 2-way ANOVA on ranks test. If this analysis showed significant two-way interactions ($P<.05$), Tukey's multiple comparisons post hoc test was utilized to identify groups which varied significantly for a given parameter.

3. Results

3.1. Growth rates, and estimated food consumption and copper intakes

Male mice consuming the HFe diet grew slower than mice consuming the AdFe diet after 2 weeks, with the most significant difference noted after 4 weeks of dietary treatment (Fig. 1A). Providing supplemental copper for the last 2 weeks of a 4-week feeding period did not normalize growth rates when comparing the AdFe and HFe groups, but differences were less pronounced when 200 mg/L copper in drinking water was given to the mice (Fig. 1B). A similar trend was noted in female mice (data not shown; Iron main effect, $P<.0001$). When supplemental copper was provided at 20 mg/L for the last 3 weeks of a 7-week feeding period, the growth rate increased slightly (Fig. 1C).

Mice on both diets consumed an average of ~3 g of food and ~3 ml of water daily, except for when the water contained 200 mg/L of copper, when water intake was ~1.5 ml/d. Based upon average food intake and water consumption, we were able to calculate daily copper intake. We estimated that animals in both dietary groups that had no copper added to the water consumed 0.024 mg copper/day, those with 20 mg/L copper in the water consumed 0.084 mg/day, and those with 200 mg/L

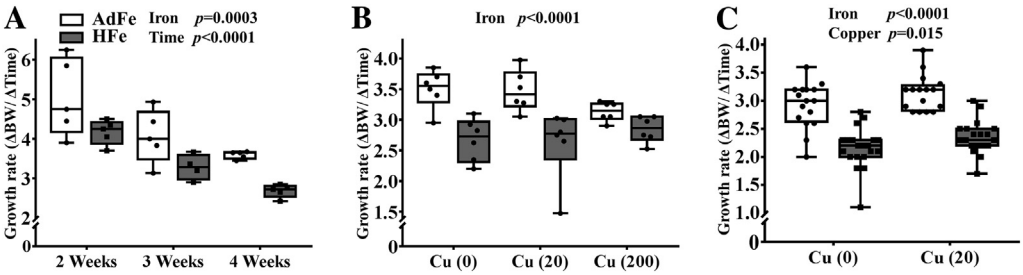

Fig. 1. Copper supplementation partially restores growth defects in high-iron fed mice. Experimental mice (weanling males) were weighed weekly to track growth. Growth rates at 2, 3 and 4 weeks of dietary treatment are shown (**A**). The influence of supplemental copper (in drinking water) on growth when administered for the last 2 weeks of a 4-week feeding period is also depicted (**B**). Also shown are growth rates in mice that received supplemental copper for the last 3 weeks of a 7-week feeding period (**C**). Data are shown as box-and-whisker plots for n=4–5 (A), 6 (B), or 16–20 (C) mice/group. Data were analyzed by two-way ANOVA. No significant 2-way interactions were noted. Individual main effects are depicted in the figure. BW, body weight. Cu(0), no copper added to water; Cu(20), 20 mg/L copper added to water; Cu(200), 200 mg/L copper added to water.

copper in the water consumed 0.324 mg/day. So, copper intake in the 20 mg/L and 200 mg/L groups was increased ~3.5- and ~13.5-fold, respectively, over the unsupplemented groups. These data thus demonstrate that consumption of the HFe diet impairs growth, consistent with our previous studies [9,10]. Providing supplemental copper tended to increase, but not fully correct, growth rates.

3.2. Time course of tissue iron loading and development of associated pathological disturbances

Consumption of a high-iron diet for 4 or more weeks leads to tissue iron accumulation and various homeostatic perturbations in growing rodents [9,10], but the time threshold for the appearance of these abnormalities has not been determined. Here, we fed weanling, male mice AdFe or HFe diets for 2–4 weeks and then assessed pathophysiological outcomes. Nonheme serum iron increased ~1.65-fold after mice had consumed the HFe diet for 2 weeks, with no additional increases noted at 3 or 4 weeks (Table S1). Liver and spleen nonheme iron content was markedly increased in mice consuming the HFe diet after 2 weeks, with further increases noted at 3 (for liver and spleen) and 4 (for spleen only) weeks (Fig. 2A-B). Total (heme plus nonheme) iron in the liver was also increased ~10.5-fold at 2 and 3 weeks, and ~9.5-fold at 4 weeks, as compared to mice fed the AdFe diet (Table S1). Renal nonheme iron was also elevated with high-iron consumption (~2.20-fold at 2 weeks, ~1.60-fold at 3 weeks and ~1.90-fold at 4 weeks) (Table S1). Furthermore, blood Hb and Hct levels were significantly lower in mice consuming the HFe diet for 2 weeks, and further depressed at 4 weeks (Fig. 2C and D). Consumption of the HFe diet thus led to tissue iron loading, as anticipated, but paradoxically, it also caused anemia.

Since our previous studies showed that high-iron consumption by growing rodents caused copper depletion [9,10], we further evaluated experimental mice (males) for signs of copper deficiency. Serum copper was markedly reduced after 2 weeks on the HFe diet, with concentrations falling below detection limits after 4 weeks on the diet (Fig. 3A). Liver copper concentrations were also significantly lower in

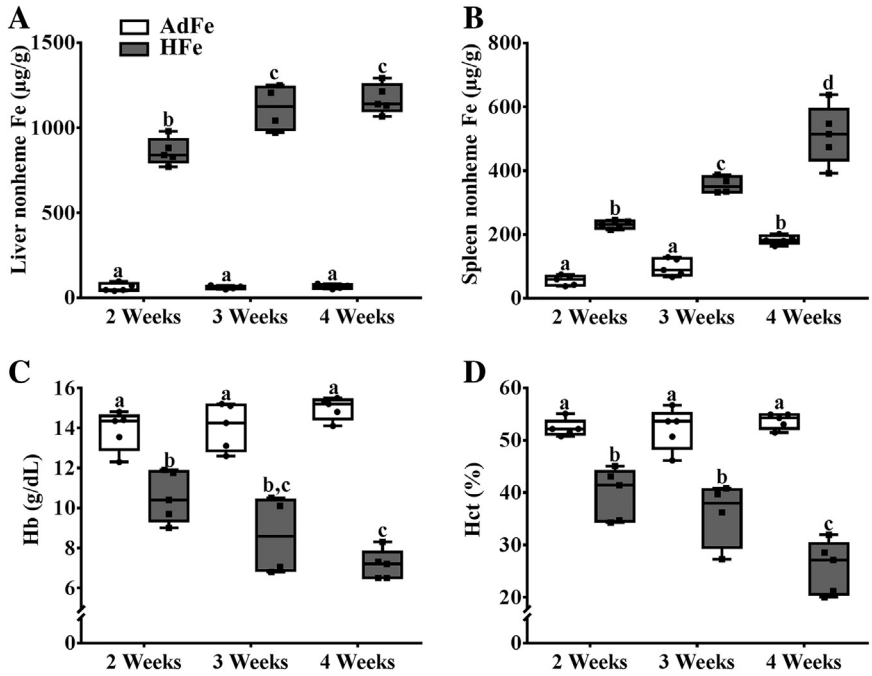

Fig. 2. High-iron consumption causes tissue iron loading and anemia in weanling mice within 2 weeks. Mice were fed diets with adequate iron (AdFe) or high iron (HFe) (both with adequate copper) for 2, 3 or 4 weeks. After the feeding regimens, tissue iron levels and hematological parameters were assessed upon sacrifice. Shown are liver (**A**) and spleen (**B**) nonheme iron concentrations, and Hb (**C**) and Hct (**D**) levels in whole blood. Data are shown as box-and-whisker plots for n=4 (3-week HFe diet group) or 5 (all others) mice/group (A-D). Data were analyzed by two-way ANOVA. Since significant two-way interactions were noted, Tukey's multiple comparisons *post hoc* test was utilized to establish significant differences between individual groups. Labeled means without a common letter differ ($P<.05$). Iron x time interactions: $P=.001$ (A), $P=.0011$ (B), $P=.0001$ (C), and $P=.0017$ (D).

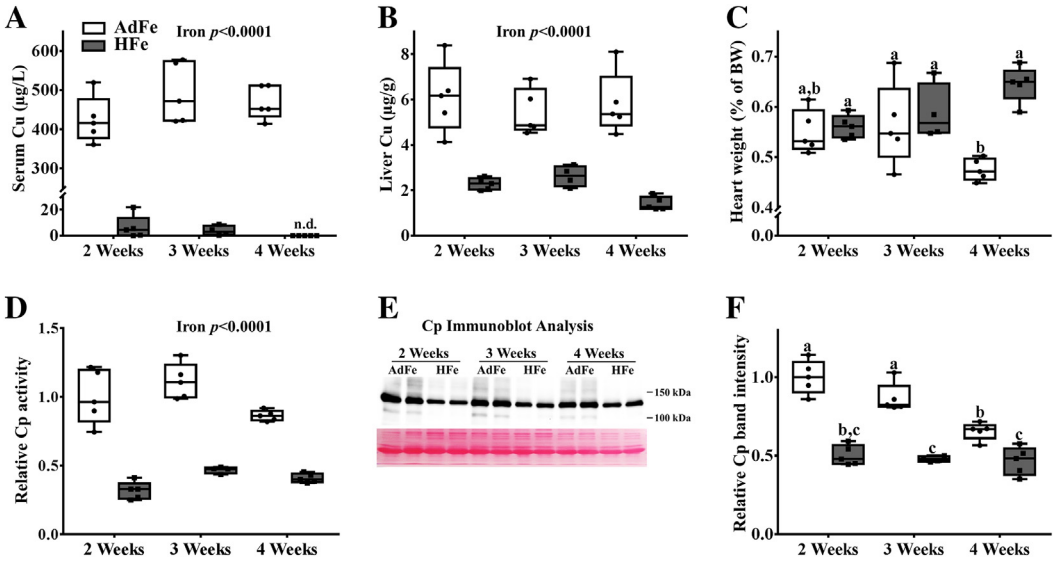

Fig. 3. High-iron diet feeding depletes serum and liver copper, causes cardiac hypertrophy and decreases serum Cp activity. Mice were fed diets with adequate iron (AdFe) or high iron (HFe) (both with adequate copper) for 2, 3 or 4 weeks. Shown are serum (**A**) and liver (**B**) copper concentrations, heart weights (**C**) and serum Cp activity (**D**). A representative immunoblot of serum Cp protein expression is also shown (**E**) as well as quantitative data (**F**) from multiple experiments. Data are shown as box-and-whisker plots for n=4 (for 3-week HFe diet group) or 5 (all others) mice/group (A-D, F). Data were analyzed by two-way ANOVA. If a significant two-way interaction was noted, Tukey's multiple comparisons *post hoc* test was utilized to establish significant differences between individual groups. Labeled means without a common letter differ ($P<.05$). Significant 2-way interactions were noted for data shown in panels C ($p=.0017$) and F ($p=.0008$). Main effects are indicated in the figure (A, B, and D). Data for heart weight (C) were $\log_{10}$ transformed prior to running statistical analysis due to unequal variance; however, for ease of interpretation, the non-transformed data are depicted in the figure. BW, body weight; n.d., not detected.

mice consuming the HFe diet after 2 weeks (Fig. 3B). Cardiac hypertrophy was, however, only noted after 4 weeks of HFe-diet consumption (Fig. 3C). Furthermore, serum Cp activity (Fig. 3D) and immunoreactive protein levels (Fig. 3E and F) were reduced in mice consuming the HFe diet for 2 weeks. Reductions in Cp expression/activity are diagnostic for moderate to severe copper deficiency [19,20]. Collectively, these observations demonstrate that consumption of the HFe diet caused moderate copper deficiency in growing mice after only 2 weeks. A more severe copper deficiency, as denoted by increases in heart size, took 4 weeks to develop. Since we sought to determine whether all pathophysiological consequences of dietary iron loading related to copper depletion, further experiments were carried out at the 4-week time point.

3.3. The effect of copper supplementation on high dietary iron-related pathologies

When copper intake is at adequate levels, high-iron consumption causes copper depletion and associated pathological disturbances. The amount of supplemental copper that will correct the copper deficiency caused by high-iron intake has, however, not been established. We thus fed growing male and female mice the HFe diet for 4 weeks, and during the last 2 weeks provided them with supplemental copper in drinking water at two concentrations (20 or 200 mg/L). In male mice, supplemental copper at both concentrations reversed the noted decreases in blood Hb and Hct caused by HFe consumption (Fig. 4A and B), and also normalized serum Cp activity (Fig. 4C). Supplemental copper at both concentrations also prevented the development of cardiac hypertrophy (Fig. 4D). In female mice, the anemia was prevented by both concentrations of supplemental copper, but Hct levels were not different between groups [no interactions or main effects] (Fig. S1A and B). Moreover, in females, serum Cp activity was normalized by supplemental copper at both concentrations, but heart weights were not different between groups [no interactions or main effects] (Fig. S1C and D). Since only males developed cardiac hypertrophy, which is indicative of more severe copper depletion, further experiments were carried out using male mice. Moreover, since the higher supplemental copper dose reduced water intake (and may have caused other physiological perturbations associated with copper toxicity), and also since both supplemental copper doses were equally effective at preventing many of the copper-depletion related pathologies, the 20 mg/L was selected for additional experimentation.

3.4. The influence of copper supplementation on the tissue iron loading caused by high-iron consumption

Growing mice consuming diets with excess iron develop iron overload and severe copper deficiency after 4 weeks, but whether the noted physiological disturbances can be reversed by copper supplementation is unknown. We thus fed weanling, male mice AdFe or HFe diets for 7 weeks, and provided mice with supplemental copper for the last 3 weeks. With consumption of the HFe diet for 7 weeks (n=5–9 mice/group for all), nonheme iron levels were higher in serum (~50% increase), liver (~16-fold increase), spleen (~5-fold increase), kidney (~2.8-fold increase), heart (~1.7-fold increase), and brain (~23% increase) (Table S2). Nonheme iron levels in these tissues were unaffected by copper supplementation [no interactions or main effects]. Total iron (heme plus nonheme) concentrations in some tissues were also elevated in mice consuming the HFe diet for 7 weeks (n=5–9 mice/group for all), including liver (~18-fold increase), spleen (~3.4-fold increase) and kidney (~50% increase) (Table S3). Copper supplementation had no effect on total iron concentrations in these tissues [no interactions or main effects]. Interestingly, total iron concentrations in heart were increased only in the group receiving supplemental copper (~20% increase). Furthermore, total iron concentrations in serum and brain were not affected by high-iron feeding or copper supplementation [no interactions or main effects] (Table S3). In bone, however, total Fe concentrations were increased ~1.8-fold by HFe consumption in the Cu (0) group, and ~2.0-fold in the Cu(20) group, and also by copper supplementation (~13% increase in AdFe group and ~30% increase in the HFe group) (Table S3). Collectively, these observations demonstrate that copper supplementation did not alter serum or tissue iron levels in mice consuming either diet.

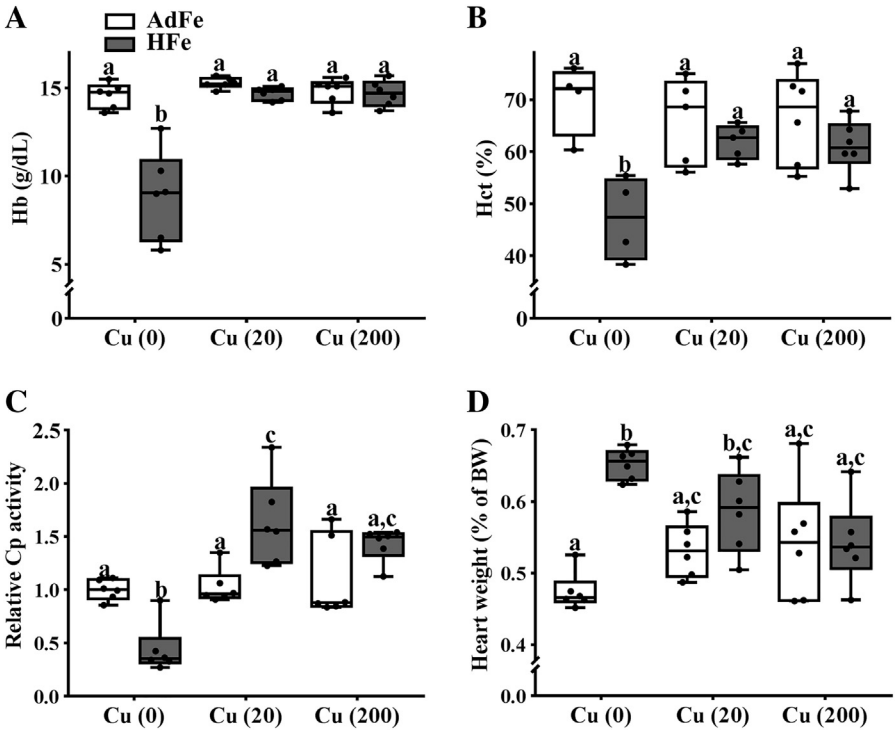

Fig. 4. Copper supplementation corrects the anemia, normalizes serum Cp activity, and prevents the development of cardiac hypertrophy in male mice. Experimental animals were fed diets with adequate iron (AdFe) or high iron (HFe) (both with adequate copper) for 4 weeks. During the last 2 weeks, animals either remained on drinking water with no added copper [Cu(0)], or were provided water with 20 [Cu(20)] or 200 [(Cu(200)] mg/L copper. Shown are Hb (**A**) and Hct (**B**) in whole blood, serum Cp activity (**C**) and heart weights, normalized to body weights (BW) (**D**). Data are shown as box-and-whisker plots for n=4–6 mice/group (A-D). Data were analyzed by two-way ANOVA. Since significant two-way interactions were noted, Tukey's multiple comparisons *post hoc* test was utilized to establish differences between individual groups. Labeled means without a common letter differ ($P<.05$) (A-D). Iron x copper interactions: $P<.0001$ (A), $P=.0003$ (B), $P<.0001$ (C), $P=.0006$ (D). Hb data (A) were analyzed after $\log_{10}$ transformation due to unequal distribution; however, for ease of interpretation, the non-transformed data are depicted in the figure.

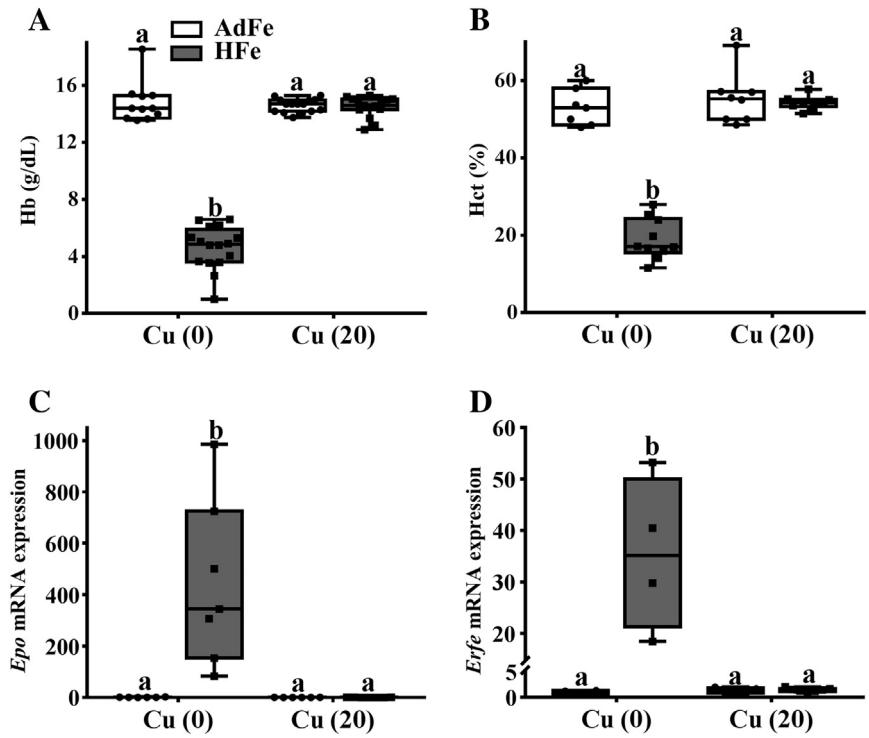

Fig. 5. Copper supplementation reverses the anemia associated with dietary iron loading in male mice. Mice were fed diets with adequate iron (AdFe) or high iron (HFe) (both with adequate copper) for 7 weeks. During the last 3 weeks, animals either remained on drinking water with no added copper [Cu(0)], or were provided water with 20 mg/L copper [Cu(20)]. Shown are Hb (**A**) and Hct (**B**) in whole blood, and relative renal *Epo* (**C**) and bone marrow *Erfe* (**D**) mRNA expression. Data are shown as box-and-whisker plots for n=11–16 (A) or 4–10 (all others) mice/group. Data were analyzed by two-way ANOVA. Since significant two-way interactions were noted, Tukey's multiple comparisons *post hoc* test was utilized to establish differences between individual groups. Labeled means without a common letter differ ($P<.05$). Iron x copper interactions: $P<.0001$ (A-D). Data for renal *Epo* (C) and bone marrow *Erfe* (D) mRNA expression were $\log_{10}$ transformed prior to running statistical analyses due to unequal variance; however, for ease of interpretation, the non-transformed data are depicted in the figure.

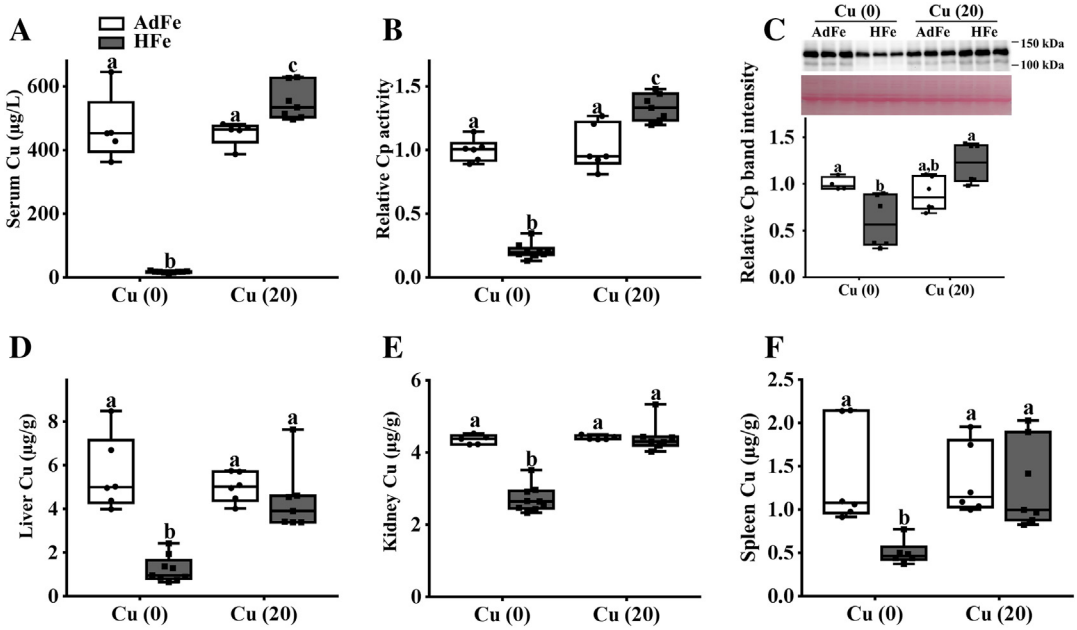

Fig. 6. Copper supplementation reestablished copper homeostasis in male mice with dietary iron overload. Mice were fed diets with adequate iron (AdFe) or high iron (HFe) (both with adequate copper) for 7 weeks. During the last 3 weeks, animals either remained on drinking water with no added copper [Cu(0)], or were provided water with 20 mg/L copper [Cu(20)]. Shown are serum copper concentrations (**A**) and Cp activity (**B**), a representative immunoblot for serum Cp protein expression and quantitative data from multiple experiments (**C**), and liver (**D**), kidney (**E**) and spleen (**F**) copper concentrations. Data are shown as box-and-whisker plots for n=4–9 mice/group. Data were analyzed by two-way ANOVA. Since significant two-way interactions were noted, Tukey's multiple comparisons *post hoc* test was utilized to establish differences between individual groups. Labeled means without a common letter differ ($P<.05$). Iron x copper interactions: $P<.0001$ (A), $P=.0008$ (B), $P<.0001$ (C), $P=.0003$ (D), $P<.0001$ (E), $P=.0292$ (F).

3.5. The influence of supplemental copper on HFe-related homeostatic perturbations

Copper supplementation did not influence tissue iron accumulation (as described above), but it remained to be established whether the pathologies noted with HFe consumption related to iron toxicity and/or copper depletion. Copper-related physiological parameters were thus assessed in male mice that consumed the AdFe or HFe diet for 7 weeks, with supplemental copper provided the final 3 weeks. High-iron consumption for 7 weeks caused hypopigmentation, as exemplified by a lightening of the coat color (Fig. S2A), which is consistent with copper deficiency [21,22]. Copper supplementation partially restored coat color (especially on the top of the head). Tissue color was in general pale with HFe consumption (likely due to anemia); copper supplementation, however, restored normal tissue coloration (Fig. S2B). Copper supplementation also corrected the severe anemia associated with high-iron consumption, as indicated by normalization of blood Hb and Hct levels, and renal *Epo* and bone marrow *Erfe* mRNA expression (Fig. 5). The anemia was, in fact, fully corrected after only one week of copper supplementation (data not shown). Furthermore, serum copper levels and Cp activity increased to above control values in HFe-fed mice provided supplemental copper (Fig. 6A and B). Immunoreactive Cp protein levels showed a similar trend (Fig. 6C). Hepatic, renal and splenic copper concentrations were also restored by copper supplementation (Fig. 6D-F). Copper supplementation also increased cardiac copper concentrations and reduced heart weight and size in iron-loaded mice, but these parameters were not fully corrected (Fig. 7). Collectively, these observations demonstrate that the anemia and depression of serum Cp activity caused by high-iron consumption are direct results of copper depletion, while hypopigmentation and cardiac hypertrophy are at least partially a result of copper depletion. Higher amounts of supplemental copper may be required to fully correct the latter conditions. In regards to the noted heart pathology, this is perhaps not unexpected as the heart is more resistant to changes in copper content than some other tissues [23,24]. Alternatively, iron toxicity could also contribute to these

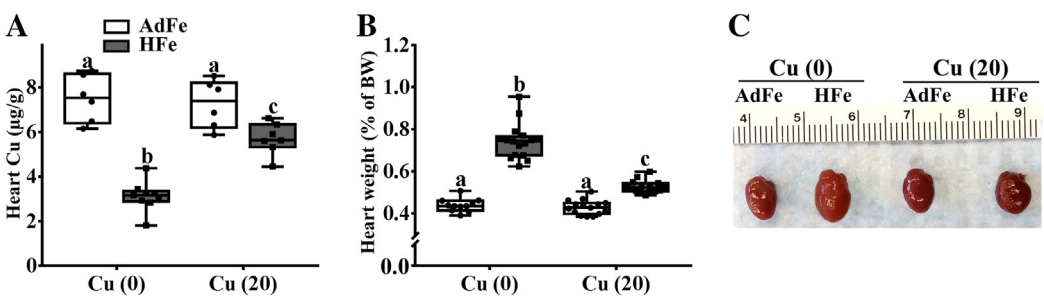

Fig. 7. Copper supplementation partially restores heart copper concentrations and reverses cardiac hypertrophy in iron-loaded mice. Mice were fed diets with adequate iron (AdFe) or high iron (HFe) (both with adequate copper) for 7 weeks. During the last 3 weeks, animals either remained on drinking water with no added copper [Cu(0)], or were provided water with 20 mg/L copper [Cu(20)]. Shown are heart copper concentrations (**A**), heart weights, normalized to body weights (BW) (**B**), and representative heart photographs (the ruler shows cm). Data are shown as box-and-whisker plots for n=6–9 mice/group (A and B). Data were analyzed by two-way ANOVA. Since significant two-way interactions were noted, Tukey's multiple comparisons *post hoc* test was utilized to establish differences between individual groups. Labeled means without a common letter differ ($P<.05$). Iron x copper interactions: $P=.0003$ (A), $P<.0001$ (B).

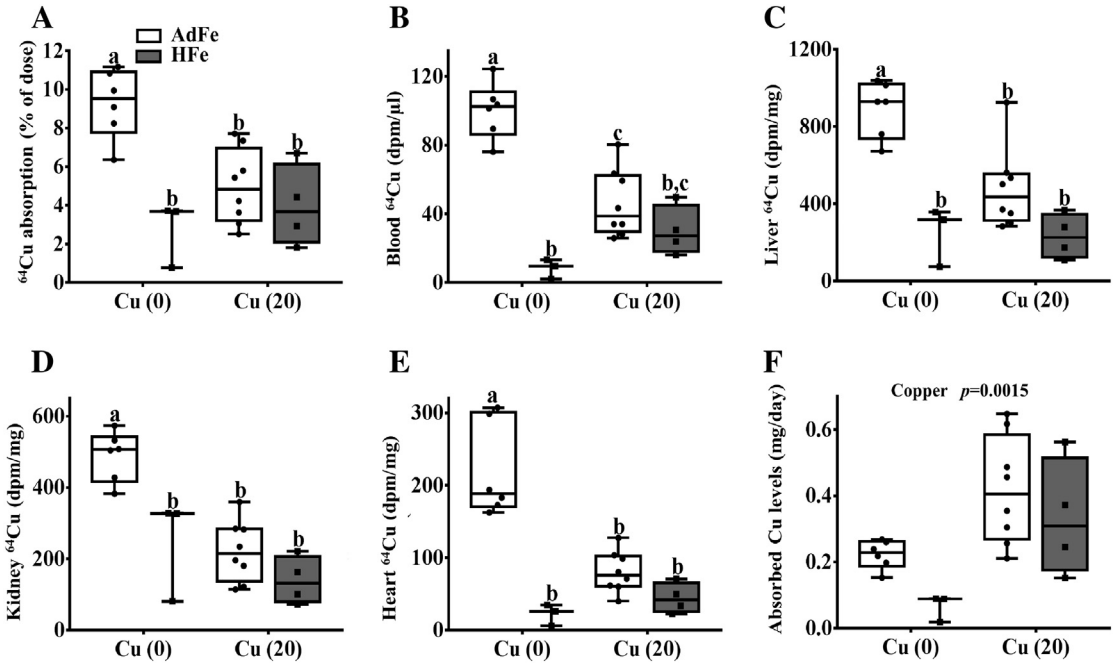

Fig. 8. High dietary iron depresses intestinal copper absorption. Mice were fed diets with adequate iron (AdFe) or high iron (HFe) (both with adequate copper) for 7 weeks. During the last 3 weeks, animals either remained on drinking water with no added copper [Cu(0)], or were provided water with 20 mg/L copper [Cu(20)]. Thereafter, copper absorption was assessed by oral, intragastric gavage of a ^{64}Cu-containing transport solution. Shown are intestinal copper absorption (**A**), and ^{64}Cu accumulation in blood (**B**), liver (**C**), kidney (**D**) and heart (**E**). Total absorbed copper is also shown (F). Data are shown as box-and-whisker plots for n=3–8 mice/group. Data were analyzed by two-way ANOVA. Since significant two-way interactions were noted, Tukey's multiple comparisons *post hoc* test was utilized to establish differences between individual groups. Labeled means without a common letter differ (P<.05) (A-D). Iron x copper interactions: P=.0062 (A), P<.0001 (B), P=.0247 (C), P=.0469 (D) and P=.0006 (E). A copper main effect is indicated in the figure (F).

perturbations, but this is perhaps less likely given that iron overload has not been previously associated with pigmentation defects or cardiac hypertrophy in mice.

3.6. Intestinal copper absorption in mice consuming a high-iron diet with and without supplemental copper

A logical postulate is that high dietary iron impairs intestinal copper absorption, given the similar physiochemical properties of iron and copper. Indeed, intestinal ^{64}Cu absorption was impaired (Fig. 8A), and tissue copper accumulation was diminished in mice consuming the HFe diet (Fig. 8B-E). Moreover, since intestinal copper absorption is known to respond to dietary intake levels [25–28], we anticipated changes in absorption in the copper-supplemented mice. Indeed, when mice were provided with supplemental copper, apparent intestinal ^{64}Cu absorption and tissue accumulation decreased in both dietary groups. Interpretation of this result, however, has to take into consideration the potential confounding influence of excess unlabeled copper in the GI tract which competes with tracer amounts of ^{64}Cu for absorption (since mice had free access to copper-supplemented water immediately after gavage). To address this concern, we calculated daily assimilation of ingested copper by multiplying the relative ^{64}Cu absorption rate by daily copper intake [0.024 mg/d in the Cu(0) groups and 0.084 mg/d in the Cu(20) groups]. Results of this analysis demonstrated that providing supplemental copper in the drinking water increased total copper assimilation (Fig. 8F), likely explaining why copper homeostasis was (at least partially) restored in copper-supplemented mice consuming the HFe diet.

4. Discussion

Growing rodents fed an otherwise normal, high-iron diet for 6–8 weeks develop distinct pathologies [9,10]. Interestingly, many of the noted physiological abnormalities, including growth retardation, anemia, hypopigmentation, depression of serum Cp activity, hepatomegaly and cardiac hypertrophy, typify copper deficiency [21,22,29]. High-iron consumption may thus disrupt copper homeostasis, as has been proposed previously [11,12]. Consistent with this, our previous studies demonstrated that high-iron diets containing excess copper (>25-fold above requirements) prevented the development of many of these abnormalities in growing rats and mice [9,10]. The current investigation sought to extend these observations to determine the time course of dietary iron loading that was associated with these physiological disturbances, and to determine whether copper supplementation could correct them once established. We provided extra copper in the drinking water since we postulated that this mode of supplementation would improve copper bioavailability. The overall intent of this investigation was to establish whether the primary lesions in high-iron-fed mice related specifically to copper depletion, and if so, to determine how much supplemental copper is required to correct the noted physiological perturbations.

Consumption of the HFe diet caused anemia, tissue copper depletion, hypopigmentation and depression of serum Cp activity after only 2 weeks, but 4 weeks on the HFe diet was necessary to induce cardiac hypertrophy. The 4-week high-iron feeding regimen was thus selected for further studies. When supplemental copper was provided to mice during the last 3 weeks of a 7-week high-iron feeding period, several physiological perturbations were partiality or fully corrected, including the noted anemia, decrements in tissue copper concentrations, pigmentation defects, cardiac hypertrophy and reductions in serum Cp activity.

One plausible mechanistic explanation for development of the above noted high-iron-related pathologies is that high dietary iron blocks intestinal copper transport (thus causing systemic copper depletion). We thus next assessed intestinal copper transport *in vivo* in experimental mice. Mice were fasted and deprived of water for 3 h prior to the transport experiment being initiated to eliminate any residual chyme and fluid from the upper GI tract. Fasted mice were given a copper transport solution

containing tracer amounts of ^{64}Cu by oral, intragastric gavage, and food and water were immediately provided thereafter. Given that mice were without food and water for 3 h prior to gavage feeding (and since this experiment was performed in the active [night] phase), we assume that the mice immediately ate food and drank water. The copper transport solution was thus mixed with normal dietary constituents and the GI tract was stimulated for optimal digestion and absorption. This approach thus nicely mimics physiological conditions. Moreover, this experimental design ensured that copper absorption was measured in the presence and absence of high dietary iron, and with and without supplemental copper.

Intestinal copper (^{64}Cu) transport was blunted in mice consuming the HFe diet, consistent with a previous study [9]. Apparent ^{64}Cu absorption was also decreased in copper-supplemented mice consuming both diets, as compared to controls (*i.e.* mice consuming the AdFe diet without copper supplementation). An important consideration regarding interpretation of these data, however, relates to the presence of competing, unlabeled copper atoms in the supplementation groups. Give the experimental design, the copper absorption rate was clearly underestimated in copper-supplemented mice. To overcome this limitation in data interpretation, we calculated daily dietary copper assimilation as the product of the apparent copper absorption rates times daily average copper intake. This data manipulation revealed that the copper supplemented mice assimilated more dietary (and water derived) copper than the unsupplemented groups. This finding is consistent with the observation that copper supplementation corrected most of the high-iron-related pathologies. Increasing copper intake thus overcame the negative influence of high-dietary iron on intestinal copper absorption.

In summary, this investigation has established the time course of high-iron consumption that impairs copper metabolism and has further defined the amount of supplemental copper and the time of administration necessary to reverse the noted HFe-related physiological abnormalities. The use of excessive dietary iron concentrations in this investigation is a clear limitation if one relates these findings to human physiology/pathophysiology. Since the current study used very high dietary iron levels and to increase translational potential, future investigations will be designed to determine the minimum amount of excess iron that disrupts copper homeostasis using pre-clinical, rodent models of human iron supplementation (*e.g.* pregnancy). This experimental approach will allow us to make logical predictions regarding whether iron supplements for human consumption should contain extra copper (as was suggested previously) [12]. In conclusion, the data presented here support the postulate that high dietary iron interferes with intestinal copper absorption, and further that increasing dietary copper intake can overcome this inhibition of copper transport.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jnutbio.2018.05.006.

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