Lee *et al. Applied Biological Chemistry* (2024) 67:10
https://doi.org/10.1186/s13765-024-00861-2

KSABC
The Korean Society for Applied Biological Chemistry

ARTICLE Open Access

High-iron consumption decreases copper accumulation and colon length, and alters serum lipids

Jisu Lee[1†], Hyun-Joo Lee[2†], Hyunsoo Jang[1], Jae-Joon Lee[3] and Jung-Heun Ha[1,4*]

Abstract

In this study, we aimed to demonstrate that a significant increase in dietary iron intake disrupts the regulation of copper availability, ultimately leading to systemic copper deficiency. To investigate this, we conducted experiments using five-week-old male weanling Sprague–Dawley rats fed diets based on AIN-93G with some modifications. These diets featured varying iron content, offering choices of adequate iron (~ 120 µg/g, near the upper limit [UL]) or high iron (~ 7544 µg/g), along with low (~ 0.3 µg/g), adequate (~ 6 µg/g), or high (~ 153 µg/g) levels of dietary copper over a 5-week period. Rats consuming the high-iron diets displayed anemia, reduced copper levels in their organs and feces, and shortened colon lengths. Increased dietary iron intake resulted in an overall reduction in copper distribution within the body, likely leading to severe copper deficiency-related disorders in the experimental rats. However, the physiological disturbances caused by a high-iron diet were prevented when additional copper was included in the rodent diet. Furthermore, high iron intake led to copper deprivation, and high iron consumption resulted in elevated serum cholesterol levels. However, increasing dietary copper consumption led to a decrease in overall serum cholesterol levels. Additionally, serum alkaline phosphate and aspartate aminotransferase levels were increased by high-iron feeding, regardless of dietary copper concentration, while alanine aminotransferase levels decreased.

Keywords High-iron, Copper deficiency, Insulin resistance, Dyslipidemia, Colon length

Introduction

Iron plays significant physiological roles in cellular oxygen delivery, DNA synthesis, and electron acquisition and donation. Iron deficiency and overload can lead to pathological disturbances, owing to the importance of iron in biology. Prolonged iron deficiency can trigger conditions such as anemia, fatigue, weakness, and pica [1–3]. The primary causes of iron deficiency include blood loss, inadequate dietary iron intake, and intestinal disorders [4–6]. Iron overload, known as hemochromatosis, is a genetic disorder caused by abnormalities in the hereditary hemochromatosis (HFE) gene [7]. Iron overload leads to the accumulation of excess iron in tissues and can result in symptoms such as joint pain [8] and insulin resistance [9] likely due to elevated oxidative stress [10]. Iron has a distinctive feature in that its intestinal absorption is crucial and tightly regulated. Therefore, almost all the required iron is biologically recycled, but a portion must be obtained through the diet. Intestinal iron absorption significantly

† Jisu Lee and Hyun-Joo Lee have contributed equally to this work.

*Correspondence:
Jung-Heun Ha
ha@dankook.ac.kr
[1] Department of Food Science and Nutrition, Dankook University, Cheonan 31116, Korea
[2] School of Wellness Industry Convergence, Major of Food & Nutrition, Hankyong National University, Anseong 17579, Korea
[3] Department of Food and Nutrition, Chosun University, Gwangju 61452, Korea
[4] Research Center for Industrialization of Natural Neutralization, Dankook University, Yongin 16890, Republic of Korea

increases in patients with low iron levels. However, in the presence of excess iron, hepatic hepcidin levels increase, further inhibiting intestinal iron absorption and/or macrophage iron recycling by suppressing the expression of ferroportin 1, an iron exporter [11, 12].

Copper also plays a vital role in promoting growth, maintaining pulmonary elasticity, regulating cardiac function, and supporting vascular health [13, 14]. Given its biological importance, copper deficiency and overload can both result in significant pathological responses. Genetic disorders such as Menkes disease (associated with copper deficiency) and Wilson's disease (linked to copper overload) are caused by the dysregulation of copper-transporting ATPases in the cellular *trans*-Golgi network [15–17]. Menkes disease, which results from an intestinal ATP7A mutation, is an X-linked genetic disorder that leads to growth retardation and severe neurodegenerative conditions in young children, often accompanied by abnormal iron absorption [18]. Wilson's disease is an autosomal recessive hereditary disorder caused by mutations in the ATP7B gene [19]. Wilson's disease can lead to liver cirrhosis and neuronal degeneration, which are primarily associated with copper metabolism [20]. Inappropriate dietary supplementation may also contribute to copper deficiency and its associated symptoms, such as anemia, irregular heartbeats, and loss of pigmentation in the integumentary system [21].

Biological interactions between iron and copper likely stem from their positive charges, involvement in oxidation–reduction reactions, and shared metabolic regulation [22]. Both dietary iron and copper are absorbed in the small intestine, within the brush border of small intestinal epithelial cells, and both minerals must undergo a reduction process [23]. After acquiring divalent forms, they must be oxidized before being exported into interstitial fluids and bound to their respective transport proteins [24]. Additionally, both divalent minerals act as cofactors in numerous enzymatic reactions and participate in electron donation and withdrawal [25]. There is a reciprocal relationship between biological iron and copper. Under iron deficiency conditions, copper accumulates in the liver to assist in iron utilization [26], whereas in cases of copper deficiency, iron accumulates in the liver [27]. Recent studies have focused on how excess dietary iron can lead to copper deficiency in experimental animals [28–30]. Excessive dietary iron intake results in growth retardation [21], increased erythropoietic demand (with decreased hemoglobin levels and elevated erythropoietin levels) [31], and copper deficiency [32], marked by reduced tissue copper levels and lower serum ceruloplasmin activity [33]. Importantly, all pathological disturbances induced by high iron intake can be significantly and gradually mitigated when additional dietary copper is provided [34].

In this study, we used adequate (AdFe) or high iron (HFe) based on previous in vivo studies [28, 29] with some modifications. We supplemented AdFe at levels close to the UL for rodents (approximately 120 μg/g), which, when extrapolated to clinical applications, surpasses the recommended iron levels (39 μg/g). Hence, we used higher AdFe levels compared to previous studies, which were set at approximately 80 μg/g [29, 35]. Our aim was to investigate how high dietary iron consumption affects responses in comparison to consuming iron levels near the UL. While previous studies have suggested that high dietary iron levels appear to influence various pathophysiological responses, potentially due to copper deprivation, their experimental designs focused primarily on iron- and copper-related metabolism. In this study, we also examined the effects of high iron consumption on glucose homeostasis, lipid profiles, and colon length.

Materials and methods

Animal experiments

All animal experimental protocols were carefully reviewed and approved by the Institutional Animal Care and Use Committee (IACUC; protocol number DKU-21–043) of Dankook University. Five-week-old, male, Sprague–Dawley (SD) rats (Doo Yeol Biotech, Inc., Seoul, Korea) were housed in overhanging wired mesh-bottomed stainless-steel cages for a 5-week dietary intervention period. The introduction age and dietary intervention periods were determined based on previous in vivo experiments with SD rats [16, 33, 36, 37], which demanded extra iron and copper due to rapid growth.

The experimental SD rats had *ad libitum* access to the designated diet, which was filtered through distilled water. The experimental diets were formulated based on the AIN-93G formulation with slight modifications [38, 39] (Doo Yeol Biotech, Inc.), and contained either AdFe or HFe in combination with low (LCu), adequate (AdCu), or high copper (HCu) (Tables 1, 2 and 3). In

Table 1 Iron and copper concentration in experimental diets

Diet	Fe (ppm)*	Cu (ppm)*
AdFe/LCu[#]	92.245	0.310
AdFe/AdCu	76.982	5.047
AdFe/HCu	109.614	163.518
HFe/LCu	6740.120	0.372
HFe/AdCu	7544.655	4.385
HFe/HCu	7462.401	164.795

[#] *H* high; *Ad* adequate; *L* low

[*] determined by ICP/MS

Table 2 Constant ingredients in the 6 experimental diets

Ingredient	Amount (g/kg)
Casein	200
Sucrose	100
Soybean oil	70
t-Butyhydroquinone	0.014
Dyetrose	132
Cellulose (micro)	50
Mineral Mix	35
Vitamin Mix	10
Choline Bitartrate	2.5
L-Cystine	3

this formulation, we raised the iron content in the AdFe diet from 39 ppm (the recommended iron content in the AIN-93G diet) to approximately 120 ppm to promote normal growth and bring the dietary iron level closer to the UL for extrapolation to the human dietary reference intake (the UL of iron is approximately three times higher than the daily recommended dietary intake of iron) for these weanling rats. Additionally, the compositions of the three different HFe diets have been noted in previous peer-reviewed studies.

The AdCu level was set at approximately 6 ppm following the recommendation for the AIN-93G diet, whereas HCu contained approximately 25 times more copper than the adequate level. This amount exceeded the gap between adequate intake and the UL for humans, which was approximately 11 times difference. We included HCu in our experimental design because we expected that the copper level in HCu would enhance intestinal copper absorption without surpassing a toxic threshold [28]. Previous reports have also indicated that elevated dietary copper levels can prevent iron-induced pathologies [28, 29].

Since carbonyl iron is generally not palatable, 100 g/kg sucrose was added to all formulated diets to enhance their palatability. It is worth noting that all diets containing HFe had slightly less energy (3726 kcal/kg) than AdFe-containing diets (3762 kcal/kg), since carbonyl iron was substituted for a small portion of the energy yield from corn starch. Nevertheless, the AdFe and HFe diets were considered almost isocaloric because the energy difference between the two formulations was less than 1.0%. In addition, the gross body weight of the animals was measured weekly, and food consumption was estimated by measuring the amount of food provided to each rat cage. The experimental animals were then humanely euthanized through thoracotomy after being subjected to carbon dioxide narcosis. Cardiac puncture was performed to obtain whole blood. Subsequently, the collected whole blood was centrifuged at 3000 × g and 4 °C for 15 min to separate the serum. The liver, spleen, heart, small intestine, kidney, and feces were collected, weighed, and stored at − 80 °C until further analysis. Colon length was measured using a ruler. The following are the equations employed in this section.

$$Delta\ body\ weight = Final\ body\ weight\ after\ dietary\ feeding - Initial\ body\ weight$$

$$Daily\ delta\ body\ weight = (Final\ body\ weight\ after\ dietary\ feeding - Initial\ body\ weight)/35\ days$$

$$Food\ efficiency\ ratio = Final\ body\ weight\ after\ dietary\ feeding/Food\ intake$$

Table 3 Variable ingredients in the 6 experimental diets

Ingredient	AdFe/LCu[#]	AdFe/AdCu	AdFe/HCu	HFe/LCu	HFe/AdCu	HFe/HCu
Cornstarch (g/kg)	397.01	397.01	396.71	386.79	386.78	386.48
Fe (Ferric citrate)	0.472	0.472	0.472	0.472	0.472	0.472
Carbonyl Fe	–	–	–	10.227	10.227	10.227
Cu (Cupric carbonate)	–	0.013	0.312	–	0.013	0.312
Kcal/kg	3763	3763	3763	3726	3726	3726

[#] *H* high; *Ad* adequate; *L* low

Hematological indices

After necrosis, fasting serum glucose and insulin were measured using commercial kits (Thermo Fisher Scientific, Waltham, MA, USA) and the homeostatic index of insulin resistance (HOMA-IR) was calculated by the prescribed equation [40]: HOMA-IR = serum insulin (μg/mL) · serum glucose (mg/dL)/405. Lipid panels including triglycerides (TG), total cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C) levels were measured using commercially available kits (Embiel, Gunpo, Korea). Subsequently, non-HDL-C levels and the cardiac risk factor (CRF) was determined by previously reported methods [41, 42]. Non-HDL-C was calculated by subtracting HDL-C from TC and CRF was calculated by dividing TC by HDL-C. To determine hepatotoxic responses the enzymatic activities of aspartate aminotransferase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) from serum were measured using their respective commercial kits (Embiel).

Iron and copper status

Hemoglobin (Hb) and hematocrit (Hct) levels were measured using a Cera-Chek Hb Plus reader (Green Cross, Yongin, Korea). Serum non-heme iron levels and total iron-binding capacity (TIBC) were quantified using a colorimetric analytical method [43, 44]. Subsequently, transferrin (Tf) saturation was calculated using the following equation: serum non-heme iron/TIBC × 100. The levels of iron and copper in the tissues were determined by inductively coupled plasma mass spectrometry (ICP-MS). In brief, a specimen weighing approximately 0.1 to 0.5 g was placed in a digestion vessel, and 5 mL of nitric acid (Daejung, Siheung, Korea) was added. Subsequently, the samples were processed using Titan MPS (PerkinElmer, Inc., Waltham, MA, USA). All prepared samples were then analyzed using an ICP-MS system (NexION 350X; PerkinElmer, Inc.) with appropriate dilutions using a standard solution, ICP-MS Calibration Standard 2 (AccuStandard, Inc., New Haven, CT, USA), and nitric acid, as required.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the liver, and cDNA synthesis and relative qRT-PCR analyses were conducted according to methods described in previous studies [42, 45, 46]. Primers used for qRT-PCR are from previous reports for hepcidin [28, 35] and GAPDH [47].

Statistical analysis

The results are presented as means and standard deviations, or as box-and-whisker plots. All figures were created using the GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). Data were statistically analyzed using two-way ANOVA with Tukey's post hoc test performed using XLSTAT 2012 (Addinsoft, Inc., Paris, France) to examine the effects of iron and copper, as well as their interactions. Statistical significance was set at $P < 0.05$. A summary of the two-way ANOVA results for both the main effects and interactions is provided in Table 4. Furthermore, the Pearson product-moment correction coefficient (r) was calculated to clarify the relationship between intestinal and fecal mineral content using XLSTAT 2012.

Results and discussion

Diet analysis

The iron concentrations in three different AdFe diets ranged from 117.0 to 122.2 μg/g, as determined by ICP-MS analysis. In HFe diets, the iron concentrations ranged from 6740 to 7544 μg/g. When we extrapolated our dietary iron concentration to the dietary reference intake for humans, it appeared that the dietary iron concentration may reach the UL, as it was approximately three times higher than the recommended iron intake for rapidly growing murine models.

The dietary copper levels in LCu were 0.31 and 0.37 μg/g in AdFe/LCu and HFe/LCu, respectively, as determined by ICP-MS analysis. In AdCu, the dietary copper levels were 6.05 and 6.39 μg/g in AdFe/AdCu and HFe/AdCu, respectively, and in HCu, the dietary copper levels were 153.5 and 154.8 μg/g in AdFe/AdCu and HFe/AdCu, respectively, according to ICP-MS analysis. The LCu diets contained approximately 20-fold less dietary copper, whereas the HCu diets had approximately 25-fold higher copper levels than the AdCu diets.

Body weight and food intake

As mentioned earlier, we expected that high iron consumption would result in a reduction in final body weight, delta body weight, and daily delta body weight due to copper depletion in experimental animals [28, 29]. However, in this experimental setup, high iron consumption had no effect on final body weight, delta body weight, or daily delta body weight (Fig. 1A–C). To investigate whether variations in dietary iron and copper levels affected energy intake, we assessed daily food intake, daily energy intake, and the food efficiency ratio. However, no significant differences were observed (Fig. 1D–E).

In this experiment, our failure to detect significant growth retardation from HFe feeding may be attributed to two dietary factors: higher AdFe and lower HFe levels compared with previous studies. AdFe levels were

Table 4 Summary of statistical analysis by two-way analysis variance (ANOVA) for main effects and interactions

Parameter	Fe main effect	Cu main effect	Fe X Cu interaction
Final body weight	* $p<0.05$	ns $p=0.591$	ns $p=0.658$
Δ body weight	* $p<0.05$	ns $p=0.501$	ns $p=0.590$
Daily Δ body weight	* $p<0.05$	ns $p=0.501$	ns $p=0.590$
Food intake	* $p<0.05$	**** $p<0.0001$	ns $p=0.669$
Energy intake	* $p<0.05$	**** $p<0.0001$	ns $p=0.669$
Food efficiency ratio	ns $p=0.200$	* $p<0.05$	ns $p=0.752$
Heart/Body weight	**** $p<0.0001$	**** $p<0.0001$	**** $p<0.0001$
Liver/Body weight	**** $p<0.0001$	** $p<0.01$	* $p<0.05$
Kidney/Body weight	**** $p<0.0001$	ns $p=0.554$	ns $p=0.877$
Spleen/Body weight	ns $p=0.589$	ns $p=0.059$	ns $p=0.440$
Pancreas/Body weight	** $p<0.01$	**** $p<0.0001$	**** $p<0.0001$
EAT/Body weight	** $p<0.01$	** $p<0.01$	ns $p=0.350$
Colon length	**** $p<0.0001$	ns $p=0.125$	**** $p<0.0001$
Serum glucose	* $p<0.05$	ns $p=0.069$	ns $p=0.800$
Serum insulin	ns $p=0.240$	ns $p=0.531$	** $p<0.01$
HOMA-IR	ns $p=0.286$	ns $p=0.870$	ns $p=0.197$
Serum triglyceride	ns $p=0.733$	ns $p=0.629$	ns $p=0.756$
Serum total cholesterol	ns $p=0.320$	* $p<0.05$	**** $p<0.0001$
Serum HDL-cholesterol	**** $p<0.0001$	**** $p<0.0001$	**** $p<0.0001$
Serum Non-HDL-cholesterol	ns $p=0.434$	ns $p=0.601$	*** $p<0.001$
Cardiac risk factor	**** $p<0.0001$	ns $p=0.216$	* $p<0.05$
Serum ALP	**** $p<0.0001$	* $p<0.05$	ns $p=0.074$
Serum AST	**** $p<0.0001$	* $p<0.05$	ns $p=0.180$
Serum ALT	**** $p<0.0001$	ns $p=0.589$	ns $p=0.307$
Hb	* $p<0.05$	**** $p<0.0001$	**** $p<0.0001$
HCT	**** $p<0.0001$	**** $p<0.0001$	**** $p<0.0001$
Serum non-heme Fe	ns $p=0.166$	** $p<0.01$	* $p<0.05$
TIBC	ns $p=0.932$	ns $p=0.401$	* $p<0.05$
Tf saturation	ns $p=0.588$	**** $p<0.0001$	** $p<0.01$
Liver hepcidin mRNA expression	**** $p<0.0001$	**** $p<0.0001$	**** $p<0.0001$
Liver Fe	**** $p<0.0001$	ns $p=0.259$	ns $p=0.944$
Spleen Fe	**** $p<0.0001$	**** $p<0.0001$	** $p<0.01$
Heart Fe	ns $p=0.473$	* $p<0.05$	* $p<0.05$
Kidney Fe	** $p<0.01$	**** $p<0.0001$	**** $p<0.0001$
Serum Fe	**** $p<0.0001$	ns $p=0.645$	ns $p=0.357$
Feces Fe	**** $p<0.0001$	** $p<0.01$	** $p<0.01$
Small intestine Fe	**** $p<0.0001$	ns $p=0.561$	ns $p=0.523$
Liver Cu	**** $p<0.0001$	**** $p<0.0001$	**** $p<0.0001$
Spleen Cu	**** $p<0.0001$	**** $p<0.0001$	**** $p<0.0001$
Heart Cu	**** $p<0.0001$	**** $p<0.0001$	**** $p<0.0001$
Kidney Cu	**** $p<0.0001$	**** $p<0.0001$	**** $p<0.0001$
Serum Cu	**** $p<0.0001$	**** $p<0.0001$	**** $p<0.0001$
Feces Cu	* $p<0.05$	**** $p<0.0001$	** $p<0.01$
Small intestine Cu	**** $p<0.0001$	**** $p<0.0001$	ns $p=0.457$

EAT epididymal adipose tissue; *HOMA-IR* homeostasis model assessment of insulin resistance; *HDL* high-density lipoprotein; *ALP* alkaline phosphatase; *AST* asparate aminotransferase; *ALT* alanine aminotransferase; *Hb* hemoglobin; *HCT* hematocrit; *TIBC* total iron-binding capacity; *Tf* transferrin; *ns* not significant

* $p<0.05$

** $p<0.01$

*** $p<0.001$

**** $p<0.0001$

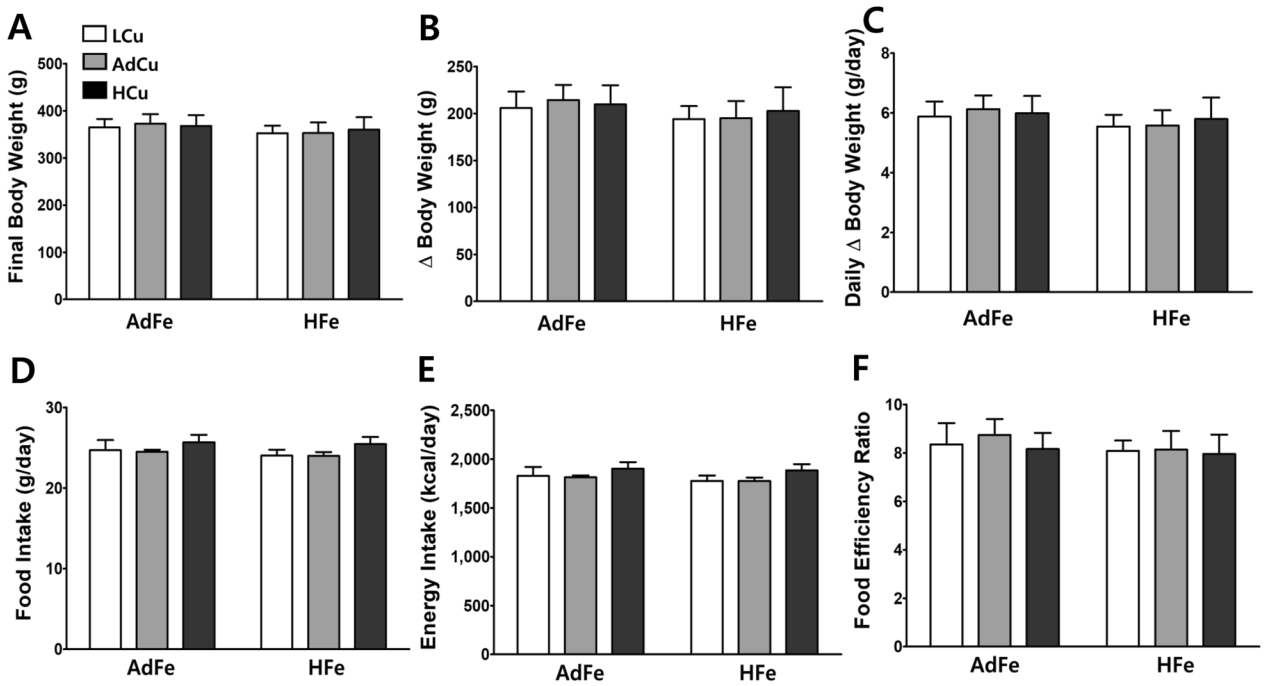

Fig. 1 Effects of high-iron feeding on the body weight, food intake, energy intake, and food efficiency ratio of rats. Five-week-old, male, SD rats were fed one of six diets, which varied only in their iron and copper content for 5 weeks *ad libitum*. Final body weight (**A**), delta body weight (the final body weight after dietary feeding—the initial body weight) (**B**), daily delta body weight (**C**), food intake (**D**), energy intake (**E**), and food efficiency ratio (**F**) were measured. The results are presented as means ± SDs; n = 10 per individual group. Data were analyzed by two-way ANOVA with Tukey's post hoc test for further comparisons. *L* low; *Ad* adequate; *H* high

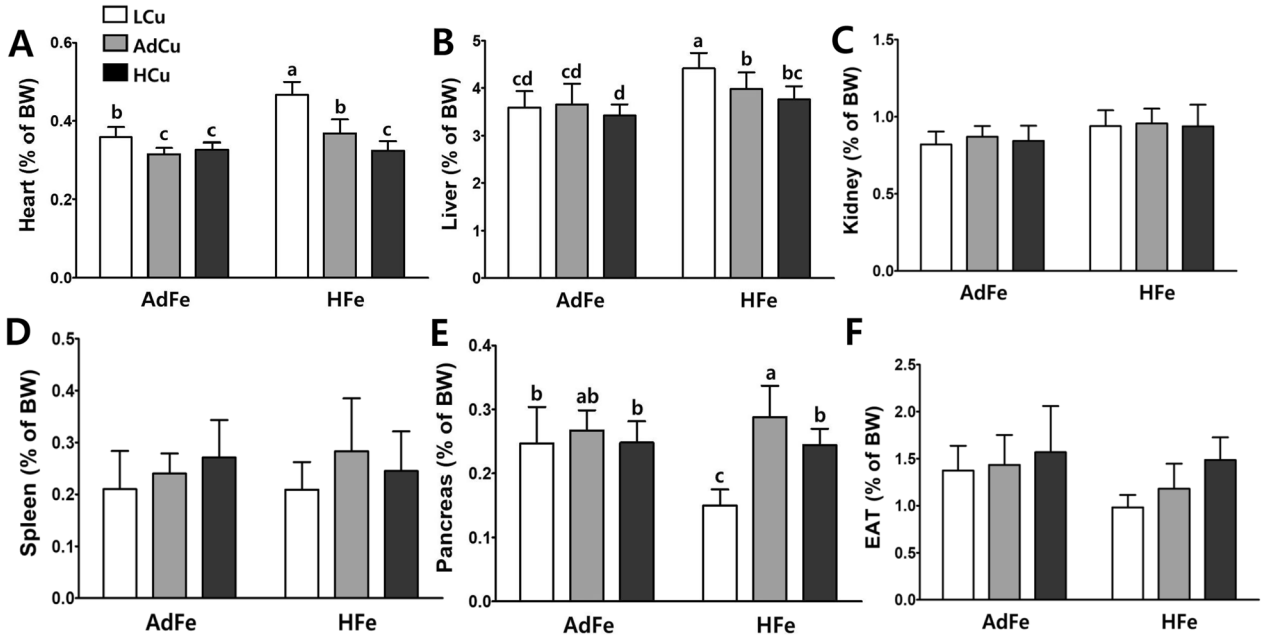

Fig. 2 Effects of high-iron feeding on relative organ weights of rats. Five-week-old, male, SD rats were fed one of six diets, which varied only in their iron and copper content for 5 weeks *ad libitum*. Relative organ weights of the heart (**A**), liver (**B**), kidney (**C**), spleen (**D**), pancreas (**E**), and EAT (**F**) were measured. The results are presented as means ± SDs; n = 10 per individual group. Data were analyzed by two-way ANOVA with Tukey's post hoc test for further comparisons. Different letters indicate significant differences ($P < 0.05$) among the labeled means. *L* low; *Ad* adequate; *H* high; *BW* body weight

close to the recommended UL for humans, and copper deficiency was noted at this concentration. Therefore, the growth rate in the AdFe group may have been lower than those reported in previous studies. Furthermore, the overall iron concentration in HFe in this study (7249 μg/g) was 18% lower than the iron concentration in HFe in the two previous reports (8820 μg/g) [28, 29], and relatively lower iron levels in HFe may not be sufficient to trigger copper-depleted growth retardation.

Organ weights

HFe-induced growth retardation was not observed in our experimental setup. However, to investigate whether HFe consumption altered the previously observed pathological responses [28, 29], we measured the relative weights of the heart, liver, kidney, spleen, pancreas, and epididymal white adipose tissue (Fig. 2A–F). As noted previously, kidney and spleen weights did not vary among the six dietary interventions, and the weight of the epididymal white adipose tissue (EAT) remained unchanged [28, 29].

Notably, the relative weights of the heart and liver confirmed those reported in our previous animal experimental protocols [28, 48]. Two distinct features were observed in the relative heart weight, indicating cardiac hypertrophy. Regardless of the dietary iron concentration, LCu consumption significantly increased the heart weight. Furthermore, consumption significantly triggered cardiac hypertrophy. However, a gradual increase in dietary copper concentration normalized heart weight compared with AdFe/AdCu, as observed in peer-reviewed reports [28, 29].

Regarding the relative liver weight, high iron consumption induced significant hepatomegaly. However, as previously observed, HFe-induced hepatomegaly was normalized by additional copper consumption. Additionally, the pancreatic weight responded dynamically to various amounts of iron and copper. Lower dietary copper intake reduced pancreatic weight, and this decline was more significant when high iron intake was combined with low copper intake. In HFe/AdCu, the weight of the pancreas was restored; however, HFe/HCu decreased its weight compared to HFe/AdCu, but maintained a weight similar to that seen in the AdFe groups.

Serum glucose, insulin, and HOMA-IR

Uncontrolled iron levels, such as iron deficiency anemia [49] and iron overload are related to insulin resistance [50]. Iron overload, such as hereditary hemochromatosis [51] and β-thalassemia [52] triggers insulin resistance by destruction of pancreatic β-cells [50] probably due to extra iron accumulation. Due to the dynamic changes in relative pancreatic weight, we logically assessed glucose metabolism-related parameters in the fasting serum, including glucose, insulin, and HOMA-IR. Serum glucose levels increased following HFe consumption (Fig. 3A). The serum insulin level was the lowest in the HFe/LCu group (Fig. 3B), and the decrease in systemic insulin in the HFe/LCu group may be attributed to the reduction in pancreatic weight (Fig. 2E). In a genetic iron overload model, patients with hemochromatosis often experienced insulin resistance, likely due to elevated oxidative stress resulting from increased iron accumulation in the pancreas. Our findings suggest that a combination of high iron accumulation and low copper concentrations can exacerbate insulin resistance. However, HOMA-IR remained consistent across the six dietary interventions, with varying iron and copper levels (Fig. 3C).

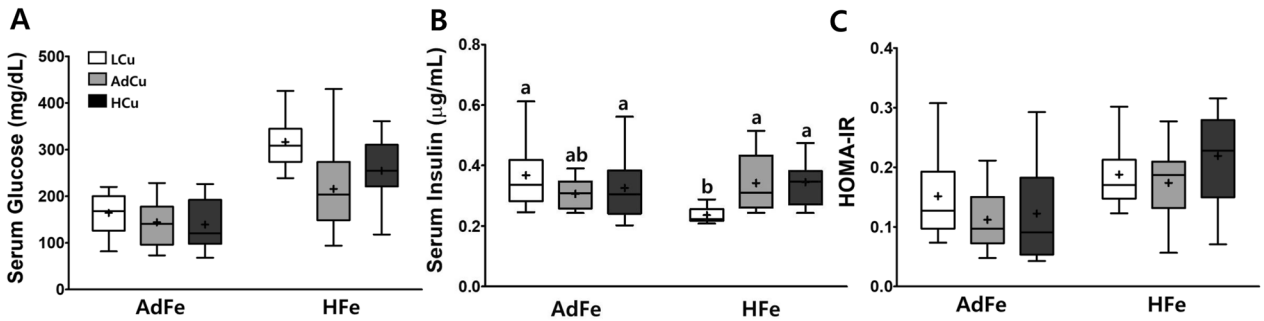

Fig. 3 Effects of high-iron feeding on serum glucose and insulin levels of rats. Five-week-old, male, SD rats were fed one of six diets, which varied only in their iron and copper content for 5 weeks *ad libitum*. Serum glucose (**A**), insulin (**B**), and HOMA-IR levels (**C**). The results are presented as means ± SDs; n = 10 per individual group. Data were analyzed by two-way ANOVA with Tukey's post hoc test for further comparisons. Different letters indicate significant differences ($P < 0.05$) among the labeled means. *L* low; *Ad* adequate; *H* high; *HOMA-IR* homeostasis model assessment-estimated insulin resistance

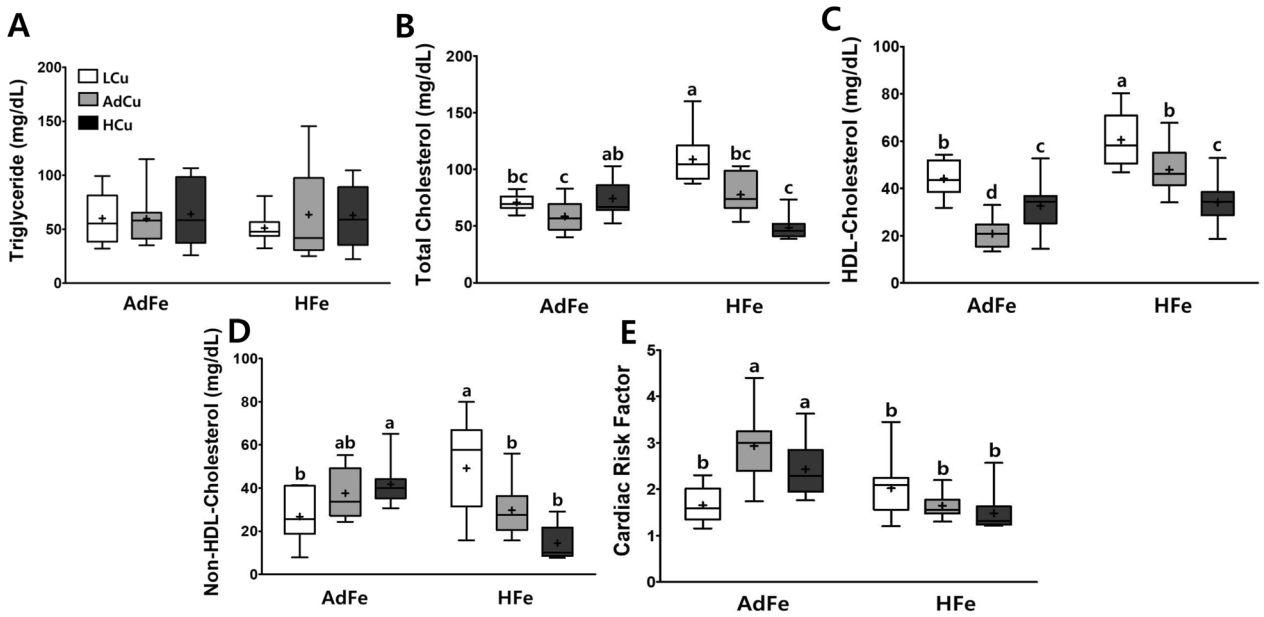

Fig. 4 Effects of high-iron feeding on serum lipid panels of rats. Five-week-old, male, SD rats were fed one of six diets, which varied only in their iron and copper content for 5 weeks *ad libitum*. Serum triglyceride (**A**), total cholesterol (**B**), HDL-cholesterol (**C**), non-HDL-cholesterol (**D**), and cardiac risk factor (**E**). The results are presented as means ± SDs; n = 10 per individual group. Data were analyzed by two-way ANOVA with Tukey's post hoc test for further comparisons. Different letters indicate significant differences ($P < 0.05$) among the labeled means. *L* low; *Ad* adequate; *H* high; *HDL* high-density lipoprotein

Lipid panels

Evidence suggests that both iron and copper are significant factors in the pathological modulation of atherosclerosis and cardiovascular diseases. However, the specific interactions that detail how dietary iron and copper affect lipid profiles have not yet been reported. In our study, we analyzed the blood lipid and cholesterol levels in the experimental animals. Various combinations of dietary iron and copper did not significantly alter the serum triglyceride (TG) levels (Fig. 4A). However, serum cholesterol levels responded dynamically to our dietary intervention (Fig. 4B). Serum TC levels were altered in a copper-sensitive manner ($P < 0.05$). In the AdFe group, extra copper consumption elevated TC levels. In the HFe groups, in combination with LCu, TC levels were the highest; however, a gradual increase in dietary copper decreased TC levels. AdFe/AdCu exhibited the lowest HDL-C levels among the various dietary interventions (Fig. 4C). In combination with LCu, HCu, or AdFe, HDL-C levels were significantly elevated. In HFe, as seen in the TC levels, HFe/LCu showed the highest HDL-C levels; however, the gradual induction of dietary copper decreased the TC levels in conjunction with HFe. In the AdFe group, non-HDL-C levels tended to increase with increasing dietary copper concentration (Fig. 4D). Therefore, AdFe/LCu exhibited the lowest non-HDL-C level, whereas AdFe/HCu exhibited the highest. However, in the HFe groups, increasing the dietary copper concentration tended to diminish the non-HDL-C levels (Fig. 4D). Therefore, HFe/LCu exhibited the highest non-HDL-C levels, whereas HFe/AdCu and HFe/HCu exhibited lower non-HDL-C levels than HFe/AdCu. It is likely that HFe consumption triggers dyslipidemia, which is generally observed in genetically iron-overloaded populations, and alterations in cholesterol levels may be a potential risk factor for the induction of cardiovascular diseases [53, 54]. Intriguingly, the gradual elevation of dietary copper significantly diminished the overall cholesterol levels, regardless of the cholesterol type. Exposure to additional divalent minerals may suppress de novo cholesterol synthesis [55]. Cardiac risk factors significantly decreased in all dietary treatments, except for AdFe/HCu, compared to AdFe/AdCu (Fig. 4E).

Enigmatically, there is a report that excess iron (i.e., non-transferrin-bound iron) exposure to primary human umbilical vein endothelial cells induces de novo cholesterol synthesis, probably via regulation of sterol regulatory element-binding protein 2 [56]. Furthermore, excess iron exposure also induces cellular apoptosis, likely through the elevation of tumor necrosis factor alpha (TNFα) [56]. These findings were verified using a cholesterol chelator (hydroxypropyl-β-cyclodextrin) since cholesterol chelation reduces excess-iron-triggered TNFα-inducible apoptosis [56].

In our study, excess iron exposure decreased overall cholesterol levels regardless of the type of cholesterol. Probably, at the cellular level, initial cholesterol synthesis may be inducible, as reported previously [56]; however, due to long-term dietary exposure, both iron and copper may also trigger cellular apoptosis, ultimately leading to a decrease in cholesterol levels. Moreover, extra copper supplementation with extra iron rather decreased overall cholesterol levels; however, based on the current gathered information, we could not conclude whether diminished overall cholesterol levels were due to excess cellular apoptosis triggered by extra copper or had preventive effects described as other biological outcomes in this study. Therefore, further intensive and specific in vivo experiments are required to understand how dietary iron and copper affect overall cholesterol metabolism.

Hepatic functional biological indices

Both iron and copper are transient divalent metals; therefore, exposure to excess iron and copper may lead to changes in hepatic enzymatic functional biological markers such as ALP, AST, and ALT activity levels in the serum. To determine whether the six different dietary interventions could alter hepatic enzymatic functional biological markers, we assessed the ALP, AST, and ALT activity levels in the serum.

Serum ALP activity levels were significantly influenced by dietary iron ($P < 0.0001$), with HFe resulting in significantly lower ALP activity than AdFe (Fig. 5A). It is noteworthy that individual with genetic conditions such as Menkes disease (copper deficiency [18]) and Wilson's disease (copper overload [57]) exhibit lower ALP levels than normal subjects. Therefore, reduced serum ALP activity may be linked to HFe-induced copper deficiency [58]. Also, serum AST levels were strongly affected by dietary iron ($P < 0.0001$), and HFe resulted in significantly lower AST activity than AdFe (Fig. 5B).

Interestingly, serum ALT activity was also significantly influenced by dietary iron ($P < 0.0001$); serum ALT activity was elevated in the HFe groups compared to that in the AdFe groups. Serum ALT activity can vary dynamically, either increasing or decreasing in conditions such as Wilson's disease [59]. Therefore, serum ALT levels may serve as sensitive markers for copper alterations, and in our experimental setting, HFe-induced copper depletion may have contributed to the reduction in serum ALT activity.

Hematological parameters

All hematological parameters have been previously validated in peer-reviewed publications [28, 60]. In the AdFe group, AdFe/LCu significantly reduced Hb levels, which could be attributed to copper deficiency (Fig. 6A). In the HFe group, HFe/LCu reduced Hb levels to a greater extent than AdFe/LCu. However, with an increase in dietary copper levels in HFe, Hb levels gradually normalized to levels similar to those in the AdFe/AdCu group. Hct levels exhibited trends similar to those of Hb as previously reported (Fig. 6B) [28, 29]. AdFe/LCu showed a decreasing trend in Hct (although not statistically significant), and HFe significantly reduced the proportion of red blood cells. However, additional copper supplementation prevented the copper-deprivation effect induced by HFe. Serum non-heme iron levels were significantly elevated in HFe/HCu mice due to a significant dietary copper interaction (Fig. 6C; $P < 0.01$). The TIBC remained consistent across all six dietary interventions (Fig. 6D). Considering the serum non-heme iron and TIBC levels, HFe/HCu exhibited the highest Tf saturation among all dietary treatments (Fig. 6E).

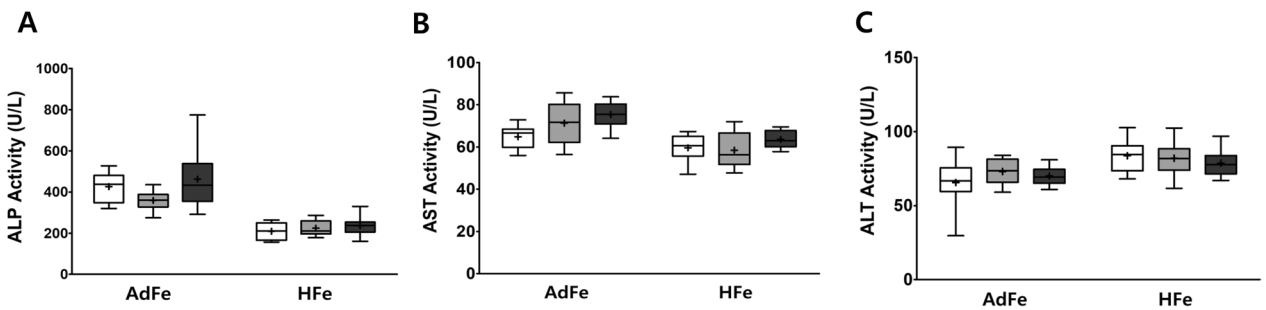

Fig. 5 Effects of high-iron feeding on the enzyme levels related to hepatic function of rats. Five-week-old, male, SD rats were fed one of six diets, which varied only in their iron and copper content for 5 weeks *ad libitum*. ALP (**A**), AST (**B**), and ALT (**C**) activities. The results are presented as means ± SDs; n = 10 per individual group. Data were analyzed by two-way ANOVA with Tukey's post hoc test for further comparisons. Different letters indicate significant differences ($P < 0.05$) among the labeled means. *L* low; *Ad* adequate; *H* high; *ALP* alkaline phosphate; *AST* aspartate amino transferase; *ALT* alanine amino transferase

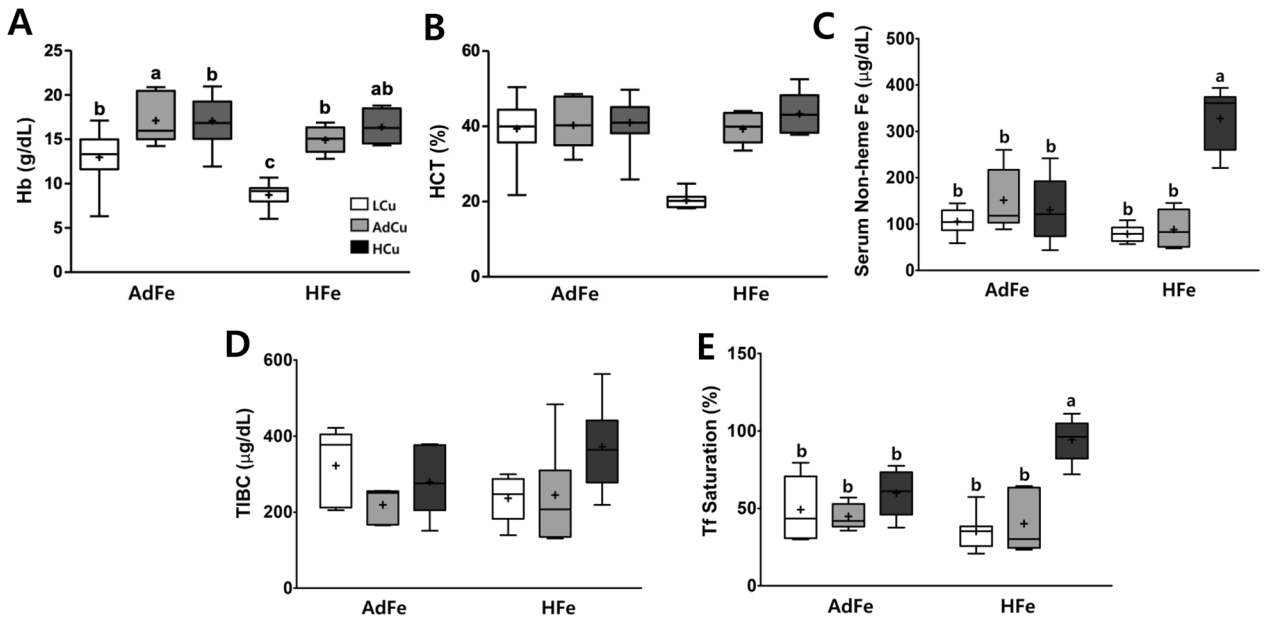

Fig. 6 Effects of high-iron feeding on hematological parameters of rats. Five-week-old, male, SD rats were fed one of six diets, which varied only in their iron and copper content for 5 weeks *ad libitum*. Hb (**A**), HCT (**B**), serum non-heme Fe (**C**), TIBC (**D**), and Tf saturation (**E**). The results are presented as means ± SDs; n = 10 per individual group. Data were analyzed by two-way ANOVA with Tukey's post hoc test for further comparisons. Different letters indicate significant differences ($P < 0.05$) among the labeled means. *L* low; *Ad* adequate; *H* high; *Hb* hemoglobin; *Hct* hematocrit; *TIBC* totaliron-binding capacity; *Tf* transferrin

In a previous communication, we reported that the expression of erythropoietin (Epo) mRNA in the kidneys of rats accurately reflected the levels of the circulating hormone [28]. Furthermore, all hematological indices, including diminished Hb and Hct, were validated according to previous experimental animal protocols [28, 29, 35, 48]. In this experimental setting, when HFe conditions were combined with LCu, erythropoietic demands were expected to be higher than in other scenarios. However, these hematological disturbances were gradually prevented when extra dietary copper content was introduced [28, 29, 35, 48]. We logically postulated that the additional copper, when combined with high iron, should lead to a decrease in erythropoietic demands (i.e. Epo expression and red blood cell (RBC) contents in whole blood).

Tissue iron loading and hepatic hepcidin mRNA expression

Intestinal iron absorption is tightly regulated, and the distribution and metabolism of iron in multiple tissues is controlled [61, 62]. To understand how the six different dietary interventions affected the distribution of iron in the systemic and peripheral tissues, iron content was analyzed using ICP-MS. Hepatic iron loading was influenced by both dietary iron ($P < 0.0001$) and copper ($P < 0.01$). HFe treatment resulted in a significantly higher hepatic iron loading. Interestingly, regardless of the dietary iron concentration, extra copper decreased hepatic copper loading. This slight but significant reduction in hepatic copper may be due to the innate ferroxidase activity of biological copper, which enhances iron utilization.

Hepatic hepcidin mRNA expression is intricately linked to hepatic iron loading, therefore, we measured hepatic hepcidin mRNA expression using qRT-PCR (Table 5). However, only HFe/HCu increased hepatic hepcidin mRNA expression. Our findings which also showed the highest hepatic hepcidin mRNA expression among all HFe groups were not consistent with those of previous reports [28]. In previous studies, adequate iron levels likely targeted the recommended intake, whereas in our study adequate dietary iron was aimed at the UL. Therefore, high dietary iron levels may increase hepatic hepcidin mRNA expression. Differences in dietary settings may explain why this study did not replicate previous findings [28, 35]. However, a clear and evident finding is that high iron levels, when combined with high copper levels, can maximize hepatic hepcidin expression.

Splenic iron accumulation was influenced by both dietary iron ($P < 0.0001$) and copper ($P < 0.0001$). No significant differences were observed when AdFe was combined with either LCu or AdCu. However, AdFe/HCu significantly elevated splenic iron loading, likely due to the extra copper enhancing its utilization. Furthermore, in the HFe groups, HFe/LCu was loaded with similar

Table 5 Tissue iron and liver hepcidin levels

μg/g	AdFe/LCu[#]	AdFe/AdCu	AdFe/HCu	HFe/LCu	HFe/AdCu	HFe/HCu
Liver	124.98 ± 21.47	96.64 ± 33.69	80.01 ± 35.20	382.22 ± 71.82	358.47 ± 59.56	352.55 ± 76.81
Liver hepcidin mRNA expression (fold change)	1.11 ± 1.08^{b}	1.00 ± 1.72^{b}	1.04 ± 1.30^{b}	1.25 ± 1.02^{b}	2.71 ± 1.63^{b}	7.14 ± 1.61^{a}
Spleen	0.16 ± 0.02^{c}	0.32 ± 0.07^{c}	1.44 ± 0.45^{b}	0.41 ± 0.21^{c}	1.51 ± 0.24^{ab}	1.69 ± 0.25^{a}
Heart	88.98 ± 10.16^{ab}	80.74 ± 8.88^{bc}	89.28 ± 6.68^{ab}	76.66 ± 13.07^{c}	90.69 ± 8.49^{ab}	100.67 ± 10.91^{a}
Kidney	43.50 ± 4.19^{cd}	47.04 ± 8.78^{bc}	41.89 ± 7.05^{cd}	37.46 ± 5.24^{d}	51.72 ± 2.95^{b}	61.64 ± 6.65^{a}
Serum	0.22 ± 0.02	0.22 ± 0.01	0.21 ± 0.02	1.00 ± 0.07	1.15 ± 0.12	1.15 ± 0.12

The results are presented as means ± SDs; n = 10 per individual group. Data were analyzed by two-way ANOVA with Tukey's post hoc test for further comparisons. Different letters indicate significant differences (P < 0.05) among the labeled means

[#] *H* high; *Ad* adequate; *L* low

levels of iron as AdFe/LCu and AdFe/AdCu. However, HFe/AdCu and HFe/HFe showed the highest splenic iron content among all groups. Cardiac iron loading was influenced not by dietary iron, but by dietary copper (P < 0.05), and only HFe/LCu loaded significantly less cardiac iron than the other dietary interventions. Renal iron accumulation was affected by both dietary iron (P < 0.01) and copper (P < 0.0001) levels. All the AdFe and HFe/LCu groups had statistically identical renal iron levels. However, in the HFe group, increasing dietary copper levels significantly elevated renal iron loading, which is consistent with previous reports [28, 35]. Serum iron levels were not influenced by dietary copper but were influenced by iron levels (P < 0.0001). The HFe group exhibited significantly higher serum iron levels than the AdFe group, regardless of the dietary copper concentration (Table 5).

Splenic iron acquisition plays a crucial role in the overall regulation of the body's iron metabolism [63, 64]. During iron deprivation, renal Epo expression is induced due to increased erythropoietic demands. Consequently, renal Epo upregulates splenic erythroferrone expression, which, in turn, may counteract hepatic hepcidin expression to facilitate additional iron acquisition [65]. In β-thalassemia patients, the specific role of splenic erythroferrone is emphasized, with erythroferrone exacerbating iron loading [66]. A previous study highlighted the sensitivity of splenic erythroferrone expression to iron-deprived conditions but not in conditions of HFe [28]. Additionally, in HFe groups, splenic erythroferrone expression showed no significant variation with different dietary copper contents [28]. Although, erythroferrone serves as a highly responsive regulatory hormone in iron metabolism, its role may be minimal in the context of high dietary iron consumption, potentially being more closely associated with splenic iron content.

Tissue copper loading

To understand how various dietary iron and copper levels influence copper loading in multiple tissues (such as the liver, spleen, heart, and kidney) and circulating biological copper content, we analyzed them using ICP-MS systems. Interestingly, copper levels in selected tissues and serum exhibited a similar pattern of response, influenced by dietary iron (P < 0.0001), copper (P < 0.0001), and the interaction between dietary iron and copper (P < 0.0001). All LCu groups loaded less copper than the other groups. However, when LCu was combined with HFe, the hepatic and renal copper loadings were further reduced. In general, HFe results in lower copper levels in the tissues and serum. Nevertheless, the HFe-induced copper depletion was gradually prevented by additional dietary copper

Table 6 Tissue copper levels

μg/g	AdFe/LCu[#]	AdFe/AdCu	AdFe/HCu	HFe/LCu	HFe/AdCu	HFe/HCu
Liver	1.80 ± 0.29^{c}	4.82 ± 0.32^{b}	5.42 ± 0.73^{a}	0.44 ± 0.08^{d}	1.83 ± 0.65^{c}	4.98 ± 0.85^{b}
Spleen	0.41 ± 0.21^{b}	1.44 ± 0.45^{a}	1.69 ± 0.25^{a}	0.16 ± 0.02^{b}	0.32 ± 0.07^{b}	1.51 ± 0.24^{a}
Heart	1.67 ± 0.20^{b}	6.13 ± 0.60^{a}	6.28 ± 0.98^{a}	1.47 ± 0.92^{b}	1.67 ± 0.37^{b}	5.87 ± 0.92^{a}
Kidney	2.33 ± 0.21^{c}	4.51 ± 0.51^{a}	4.41 ± 0.66^{a}	1.69 ± 0.18^{d}	2.13 ± 0.21^{c}	3.65 ± 0.29^{b}
Serum	0.21 ± 0.02^{c}	1.00 ± 0.06^{b}	1.10 ± 0.09^{a}	0.22 ± 0.02^{c}	0.22 ± 0.01^{c}	1.15 ± 0.12^{a}

The results are presented as means ± SDs; n = 10 per individual group. Data were analyzed by two-way ANOVA with Tukey's post hoc test for further comparisons. Different letters indicate significant differences (P < 0.05) among the labeled means

[#] *H* high; *Ad* adequate; *L* low

consumption in our experimental SD rats, as previously reported [28, 35]. The observed copper depletion in the heart owing to HFe feeding may be a significant potential cause of cardiac hypertrophy (Fig. 2A, Table 6).

Colon length, iron and copper content in small intestine and feces

Following our dietary formula, both HFe and HCu supply iron and copper in amounts significantly higher than the biological requirements, and the excess should reach the colon. Thus, unabsorbed dietary iron and copper can alter the colonic environment and physiology. To investigate our research hypothesis, we first measured and summarized colon length (Fig. 7A, B). Colon lengths exhibited different patterns in the AdFe and HFe groups. In the AdFe groups, colon length was significantly reduced only when extra copper was added. However, in the HFe/LCu group, the colon length was the shortest among all groups. Nevertheless, a gradual increase in dietary copper concentration prevented shortening of the colon length.

In our previous studies [35, 48], we consistently observed that HFe triggered copper depletion, and that HFe-induced systemic copper depletion was prevented by extra dietary copper supplementation. Therefore, the normalization of colon length in HFe when supplying extra copper, may be closely related to copper depletion and repletion. However, further in-depth-studies are required to understand the exact underlying molecular mechanisms. Intriguingly, in AdFe, extra copper decreases colon length and may elevate oxidative stress in the colon. However, further studies are required to fully elucidate these phenomena.

To assess the absorption and excretion of iron and copper in our experimental settings, we measured the iron and copper contents in the feces and small intestine. Fecal iron levels were influenced by dietary iron ($P < 0.0001$) and copper ($P < 0.01$) and were significantly higher in all HFe groups than in the AdFe groups (Fig. 7C). Interestingly, the highest fecal iron levels were observed in HFe/AdCu, with HFe/AdCu having the second-highest levels and HFe/LCu exhibiting the lowest fecal iron levels among the HFe groups. The rate of intestinal iron absorption is tightly regulated [67], which may explain the levels of fecal iron excretion. Notably, there was a positive correlation between intestinal and fecal iron content, as determined by Pearson's test ($P < 0.0001$; $r = 0.817$; Fig. 7C, D).

Copper absorption and excretion are influenced by both dietary iron and copper content. The copper content in the feces was affected by dietary iron ($P < 0.05$) and copper ($P < 0.0001$) (Fig. 7E). Again, there was a positive correlation between intestinal copper content and fecal copper content, as indicated by the Pearson's test ($P < 0.0001$; $r = 0.922$). Furthermore, the intestinal copper content was affected by dietary iron ($P < 0.0001$) and copper ($P < 0.0001$) (Fig. 7F). Although, HFe leads to Cu deficiency, intestinal Cu absorption and excretion are influenced by dietary iron and copper concentrations.

The colonial microbiome is influenced by various dietary factors, such as energy nutrients and vitamins [68, 69]. Recently, attention has been given to the influence of the microbiome on minerals, including iron [70]. In

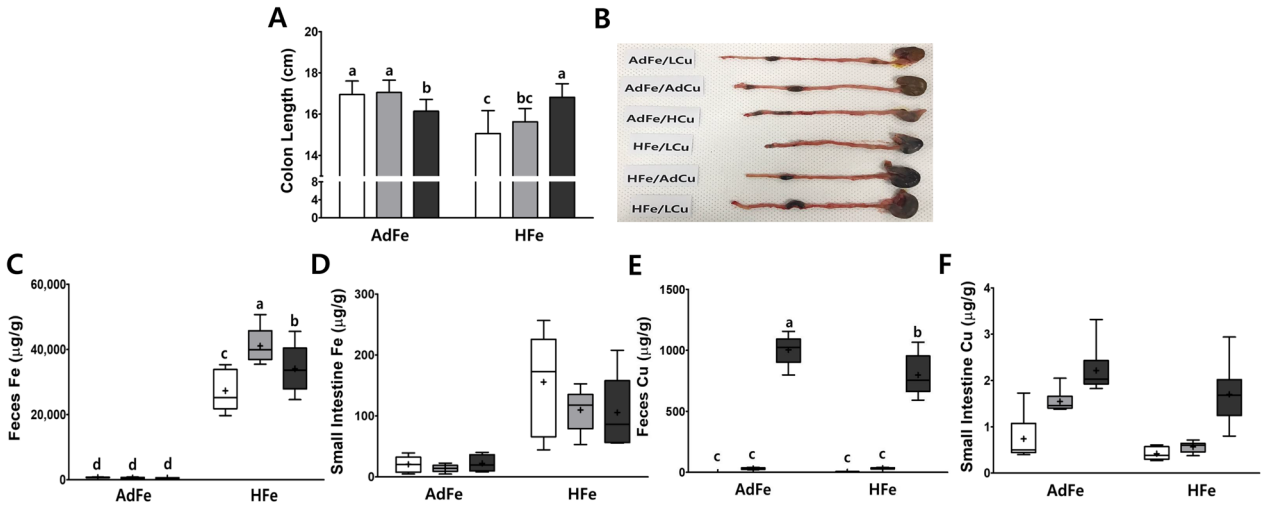

Fig. 7 Effects of high-iron feeding on colon length and iron and copper concentration in feces and the small intestine of rats. Five-week-old, male, SD rats were fed one of six diets, which varied only in their iron and copper content for 5 weeks *ad libitum*. Colon length (**A**), image of colon length (**B**), iron concentration in feces (**C**), and the small intestine (**D**), copper concentration in feces (**E**), and the small intestine (**F**). The results are presented as means ± SDs; n = 10 per individual group. Data were analyzed by two-way ANOVA with Tukey's post hoc test for further comparisons. Different letters indicate significant differences ($P < 0.05$) among the labeled means. *L* low; *Ad* adequate; *H* high

iron-deprived conditions, certain probiotics enhance intestinal iron absorption to prevent iron deprivation. For example, exposure to specific probiotics, such as *Lactobacillus plantarum* [71], *L. plantarum 299v* [72], *Streptococcus thermophiles* [73], *L. fermentum* [74], and *L. acidophilus* [75], significantly elevates iron utilization in vivo by increasing iron availability [70].

In addition to the consumption of probiotics to aid iron utilization, iron supplementation is an easy and practical means to prevent iron deficiency. However, excess iron absorption negatively alters the colonic environment due to unabsorbed iron [76], resulting in a decrease in short-chain fatty acid production and an increase in *Enterobacteriaceae* [76]. Indeed, the majority of colonic bacteria require iron to maintain normal physiology [76]. However, due to the competitiveness between beneficial and harmful colonic bacteria, harmful bacteria prefer to utilize iron over beneficial bacteria. Therefore, exposure to excess iron reduces beneficial *Bifidobacterium* and *Lactobacillus* spp., but elevates harmful *Enterobacteriaceae* spp. [76–79].

In this experiment, we found that extra iron decreased colon length, which may be closely related to colonial inflammation and/or oxidative stress. However, when extra copper was combined with HFe, colon length was normalized. This dynamic alteration in colon length is probably related to changes in the microbiome. While we expected HFe to elevate harmful bacterial population, the combination with extra copper may decrease the relative harmful bacterial population. Therefore, to answer our extended research question, we may characterize the fecal microbiome in a follow-up study.

Acknowledgements
Not applicable.

Author contributions
JL and H-JL: conceptualization, data curation, formal analysis, software, validation, investigation, writing—original draft, writing—review & editing. HJ and J-JL: formal analysis, validation, investigation, writing—review & editing. J-HH: conceptualization, data curation, methodology, software, validation, investigation, writing—original draft, writing—review & editing.

Funding
This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2021R1I1A3060357).

Availability of data and materials
The data sets utilized in this investigation can be obtained by contacting the corresponding author upon a reasonable request.

Declarations

Competing interests
The authors declare no competing interests.

Received: 20 November 2023 Accepted: 9 January 2024
Published online: 31 January 2024

References

1. Bryant BJ, Yau YY, Arceo SM, Hopkins JA, Leitman SF (2013) Ascertainment of iron deficiency and depletion in blood donors through screening questions for pica and restless legs syndrome. Transfusion 53:1637–1644. https://doi.org/10.1111/trf.12061
2. Elstrott B, Khan L, Olson S, Raghunathan V, DeLoughery T, Shatzel JJ (2020) The role of iron repletion in adult iron deficiency anemia and other diseases. Eur J Haematol 104:153–161. https://doi.org/10.1111/ejh.13345
3. Yokoi K, Konomi A (2017) Iron deficiency without anaemia is a potential cause of fatigue: meta-analyses of randomised controlled trials and cross-sectional studies. Br J Nutr 117:1422–1431. https://doi.org/10.1017/S0007114517001349
4. Camaschella C (2019) Iron deficiency. Blood 133:30–39. https://doi.org/10.1182/blood-2018-05-815944
5. Pasricha SR, Tye-Din J, Muckenthaler MU, Swinkels DW (2021) Iron deficiency. Lancet 397:233–248. https://doi.org/10.1016/S0140-6736(20)32594-0
6. Cappellini MD, Musallam KM, Taher AT (2020) Iron deficiency anaemia revisited. J Intern Med 287:153–170. https://doi.org/10.1111/joim.13004
7. Bacon BR (2012) Hemochromatosis: discovery of the HFE gene. Mo Med 109:133–136
8. Cai C, Hu W, Chu T (2022) Interplay between iron overload and osteoarthritis: clinical significance and cellular mechanisms. Front Cell Dev Biol 9:817104. https://doi.org/10.3389/fcell.2021.817104
9. Dongiovanni P, Ruscica M, Rametta R, Recalcati S, Steffani L, Gatti S et al (2013) Dietary iron overload induces visceral adipose tissue insulin resistance. Am J Pathol 182:2254–2263. https://doi.org/10.1016/j.ajpath.2013.02.019
10. Puntarulo S (2005) Iron, oxidative stress and human health. Mol Aspects Med 26:299–312. https://doi.org/10.1016/j.mam.2005.07.001
11. Guo S, Frazer DM, Anderson GJ (2016) Iron homeostasis: transport, metabolism, and regulation. Curr Opin Clin Nutr Metab Care 19:276–281. https://doi.org/10.1097/MCO.0000000000000285
12. Rishi G, Wallace DF, Subramaniam VN (2015) Hepcidin: regulation of the master iron regulator. Biosci Rep 35:e00192. https://doi.org/10.1042/BSR20150014
13. Kunutsor SK, Dey RS, Laukkanen JA (2021) Circulating serum copper is associated with atherosclerotic cardiovascular disease, but not venous thromboembolism: a prospective cohort study. Pulse 9:109–115. https://doi.org/10.1159/000519906
14. National Research Council (2000) Copper in drinking water. https://doi.org/10.17226/9782
15. Gulec S, Collins JF (2014) Silencing the Menkes copper-transporting ATPase (Atp7a) gene in rat intestinal epithelial (IEC-6) cells increases iron flux via transcriptional induction of ferroportin 1 (Fpn1). J Nutr 144:12–19. https://doi.org/10.3945/jn.113.183160
16. Ravia JJ, Stephen RM, Ghishan FK, Collins JF (2005) Menkes Copper ATPase (Atp7a) is a novel metal-responsive gene in rat duodenum, and immunoreactive protein is present on brush-border and basolateral membrane domains. J Biol Chem 280:36221–36227. https://doi.org/10.1074/jbc.M506727200
17. Prohaska JR (1986) Genetic diseases of copper metabolism. Clin Physiol Biochem 4:87–93
18. Lenartowicz M, Starzyński RR, Krzeptowski W, Grzmil P, Bednarz A, Ogorek M et al (2014) Haemolysis and perturbations in the systemic iron metabolism of suckling, copper-deficient mosaic mutant mice–an animal model of Menkes disease. PLoS ONE 9:e107641. https://doi.org/10.1371/journal.pone.0107641
19. Chang IJ, Hahn SH (2017) The genetics of Wilson disease. Handb Clin Neurol 142:19–34. https://doi.org/10.1016/B978-0-444-63625-6.00003-3
20. Roy S, McCann CJ, Ralle M, Ray K, Ray J, Lutsenko S, Jayakanthan S (2020) Analysis of Wilson disease mutations revealed that interactionfwes between different ATP7B mutants modify their properties. Sci Rep 10:13487. https://doi.org/10.1038/s41598-020-70366-7

21. Lönnerdal B (2017) Excess iron intake as a factor in growth, infections, and development of infants and young children. Am J Clin Nutr 106:1681S–1687S. https://doi.org/10.3945/ajcn.117.156042
22. Doguer C, Ha JH, Collins JF (2018) Intersection of iron and copper metabolism in the mammalian intestine and liver. Compr Physiol 8:1433–1461. https://doi.org/10.1002/cphy.c170045
23. Collins JF, Prohaska JR, Knutson MD (2010) Metabolic crossroads of iron and copper. Nutr Rev 68:133–147. https://doi.org/10.1111/j.1753-4887.2010.00271.x
24. Mills E, Dong XP, Wang F, Xu H (2010) Mechanisms of brain iron transport: insight into neurodegeneration and CNS disorders. Future Med Chem 2:51–64. https://doi.org/10.4155/fmc.09.140
25. Cassat JE, Skaar EP (2013) Iron in infection and immunity. Cell Host Microbe 13:509–519. https://doi.org/10.1016/j.chom.2013.04.010
26. Gulec S, Collins JF (2014) Molecular mediators governing iron-copper interactions. Annu Rev Nutr 34:95–116. https://doi.org/10.1146/annurev-nutr-071812-161215
27. Fox PL (2003) The copper-iron chronicles: the story of an intimate relationship. Biometals 16:9–40. https://doi.org/10.1023/a:1020799512190
28. Ha JH, Doguer C, Wan X, Flores SR, Collins JF (2016) High-iron consumption impairs growth and causes copper-deficiency anemia in weanling Sprague-Dawley rats. PLoS ONE 11:e0161033. https://doi.org/10.1371/journal.pone.0161033
29. Ha JH, Doguer C, Collins JF (2017) Consumption of a high-iron diet disrupts homeostatic regulation of intestinal copper absorption in adolescent mice. Am J Physiol Gastrointest Liver Physiol 313:G353–G360. https://doi.org/10.1152/ajpgi.00169.2017
30. Prohaska JR, Heller LJ (1982) Mechanical properties of the copper-deficient rat heart. The J Nutr 112:2142–2150. https://doi.org/10.1093/jn/112.11.2142
31. Ganz T (2018) Erythropoietin and iron—a conflicted alliance? Kidney Int 94:851–853. https://doi.org/10.1016/j.kint.2018.07.027
32. Klevay LM (2016) IHD from copper deficiency: a unified theory. Nutr Res Rev 29:172–179. https://doi.org/10.1017/S0954422416000093
33. Ranganathan PN, Lu Y, Jiang L, Kim C, Collins JF (2011) Serum ceruloplasmin protein expression and activity increases in iron-deficient rats and is further enhanced by higher dietary copper intake. Blood 118:3146–3153. https://doi.org/10.1182/blood-2011-05-352112
34. Wang T, Xiang P, Ha JH, Wang X, Doguer C, Flores SR et al (2018) Copper supplementation reverses dietary iron overload-induced pathologies in mice. J Nutr Biochem 59:56–63. https://doi.org/10.1016/j.jnutbio.2018.05.006
35. Ha JH, Doguer C, Flores SR, Wang T, Collins JF (2018) Progressive increases in dietary iron are associated with the emergence of pathologic disturbances of copper homeostasis in growing rats. J Nutr 148:373–378. https://doi.org/10.1093/jn/nxx070
36. Collins JF, Franck CA, Kowdley KV, Ghishan FK (2005) Identification of differentially expressed genes in response to dietary iron deprivation in rat duodenum. Am J Physiol Gastrointest Liver Physiol 288:G964–G971. https://doi.org/10.1152/ajpgi.00489.2004
37. Collins JF, Hu Z, Ranganathan PN, Feng D, Garrick LM, Garrick MD, Browne RW (2008) Induction of arachidonate 12-lipoxygenase (Alox15) in intestine of iron-deficient rats correlates with the production of biologically active lipid mediators. Am J Physiol Gastrointest Liver Physiol 294:G948–G962. https://doi.org/10.1152/ajpgi.00274.2007
38. Reeves PG, Nielsen FH, Fahey GC Jr (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr 123:1939–1951. https://doi.org/10.1093/jn/123.11.1939
39. Reeves PG, Rossow KL, Lindlauf J (1993) Development and testing of the AIN-93 purified diets for rodents: results on growth, kidney calcification and bone mineralization in rats and mice. The J Nutr 123:1923–1931. https://doi.org/10.1093/jn/123.11.1923
40. Zheng J, Lee J, Byun J, Yu D, Ha JH (2023) Partial replacement of high-fat diet with n-3 PUFAs enhanced beef tallow attenuates dyslipidemia and endoplasmic reticulum stress in tunicamycin-injected rats. Front Nutr 10:1155436. https://doi.org/10.3389/fnut.2023.1155436
41. Son HK, Kim BH, Lee J, Park S, Oh CB, Jung S et al (2022) Partial replacement of dietary fat with krill oil or coconut oil alleviates dyslipidemia by partly modulating lipid metabolism in lipopolysaccharide-injected rats on a high-fat diet. Int J Environ Res Public Health 19:843. https://doi.org/10.3390/ijerph19020843
42. Son HK, Xiang H, Park S, Lee J, Lee JJ, Jung S, Ha JH (2021) Partial replacement of dietary fat with polyunsaturated fatty acids attenuates the lipopolysaccharide-induced hepatic inflammation in Sprague-Dawley rats fed a high-fat diet. Int J Environ Res Public Health 18:10986. https://doi.org/10.3390/ijerph182010986
43. Ramsay WNM (1957) The determination of the total iron-binding capacity of serum. Clin Chim Acta 2:221–226. https://doi.org/10.1016/0009-8981(57)90106-7
44. Babson AL, Kleinman NM (1967) A source of error in an autoanalyzer determination of serum iron. Clin Chem 13:163–166
45. Jeong S, Bae S, Yu D, Yang HS, Yang MJ, Lee JH, Ha JH (2023) Dietary intervention with quercetin attenuates diesel exhaust particle-instilled pulmonary inflammation and behavioral abnormalities in mice. J Med Food 26:93–103. https://doi.org/10.1089/jmf.2022.K.0104
46. Lee J, Lee JK, Lee JJ, Park S, Jung S, Ha JH et al (2022) Partial replacement of high-fat diet with beef tallow attenuates dyslipidemia and endoplasmic reticulum stress in db/db mice. J Med Food 25:660–674. https://doi.org/10.1089/jmf.2022.K.0019
47. Lee BK, Xu P, Mageswaran UM, Jeong WS, Engku-Husna EI, Muhammad-Nashriq K et al (2023) Probiotic improves skin oxidation, elasticity, and structural properties in aging rats. Prev Nutr Food Sci 28:293–301. https://doi.org/10.3746/pnf.2023.28.3.293
48. Lee JK, Ha JH, Collins JF (2021) Dietary iron intake in excess of requirements impairs intestinal copper absorption in Sprague Dawley rat dams, causing copper deficiency in suckling pups. Biomedicines 9:338. https://doi.org/10.3390/biomedicines9040338
49. Soliman AT, Sanctis VD, Yassin M, Soliman N (2017) Iron deficiency anemia and glucose metabolism. Acta Biomed 88:112–118. https://doi.org/10.23750/abm.v88i1.6049
50. Fillebeen C, Lam NH, Chow S, Botta A, Sweeney G, Pantopoulos K (2020) Regulatory connections between iron and glucose metabolism. Int J Mol Sci 21:7773. https://doi.org/10.3390/ijms21207773
51. Mithila M, Islam MR, Khan MR, Gazi MS, Hossain SJ (2023) Sonneratia apetala (Buch.-Ham.) fruit extracts ameliorate iron overload and iron-induced oxidative stress in mice. Prev Nutr Food Sci 28:278–284. https://doi.org/10.3746/pnf.2023.28.3.278
52. Wessling-Resnick M (2017) Excess iron: considerations related to development and early growth. Am J Clin Nutr 106:1600S–1605S. https://doi.org/10.3945/ajcn.117.15587
53. Kim SH, Yadav D, Kim SJ, Kim JR, Cho KH (2017) High consumption of iron exacerbates hyperlipidemia, atherosclerosis, and female sterility in zebrafish via acceleration of glycation and degradation of serum lipoproteins. Nutrients 9:690. https://doi.org/10.3390/nu9070690
54. Kim YE, Kim DH, Roh YK, Ju SY, Yoon YJ, Nam GE et al (2016) Relationship between serum ferritin levels and dyslipidemia in Korean adolescents. PLoS ONE 11:e0153167. https://doi.org/10.1371/journal.pone.0153167
55. Alarcon-Corredor OM, Guerrero Y, Ramirez de Fernandez M, D'Jesús I, Burguera M, Burguera JL et al (2004) Effect of copper supplementation on lipid profile of Venezuelan hyperlipemic patients. Arch Latinoam Nutr 54:413–418
56. Fisher AL, Srole DN, Palaskas NJ, Meriwether D, Reddy ST, Ganz T, Nemeth E (2021) Iron loading induces cholesterol synthesis and sensitizes endothelial cells to TNFα-mediated apoptosis. J Biol Chem 297:101156. https://doi.org/10.1016/j.jbc.2021.101156
57. Hayashi H, Hattori A, Tatsumi Y, Hayashi K, Katano Y, Ueyama J et al (2013) Various copper and iron overload patterns in the livers of patients with Wilson disease and idiopathic copper toxicosis. Med Mol Morphol 46:33–140. https://doi.org/10.1007/s00795-013-0015-2
58. Diamond E, Newman J, Schalet R, Lap CJ, Abutaleb AO (2023) Dysregulation of copper metabolism in a patient with acute-on-chronic liver failure worked up for fulminant wilson disease. ACG Case Rep J 10:e01084. https://doi.org/10.14309/crj.0000000000001084
59. Hayashi H, Watanabe K, Inui A, Kato A, Tatsumi Y, Okumura A et al (2019) Alanine aminotransferase as the first test parameter for Wilson's disease. J Clin Transl Hepatol 7:293. https://doi.org/10.14218/JCTH.2019.00042
60. Gulec S, Collins JF (2013) Investigation of iron metabolism in mice expressing a mutant Menke's copper transporting ATPase (Atp7a) protein with diminished activity (Brindled; Mo Br/y). PLoS ONE 8:e66010. https://doi.org/10.1371/journal.pone.0066010

61. Sharp P, Srai SK (2007) Molecular mechanisms involved in intestinal iron absorption. World J Gastroenterol 13:4716. https://doi.org/10.3748/wjg.v13.i35.4716
62. Anderson GJ, Frazer DM (2017) Current understanding of iron homeostasis. Am J Clin Nutr 106(suppl_6):1559S-1566S. https://doi.org/10.3945/ajcn.117.155804
63. Vogt ACS, Arsiwala T, Mohsen M, Vogel M, Manolova V, Bachmann MF (2021) On iron metabolism and its regulation. Int J Mol Sci 22:4591. https://doi.org/10.3390/ijms22094591
64. Sorokin EP, Basty N, Whitcher B, Liu Y, Bell JD, Cohen RL, Thomas EL et al (2022) Analysis of MRI-derived spleen iron in the UK Biobank identifies genetic variation linked to iron homeostasis and hemolysis. Am J Hum Genet 109:1092–1104. https://doi.org/10.1016/j.ajhg.2022.04.013
65. Kautz L, Jung G, Valore EV, Rivella S, Nemeth E, Ganz T (2014) Identification of erythroferrone as an erythroid regulator of iron metabolism. Nat Genet 46:678–684. https://doi.org/10.1038/ng.2996
66. Olivera J, Zhang V, Nemeth E, Ganz T (2023) Erythroferrone exacerbates iron overload and ineffective extramedullary erythropoiesis in a mouse model of β-thalassemia. Blood Adv 7:3339–3349. https://doi.org/10.1182/bloodadvances.2022009307
67. Morgan EH, Oates PS (2002) Mechanisms and regulation of intestinal iron absorption. Blood Cells Mol Dis 29:384–399. https://doi.org/10.1006/bcmd.2002.0578
68. Moschen AR, Wieser V, Tilg H (2012) Dietary factors: major regulators of the gut's microbiota. Gut Liver 6:411. https://doi.org/10.5009/gnl.2012.6.4.411
69. Akimbekov NS, Digel I, Yerezhepov AY, Shardarbek RS, Wu X, Zha J (2022) Nutritional factors influencing microbiota-mediated colonization resistance of the oral cavity: a literature review. Front Nutr 9:1029324. https://doi.org/10.3389/fnut.2022.1029324
70. Rusu IG, Suharoschi R, Vodnar DC, Pop CR, Socaci SA, Vulturar R et al (2020) Iron supplementation influence on the gut microbiota and probiotic intake effect in iron deficiency—a literature-based review. Nutrients 12:1993. https://doi.org/10.3390/nu12071993
71. Adeyanju AA, Kruger J, Taylor JR, Duodu KG (2019) Effects of different souring methods on the protein quality and iron and zinc bioaccessibilities of non-alcoholic beverages from sorghum and amaranth. Int J Food Sci Technol 54:798–809. https://doi.org/10.1111/ijfs.13998
72. Adiki SK, Perla CK, Saha G, Katakam P, Theendra V (2019) Enhancement in iron absorption on intake of chemometrically optimized ratio of probiotic strain *Lactobacillus plantarum* 299v with iron supplement pearl millet. Biol Trace Elem Res 190:150–156. https://doi.org/10.1007/s12011-018-1541-5
73. Gomaa MA, Allam MG, Haridi AA, Eliwa AEM, Darwish AM (2022) High-protein concentrated pro-yogurt (Pro-WPI) enriched with whey protein isolate improved athletic anemia and performance in a placebo-controlled study. Front Nutr 8:788446. https://doi.org/10.3389/fnut.2021.788446
74. Garcés V, Rodríguez-Nogales A, González A, Gálvez N, Rodríguez-Cabezas ME, García-Martin ML et al (2018) Bacteria-carried iron oxide nanoparticles for treatment of anemia. Bioconjug Chem 29:1785–1791. https://doi.org/10.1021/acs.bioconjchem.8b00245
75. Khodaii Z, Zadeh MN, Kamali J, Natanzi MM (2019) Enhanced iron absorption from lactic acid fermented bread (an in vivo/ex vivo study). Gene Rep 15:100389. https://doi.org/10.1016/j.genrep.2019.100389
76. Monteagudo-Mera A, Shalunkhe A, Duhduh A, Walton GE, Gibson GR, Pereira DI et al (2023) Impact of inorganic iron and haem on the human gut microbiota; an in vitro batch-culture approach. Front Microbiol 14:1074637. https://doi.org/10.3389/fmicb.2023.1074637
77. Zimmermann MB, Chassard C, Rohner F, N'goran EK, Nindjin C, Dostal A et al (2010) The effects of iron fortification on the gut microbiota in African children: a randomized controlled trial in Cote d'Ivoire. Am J Clin Nutr 92:1406–1415. https://doi.org/10.3945/ajcn.110.004564
78. Jaeggi T, Kortman GA, Moretti D, Chassard C, Holding P, Dostal A et al (2015) Iron fortification adversely affects the gut microbiome, increases pathogen abundance and induces intestinal inflammation in Kenyan infants. Gut 64:731–742. https://doi.org/10.1136/gutjnl-2014-307720
79. Parmanand BA, Kellingray L, Le Gall G, Basit AW, Fairweather-Tait S, Narbad A (2019) A decrease in iron availability to human gut microbiome reduces the growth of potentially pathogenic gut bacteria; an in vitro colonic fermentation study. J Nutr Biochem 67:20–27. https://doi.org/10.1016/j.jnutbio.2019.01.010

Publisher's Note