Copper- Fructose interaction and NAFLD

Kupffer cell depletion protects against the steatosis, but not the liver damage, induced by marginal copper, high fructose diet in male rats

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ABSTRACT

High fructose feeding impairs copper status and leads to low copper availability, which is a novel mechanism in obesity related fatty liver. Copper deficiency associated hepatic iron overload likely plays an important role in fructose-induced liver injury. Excess iron in the liver is distributed throughout hepatocytes and Kupffer cells (KCs). The aim of this study was to examine the role of KCs in the pathogenesis of nonalcoholic fatty liver disease (NAFLD) induced by a marginal copper high fructose diet (CuMF). Male weanling Sprague-Dawley rats were fed either a copper adequate or a marginally copper deficient diet for 4 weeks. Deionized water or deionized water containing 30% fructose (w/v) was also given ad lib. KCs were depleted by intravenous administration of gadolinium chloride (GdCl₃) before and/or in the middle of the experimental period. Hepatic triglyceride accumulation was completely eliminated with KC depletion in CuMF consumption rats, which was associated with the normalization of elevated plasma monocyte chemoattractant protein-1 (MCP-1) and increased hepatic sterol regulatory element binding protein-1 (SREBP-1) expression. However, hepatic copper and iron content were not significantly affected by KC depletion. In addition, KC depletion reduced body weight and epididymal fat weight as well as adipocyte size. Plasma endotoxin and gut permeability were markedly increased in CuMF rats. Moreover, MCP-1 was robustly increased in the culture medium when isolated KCs from CuMF rats were treated with LPS. Our data suggest that KCs play a critical role in the development of hepatic steatosis induced by marginal copper high fructose diet.

KEYWORDS: Fructose; Copper; Iron; Kupffer cell; Adipose tissue.
GLOSSARY: KC, Kupffer cell; NAFLD, nonalcoholic fatty liver disease; GdCl₃, gadolinium chloride; SREBP-1, sterol regulatory element binding protein-1; MCP-1(CCL2), monocyte chemoattractant protein-1; NASH, nonalcoholic steatohepatitis; IRHIO, insulin resistance-associated hepatic iron overload; AAS, atomic absorption spectrometer; ALT, alanine aminotransferase; AST, aspartate aminotransferase; NEFA, non-esterified fatty acids; Emr1, EGF-like module containing, mucin-like, hormone receptor-like 1; VLDL, very low density lipoprotein; Acacb, acetyl-CoA carboxylase beta; Scd1, stearoyl-Coenzyme A desaturase 1; Fasn, fatty acid synthase; Srebf1, sterol regulatory element binding transcription factor 1; Cpt1a, carnitine palmitoyltransferase 1a, liver; Acadl, acyl-CoA dehydrogenase, long chain; Acadvl, acyl-CoA dehydrogenase, very long chain; Acadm, acyl-CoA dehydrogenase, C-4 to C-12 straight chain; Hmgcs2, 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (mitochondrial); Cpy4a1, Cytochromes P450 4A1; Mttp, microsomal triglyceride transfer protein; Apob, apolipoprotein B; Actb, actin, beta; TNF α, tumor necrosis factor α; IL, interleukin; TLR4, Toll-like receptor 4. ATM, adipose tissue macrophage; WAT, white adipose tissue.
INTRODUCTION

NAFLD is now the most common liver disease in the United States. As of 2011, the prevalence of NAFLD and nonalcoholic steatohepatitis (NASH) was reported to be 46% and 12.2%, respectively, in middle-aged adults in the United States (47). The rapid rise in NAFLD suggests a possible role for environmental factors in the pathogenesis of this disease.

Inadequate copper intake (23, 26, 27) and increased fructose consumption represent two important nutritional problems in the United States (45). Recent studies suggest that high fructose intake may be an important risk factor for the development of NAFLD (3, 33). Decreased copper availability has also been observed in NAFLD patients, and a copper deficient diet induces fatty liver in rodents (1, 2). Moreover, dietary fructose-copper interactions worsen copper status and are associated with the metabolic syndrome (13-15). Our recent research clearly showed that not only high dietary fructose, but also modest fructose consumption worsened copper status in marginally copper deficient rats, and caused liver injury and fat accumulation (39, 40). One potential underlying mechanism for the hepatic abnormalities caused by low copper/high fructose diets is hepatic iron overload (7, 17, 39, 40). The release of iron from macrophages and hepatocytes is a copper-dependent process which involves ceruloplasmin, a copper-dependent ferroxidase (9). In fact, hepatic iron accumulation is observed in up to 34.5% of NAFLD patients (31). The term “insulin resistance-associated hepatic iron overload” (IRHIO) syndrome was coined a decade ago to describe the association between insulin resistance, NASH and iron overload (29).
Maintenance of systemic iron homeostasis involves the balance between iron absorption, storage and recycling. Most of the iron in the body is in the hemoglobin of erythrocytes. Senescent erythrocytes are phagocytosed by macrophages and recycled at a rate of 20-25mg iron per day (20). Hepcidin is the master regulator of iron homeostasis, and it is secreted mainly by hepatocytes in response to iron stores, inflammation and hypoxia (34). It regulates cellular iron release by acting on the iron exporter, ferroportin-1 (12, 32). In addition, efficient iron release from hepatocytes/macrophages and enterocytes also requires ceruloplasmin and hephaestin, respectively, which are multicopper oxidases which oxidize Fe(II) to Fe(III), the form of iron that binds to Apo-transferrin (9). Therefore, copper deficiency may lead to iron accumulation in the liver (22). Excess iron in the liver secondary to copper deficiency is distributed throughout hepatocytes and KCs. Human research suggests that the cellular pattern of hepatic iron deposition is correlated with the histological severity in NAFLD patients (31).

KCs are the resident macrophages of liver, and they play critical roles in host defense and iron recycling. However, the exact role of KCs in the pathogenesis of NAFLD is unclear. Our previous studies clearly documented increased hepatic iron deposition in the CuMF rat model (39, 40). However, the iron deposition pattern and the role of iron deposition in different cell types have not been defined in an animal model of NAFLD. The present study was conducted to examine the role of KCs in the development of NAFLD and the role of cell type specific iron deposition in disease progression.
MATERIALS AND METHODS

Animal experiments. Male weanling Sprague-Dawley rats (35-45g) from the Harlan Laboratories (Indianapolis, IN) were fed (ad lib) a purified AIN-76 diet with a defined copper content. The copper adequate rats received 6 mg/kg copper, and the marginal copper rats received 1.6 mg/kg of copper for 4 weeks to achieve marginal copper deficiency. The animals were housed in stainless steel cages in a temperature and humidity controlled room with a 12:12h light-dark cycle. Animals had free access to either deionized water or deionized water containing 30% fructose (w/v). Fructose enriched drinking water was changed twice a week. KCs were depleted by tail vein injection of GdCl₃ (10mg/kg body weight) twice a week before (Pre-GdCl₃) or after 2 weeks (Post-GdCl₃) of CuMF diet (35). In the Pre-GdCl₃ group, animals received GdCl₃ or saline solution injection three times prior to the beginning of experimental diet (CuMF) at 72h, 48h, 24h; then followed by twice weekly injections until the end of the experiment (total 4 weeks). In the Post-GdCl₃ group, animals received GdCl₃ injection twice weekly starting from two weeks exposure to CuMF diet until the end of the experiment (total 2 weeks). After fasting overnight, all the animals were killed under anesthesia with pentobarbital (50mg/kg I.P. injection). Blood was collected from the inferior vena cava, and citrated plasma was stored at -80°C for further analysis. Portions of liver tissue were fixed with 10% formalin for subsequent sectioning, while others were snap-frozen with liquid nitrogen. All studies were approved by the University of Louisville Institutional Animal Care and Use Committee, which is certified by the American Association of Accreditation of Laboratory Animal Care.
Hepatocyte and KC isolation and culture. Primary hepatocytes were isolated from experimental rats according to a modified two-step collagenase perfusion method (38). KCs were isolated by Percoll gradient centrifugation as described previously (38). The purity of KCs was 80%. KCs were suspended with RPMI1640 containing 5 U/ml penicillin and 50 μg/ml streptomycin, supplemented with 10% FBS at 37 °C in a humidified O2/CO2 (19:1) atmosphere, and seeded into 24-well plate at a density of 1×10^6/well.

Assessment of copper and iron status. Plasma ceruloplasmin was measured on the basis of its oxidase activity (37). Plasma copper and liver copper were measured by Varian SpectrAA 880/GTA-100 graphite furnace atomic absorption spectrometer (AAS) (Worcester Polytechnic Institute, Worcester, MA). Liver iron concentration was measured by iCE3000 series flame AAS (Thermo Fisher Scientific, Waltham, MA).

Hepatic triglyceride assay. Liver tissues were homogenized in ice-cold phosphate buffered saline. Hepatic total lipids were extracted with chloroform/methanol (2:1) according to the method described by Bligh and Dyer (6). The lipid in chloroform was dried and redissolved in 2% Triton X-100 in water. Hepatic triglyceride content was determined by commercially available kits (Infinity, Thermo Electron, Melbourne, Australia).

Liver enzyme and plasma biochemical assays. Liver enzyme and the plasma biochemical assays were performed with commercially available kits: AST, glucose, cholesterol, triglyceride (Infinity, Thermo Electron, Melbourne, Australia), non-esterified fatty acids (NEFA) (Wako Chemicals, Richmond, VA), insulin (Lino Research, St.
Charles, MO), hepcidin (Uscn Life Science Inc. Houston, TX), and MCP-1 (Invitrogen Corporation, Camarillo, CA).

**Determination of gut permeability and plasma endotoxin.** For ex vivo detection of intestinal permeability, a freshly isolated 10 cm section of ileum was rinsed with modified Krebs-Henseleit bicarbonate buffer (KHBB, pH 7.4). 100 µl FITC-dextran (molecular weight 4,000, FD-4, 40 mg/ml) was injected into the lumen before the gut was ligated to form a sac, as described previously (48). The gut sac was then placed in KHBB and incubated at 37°C for 20 min. The FD-4 that penetrated from the lumen into the incubation buffer was measured spectrofluorometrically with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The FD-4 permeability was expressed as micrograms per centimeter per minute. Plasma samples were diluted 2-fold in pyrogen-free water and heated at 75°C for 10 min and cooled down to room temperature (48). Endotoxin was measured using the limulus amebocyte lysate kit (Lonza Walkersville, MD) according to the manufacturer’s instructions.

**Histology and immunohistochemistry.** Formalin-fixed, paraffin-embedded liver and adipose tissue sections were cut at 5 µm thickness using a routine procedure. Sections were stained with hematoxylin and eosin (H&E). Adipocyte size was measured using Image J software (http://rsb.info.nih.gov/ij/). For immunohistochemical analysis, sections were incubated with anti-CD68 antibody (AbD Serotec, Raleigh, NC) overnight at 4°C. Staining was visualized using the horseradish peroxidase-conjugated DAKO staining system (DAKO InVision, Carpenteria, CA).

**Isolation of RNA and real time RT-PCR.** Total RNA was extracted from liver and adipose tissues using TRIZOL (Invitrogen, Carlsbad, CA). Primers were designed using
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Primer-BLAST except acetyl-CoA carboxylase beta (Acacb, PPR49745F) (SABiosciences, Valencia, CA) (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) (Table1). Real-time PCR was performed with an ABI prism 7500 sequence detection system and SYBR green I dye reagents. The relative gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method.

Western blot. Equal amounts of protein extracted from liver homogenate were loaded and resolved on 4%-15% SDS-polyacrylamide gels, and transferred to PVDF membrane (Millipore, Bedford, MA). The membrane was blocked and probed with primary antibody for SREBP1 (Santa Cruz Biotechnology, INC., Santa Cruz, CA) overnight at 4°C, and incubated with the corresponding horseradish peroxidase-conjugated secondary antibody. Protein signals were visualized using the enhanced chemiluminescence system (Amersham Biosciences, Little Chalfont, UK). Band intensities were quantified using Image J software (http://rsb.info.nih.gov/ij/).

Statistical analysis. All data were expressed as mean ± SD (Standard Deviation) and analyzed by one-way analysis of variance (ANOVA) followed by Newman Keuls’ Multiple Comparison Test and Student’s $t$-test. The interactions between copper and fructose were examined by two-way ANOVA. Differences at $P \leq 0.05$ were considered to be statistically significant.

RESULTS

Dietary marginal copper deficiency and high fructose feeding induces liver injury and fat accumulation. As expected, rats fed on the CuMF diet developed obvious liver injury and fat accumulation at 4 weeks, as shown by elevated plasma AST (Fig. 1A), increased hepatic triglyceride level (Fig. 1B), and histology (H&E staining) (Fig. 1C).
These findings are consistent with our previous data, and further confirmed our previous results (39).

*KC depletion completely eliminates hepatic fat accumulation, but not liver injury, in CuMF rats.* CuMF-induced fat accumulation was characterized mainly by macrovesicular steatosis and located around the portal areas as shown by H&E. The increased hepatic fat accumulation in CuMF rats was not apparent with KC depletion (Fig. 2A). Consistent with histologic findings, the hepatic triglyceride level was significantly increased in CuMF rats compared to controls. KC depletion, either before or in the middle of the CuMF protocol, completely prevented or resolved fat accumulation in the liver (Fig. 2B).

Liver injury was assessed by plasma levels of liver enzymes (ALT and AST). While ALT level was not significantly changed in CuMF rats compared to controls (data not shown), AST level was significantly increased. Moreover, this increase was not attenuated by KC depletion (Fig. 2C).

*Hepatic expression of KC marker and proinflammatory genes with KC depletion.* The expression of hepatic KC marker genes, Emr1 (F4/80), CD68 and CD163 were each significantly decreased (by ≈50%, 55%, and 35%, respectively) with GdCl3 injection prior to fructose feeding (Pre-GdCl3). A similar effect was also observed at the later time point (Post-GdCl3), suggesting the successful depletion of KCs both before and after/during fructose feeding (Fig. 3A). It was further confirmed by markedly decreased hepatic CD68 expression with KC depletion, as shown by immunohistochemical staining (Fig. 3B). TNF-α and Il-1β mRNA expression were significantly down-regulated with KC depletion in both Pre-GdCl3 and Post-GdCl3 group. TLR4 mRNA was markedly down-regulated in Post-GdCl3 group (Fig. 3C).
Effects of KC depletion on body weight, liver weight, epididymal fat weight and plasma metabolic indices. After 4 weeks exposure to dietary CuMF, rats in Pre-GdCl₃ group showed significantly lower body weight and body weight gain compared to controls. While body weight and weight gain were not significantly reduced in rats from the Post-GdCl₃ group compared to controls, these variables were significantly higher in the Post-GdCl₃ animals than in the Pre-GdCl₃ group. Liver weight was significantly decreased with KC depletion in rats from Pre-GdCl₃ group compared to control. However, liver/body weight ratio did not differ significantly. KC depletion also led to a significant decrease of epididymal fat weight as well as epididymal fat weight/body weight ratio compared to controls. Although the difference between groups in plasma indices did not reach the statistical significance, there is a trend toward decreased plasma triglyceride, glucose and HOMA-IR in Pre-GdCl₃ group as compared to controls (Table 2).

KC depletion reduces adipocyte size with altered adipose tissue proinflammatory profile in epididymal fat. As expected, intravenous injection of GdCl₃ successfully depleted hepatic KCs, whereas adipose tissue macrophages were not significantly affected, as shown by the macrophage marker gene expression (Fig. 4A). Mean epididymal adipocyte size was significantly smaller in the two groups with KC depletion, with the more profound effect seen in the Pre-GdCl₃ group (Fig. 4B). While some of the proinflammatory cytokine gene expression was down-regulated (IL-6) or had a trend toward being decreased (CCL2), however, it seems that the adipose tissue inflammation was not markedly improved with KC depletion, at least in the limited time period, from the profile of proinflammatory gene expression (Fig. 4C).
Effects of KC depletion on copper and iron status. As expected, the plasma ceruloplasmin activity, as well as the plasma and liver copper levels, were significantly decreased in the CuMF rats compared to controls. KC depletion with GdCl₃ slightly decreased the plasma ceruloplasmin activity, as well as the plasma and liver copper levels. However, the differences did not reach the statistical significance (Fig. 5A). Moreover, liver iron was significantly increased and plasma iron was significantly decreased in the CuMF rats, and they were not modified by KC depletion (Fig. 5B).

Increased hepatocyte iron and decreased spleen iron is associated with plasma hepcidin deficiency in CuMF rats. KC depletion did not affect iron content in the whole liver tissue. To further determine the iron level in each cell type, we isolated hepatocytes and KCs from CuMF treated rats. Hepatocyte iron was markedly increased (Fig. 6A), and spleen iron was significantly decreased in CuMF rats (Fig. 6C), suggesting iron redistribution from spleen to hepatocytes after exposure to CuMF diet. However, we did not detect a significant change in iron levels in KCs from CuMF rats compared to control, at least at this time point (Fig. 6B). Plasma hepcidin, an antimicrobial small peptide which regulates iron homeostasis, was significantly decreased in CuMF rats (Fig. 6D).

Effect of KC depletion on hepatic expression of genes involved in lipid metabolism and SREBP-1 protein. To further explore the mechanism(s) by which KC depletion prevented hepatic fat accumulation, hepatic expression of genes involved in fatty acid synthesis, fatty acid oxidation and very low density lipoprotein (VLDL) secretion were determined by real time RT-PCR. Among the multiple genes related to fatty acid oxidation, most genes regulate mitochondrial β-oxidation; only AcadVL and Cyp4a1 genes regulate peroxisomal and microsomal oxidation, respectively. While most genes
related to fatty acid oxidation were suppressed in CuMF rats as well as in CuMF rats with KC depletion, some (Hmgcs2 and Cyp4a1) were further down-regulated with KC depletion (Fig. 7A). Srebp1 gene expression was not significantly changed in CuMF rats with or without KC depletion. While fasn gene expression was not changed with diet, it was significantly up-regulated by KC depletion in CuMF rats. Scd1 expression was inhibited in CuMF rats, and it was further down-regulated by KC depletion (Fig. 7B).

The expression of genes involved in VLDL secretion was also suppressed in CuMF rats as well as in CuMF rats with KC depletion. Moreover, ApoB expression was further down-regulated with KC depletion (Fig. 7C). SREBP-1 is a critical transcriptional factor in hepatic lipogenesis. As shown in Fig. 7D, CuMF feeding led to an obvious increase of mature SREBP-1 compared to controls and this increase was blocked with KC depletion.

**Increased gut permeability in CuMF rats.** Gut permeability was evaluated by plasma endotoxin level and *ex vivo* measurement of ileum permeability to FD-4. As shown in Fig. 8A, plasma endotoxin was significantly increased in CuMF rats. Consistent with plasma endotoxin level, FD-4 permeability of ileum was also markedly increased in CuMF rats compared to control (Fig. 8B).

**KC depletion prevents elevated plasma MCP-1 in CuMF rats.** Plasma MCP-1 level was significantly increased in CuMF rats. However, this increase was blocked by KC depletion, suggesting KCs may directly or indirectly contribute to the increased plasma MCP-1 in CuMF rats (Fig. 9A).

**KC MCP-1 Production in Response to LPS and Iron Chelation.** To further determine KC function in response to LPS and the role of iron in this response, freshly isolated KCs from CuMF rats were treated with LPS in the presence/absence of an iron chelator. MCP-
levels were predictably increased in response to LPS. Interestingly, this increase was significantly attenuated by pretreatment with an intracellular lysosomal iron chelator, suggesting that the MCP-1 signaling pathway was mediated, at least partially, by intracellular iron (Fig. 9B).

DISCUSSION

KCs are the resident macrophages in the liver, and they are a critical component of the innate immune response. In addition, KCs play a role in iron recycling. However, the role of KCs in the pathogenesis of NAFLD remains poorly understood. In the current study, we found that KC depletion completely blocked hepatic fat accumulation in CuMF rats, but did not block the liver injury, suggesting that KCs play a pivotal role in hepatic lipid metabolism. Particularly, we found that KC depletion reduced body weight and epididymal fat weight as well as adipocyte size, predominantly in the Pre-GdCl₃ group. Moreover, elevated plasma MCP-1 was inhibited with KC depletion in CuMF rats, suggesting that KCs may directly or indirectly contribute to the elevated levels of circulating MCP-1.

It is well documented that hepatic iron overload is a common feature associated with copper deficiency (17, 22). Excess iron in the liver was diffusely distributed throughout hepatocytes and KCs (22). To further dissect the differential role of iron deposition in KCs and hepatocytes in CuMF induced liver injury and fat accumulation, we depleted KCs before or in the middle of feeding rats a CuMF diet. Interestingly, we found that while fat accumulation was completely prevented with KC depletion at both time points, hepatic copper and iron were not significantly affected by KC depletion. Next, we
isolated KCs and hepatocytes and measured iron content. We did not detect significantly increased iron in the KCs of CuMF rats, at least at the time of sacrifice, compared to controls, probably due to the limited time period of the experiment. Although the KC iron level was not increased in rats fed with CuMF diet for 4 weeks, the plasma endotoxin level was significantly increased. In fact, previous studies have shown that high fructose feeding may lead to bacterial overgrowth and increased gut permeability, with subsequent endotoxemia, which may contribute to KC activation and MCP-1 secretion (5, 41). To further demonstrate this, isolated KCs from CuMF rats were exposed to exogenous LPS in the presence or absence of an iron chelator. We found that MCP-1 was dramatically increased in the culture medium in response to LPS, and this increase was significantly attenuated by pretreatment with an iron chelator, suggesting that KCs may be one of the sources of elevated circulating MCP-1, and that LPS induced MCP-1 secretion is, at least partially, iron dependent.

While KC iron content was not significantly affected, hepatocyte iron was significantly increased, and spleen iron was markedly decreased in CuMF rats compared to controls. Moreover, plasma hepcidin, the master regulator of iron homeostasis, was significantly decreased in CuMF rats, suggesting that iron was redistributed under the condition of hepcidin deficiency (19, 21). Gender differences in the metabolic effects of fructose and/or copper deficiency have long been noted in the animal studies (16, 18) as well as in humans (4, 10), with male gender being sensitive to the deleterious effects of fructose and/or copper deficiency, and female gender being protective. Hepatic iron overload is a likely mechanism underlying fructose/copper deficiency-induced metabolic
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syndrome. It is also well-documented that the expression of hepcidin is suppressed by testosterone (28). Taken together, gender-related discrepancy in the metabolic effects of fructose might attribute to the dysregulated metabolism of copper and/or iron. Further studies on the potential molecular mechanisms are warranted.

A growing body of evidence has suggested that KCs may promote the development of diet-induced hepatic steatosis by suppression of hepatocyte fatty acid β-oxidation via secreting TNF-α and IL-1β (24, 42), or they may trigger NASH development via recruitment of monocytes/macrophages through MCP-1(30, 44). In the current study, we found that KC depletion not only protects against the development of NAFLD, but also reduces total body weight as well as white adipose tissue fat mass and weight. Moreover, KC depletion was associated with the normalization of plasma MCP-1 and hepatic SREBP-1 expression, both of which play important roles in obesity-related hepatic steatosis and insulin resistance. The role of MCP-1 in the onset of metabolic disorders is attributed to macrophage recruitment in either adipose tissue (25) or liver (30, 44). In agreement with this, our data showed that hepatic TNF-α and IL-1β, as well as TLR4, mRNA were significantly down-regulated by KC depletion, which is consistent with previous findings (24, 42). In adipose tissue, IL-6 mRNA, but not TNF-α mRNA, was markedly down-regulated. IL-6 is mainly produced from adipocytes, and adipose tissue macrophage (ATM) is the major source of TNF-α (36, 43, 46). Both of these factors support the concept that the alteration of adipose tissue proinflammatory profile is likely due to the reduced adipocyte size. Inhibition of hepatic SREBP-1 expression by KC
depletion suggests that hepatic lipogenesis may be regulated by KCs. However, how KC regulate SREBP-1 expression remains to be determined.

The fact that KC depletion leads to hepatic steatosis but not liver injury supports the concept that metabolic endotoxemia is the initiator of hepatic steatosis and obesity (“first hit”) (8, 11). However, low copper status and hepatic iron overload were not improved by KC depletion, suggesting they might be the “second hit” leading to the liver injury and NASH progression (11).

In summary, dietary fructose and marginal copper deficiency interaction further impaired copper status and led to iron redistribution with increased deposition in hepatocytes and decreased levels in the spleen under the condition of hepcidin deficiency (21). KC depletion prevented hepatic steatosis with reduced white adipose tissue fat mass, suggesting a critical role of KCs in the onset of hepatic steatosis and adiposity induced by marginal copper and high fructose diet (Fig. 10). However, copper and iron status were not improved by KC depletion, suggesting the possible link between low copper, iron overload and liver injury. Our data provided novel insights into the better understanding of NAFLD.
**Figure Legends**

**Fig.1.** Effect of CuMF feeding on liver injury and fat accumulation. (A) Plasma AST. (B) Hepatic triglyceride. (C) Representative photomicrographs of the H&E staining of liver section (200×). Data represent means ± SD (n=5-8). * versus CuA; ‡ versus CuM; § versus CuAF (p<0.05). †, interaction between copper and fructose is significant (p<0.05, two-way ANOVA). CuA, adequate copper diet; CuM, marginal copper deficient diet; CuAF, adequate copper diet+30% fructose drinking; CuMF, marginal copper deficient diet+30% fructose drinking. H&E, hematoxylin and eosin.

**Fig.2.** Effect of KC depletion on the CuMF-induced hepatic fat accumulation and liver injury. (A) Representative photomicrographs of the H&E staining of liver section (200×). (B) Hepatic triglyceride. (C) Plasma AST. Data represent means ± SD (n=6-8). * versus CuMF; § versus CuMF+saline (p<0.05, one-way ANOVA). CuMF, marginal copper deficient diet+30% fructose drinking; GdCl₃, gadolinium chloride. Pre-GdCl₃, GdCl₃ injection before CuMF; Post-GdCl₃, GdCl₃ injection after 2 weeks of CuMF; H&E, hematoxylin and eosin.

**Fig.3.** Effect of KC depletion on KC markers and proinflammatory gene expression in liver. (A) Liver Emr1 (F4/80), CD68, CD163 mRNA expression. (B) Representative photomicrographs of immunohistochemical staining for rat liver CD68 (100×). (C) Proinflammatory gene expression. Data represent means ± SD (n=6-8). * versus CuMF+saline; § versus Pre-GdCl₃ (p<0.05, one-way ANOVA). CuMF, marginal copper deficient diet+30% fructose drinking; GdCl₃, gadolinium chloride. Pre-GdCl₃, GdCl₃ injection before CuMF; Post-GdCl₃, GdCl₃ injection after 2 weeks of CuMF.
Fig. 4. Effect of KC depletion on macrophage markers, adipocyte size and proinflammatory gene expression in white adipose tissue. (A) Emr1 (F4/80), CD68, CD163 mRNA expression. (B) Representative photomicrographs of the H&E staining of epididymal fat section (100×) and measurement of adipocyte size. Adipocyte size (µm²) was measured and average cell size of >100 cells for each group was calculated. (C) Proinflammatory gene expression. Data represent means ± SD (n=6-8). * versus CuMF+saline; $ versus Pre-GdCl₃ (p<0.05, one-way ANOVA). CuMF, marginal copper deficient diet+30% fructose drinking; GdCl₃, gadolinium chloride; Pre-GdCl₃, GdCl₃ injection before CuMF; Post-GdCl₃, GdCl₃ injection after 2 weeks of CuMF; WAT, white adipose tissue.

Fig. 5. Effect of KC depletion on copper and iron status. (A) Plasma ceruloplasmin, copper and liver copper. (B) Plasma iron and liver iron. Data represent means ± SD (n=6-8). * versus CuA (p<0.05, one-way ANOVA). CuA, adequate copper diet; CuMF, marginal copper deficient diet+30% fructose drinking; GdCl₃, gadolinium chloride. Pre-GdCl₃, GdCl₃ injection before CuMF; Post-GdCl₃, GdCl₃ injection after 2 weeks of CuMF.

Fig. 6. Iron content in hepatocytes, KCs, spleen and plasma hepcidin. (A) Hepatocyte iron. (B) KC iron. (C) Spleen iron. (D) Plasma hepcidin. Data represent means ± SD (n=6-8). *p≤0.05, Student’s t-test. AAS, atomic absorption spectrometer; CuA, adequate copper diet; CuMF, marginal copper deficient diet+30% fructose drinking.

Fig. 7. Effect of KC depletion on hepatic gene expression of lipid metabolism and SREBP-1 protein expression. (A) Fatty acid oxidation. (B) Fatty acid synthesis. (C) VLDL secretion. Data represent means ± SD (n=6-8). * versus CuA; # versus CuMF;
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$ vs CuMF+saline (p<0.05). (D) Hepatic SREBP-1 expression was determined by Western Blots. Optical density of the band was quantified by ImageJ software. The ratio to β-actin was calculated by assigning the value from adequate copper diet controls as one. Data represent means ± SD (n=3). * vs CuA; # vs CuMF; $ vs CuMF+saline (p<0.05, one-way ANOVA). CuMF, marginal copper deficient diet+30% fructose drinking; GdCl₃, gadolinium chloride. Pre-GdCl₃, GdCl₃ injection before CuMF; Post-GdCl₃, GdCl₃ injection after 2 weeks of CuMF; SREBP-1, sterol regulatory element binding protein 1.

Fig. 8. Plasma endotoxin and gut permeability. (A) Plasma endotoxin. Endotoxin was measured by the limulus ameobocyte lysate (LAL) kit. (B) Gut permeability. The penetration of intraluminal FD-4 to the incubation buffer was determined after incubation of ileum sac for 20 min. Data represent means ± SD (n=5-7). *p<0.05, Student's t-test.

Fig. 9. Plasma MCP-1 and KCs MCP-1 production in response to LPS and iron chelation. (A) Plasma MCP-1. Data represent means ± SD (n=5-7). * vs CuA; # vs CuMF (p<0.05, one-way ANOVA). (B) MCP-1 in KCs culture medium. KCs were seeded into 24-well plate (as described in Materials and Methods) and pretreated with L1 or DFO (iron chelators) for 20 hours, then treated with LPS 300ng/ml for 24 hours (This experiment was repeated three times). MCP-1 in the culture medium and plasma was determined by ELISA. * vs Control; # vs LPS (p<0.05, one-way ANOVA). CuA, adequate copper diet; CuMF, marginal copper deficient diet+30% fructose drinking. L1, Deferiprone; DFO, Deferoxamine.
Fig.10. Schematic diagram of the critical role of KC in marginal copper high fructose diet-induced hepatic steatosis. Dashed arrows denote mechanisms that have not been fully defined. CuMF, marginal copper deficient diet+30% fructose drinking.
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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.
REFERENCES


44. Tosello-Trampont AC, Landes SG, Nguyen V, Novobrantseva TI, and Hahn YS. Kupffer cells trigger nonalcoholic steatohepatitis development in diet-induced
Copper- Fructose interaction and NAFLD


### Table 1. Primers used for real-time PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence(5’-3’)</th>
<th>Refseq No.</th>
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<tr>
<td>Emr1</td>
<td>Forward: CATCCAGCAGATGGGAATTG</td>
<td>NM_001007557.1</td>
</tr>
<tr>
<td></td>
<td>Reverse: CTCCCAAGGGTGTGGTACA</td>
<td></td>
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<tr>
<td>CD68</td>
<td>Forward: ACCTTTGGATTCAAACAGGAC</td>
<td>NM_001031638.1</td>
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<tr>
<td></td>
<td>Reverse: GCTGAGAAATGTCCACTGTGC</td>
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<tr>
<td>CD163</td>
<td>Forward: GGCATGCAATGGAAATGAGT</td>
<td>NM_001107887.1</td>
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<tr>
<td></td>
<td>Reverse: TCAGATCGCCTCCGTCTAAG</td>
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<tr>
<td>TNF-α</td>
<td>Forward: ACTGAACTTGCGGGTGATCG</td>
<td>NM_012675.3</td>
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<tr>
<td></td>
<td>Reverse: TTTGCTACGACGTTGGCTAC</td>
<td></td>
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<tr>
<td>Il1b</td>
<td>Forward: AGCACTTTCCGACAGTGAGG</td>
<td>NM_031512.2</td>
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<tr>
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<tr>
<td>Ccl2</td>
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<td>NM_031530.1</td>
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<td>Reverse: TGCCATGCCCTTGTCTTCA</td>
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<td>Scd1</td>
<td>Forward: CCAAGAGATCTCCAGTCTACA</td>
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<td>Srebf1</td>
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<td>Cpt1a</td>
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<td>Hmgcs2</td>
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<td>NM_173094.2</td>
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<td>Reverse: GATCCTATGGGGTCTGTG</td>
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<td>Gene</td>
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<td>Reverse</td>
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<td>-------</td>
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<td>Cpy4a1</td>
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<tr>
<td>Mttp</td>
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</tr>
<tr>
<td>Apob</td>
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<td>ACGTACTTCCGGAGGTGCTTGGGA</td>
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<tr>
<td>Actb</td>
<td>GCGCAAGTACTCTGTGTGGGA</td>
<td>ACATCTGCTGGGAAGGTTGGAC</td>
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</table>
Table 2. Effects of KCs depletion on body weight, liver weight, epididymal fat weight and plasma indices

<table>
<thead>
<tr>
<th>Variable</th>
<th>CuMF</th>
<th>Saline</th>
<th>Pre-GdCl₃</th>
<th>Post-GdCl₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (BW, g)</td>
<td>242.3 ± 15.8</td>
<td>236.5 ± 22.6</td>
<td>198.7 ± 9.0*#</td>
<td>225.3 ± 15.2$</td>
</tr>
<tr>
<td>BW Gain (g)</td>
<td>190.0 ± 14.5</td>
<td>185.6 ± 21.1</td>
<td>149.5 ± 7.7*#</td>
<td>175.4 ± 13.5$</td>
</tr>
<tr>
<td>Liver Weight (g)</td>
<td>10.24 ± 1.31</td>
<td>9.58 ± 1.16</td>
<td>8.14 ± 0.71*</td>
<td>9.36 ± 1.06</td>
</tr>
<tr>
<td>Liver/BW Ratio (%)</td>
<td>4.22 ± 0.35</td>
<td>4.06 ± 0.35</td>
<td>4.09 ± 0.26</td>
<td>4.15 ± 0.30</td>
</tr>
<tr>
<td>Epididymal fat weight (EFW, g)</td>
<td>3.00 ± 0.51</td>
<td>2.53 ± 0.31</td>
<td>1.80 ± 0.31*#</td>
<td>2.06 ± 0.57*</td>
</tr>
<tr>
<td>EFW/BW Ratio (%)</td>
<td>1.23 ± 0.14</td>
<td>1.07 ± 0.10</td>
<td>0.91 ± 0.16*</td>
<td>0.91 ± 0.21*</td>
</tr>
<tr>
<td>Plasma Indices</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>43.2 ± 9.3</td>
<td>43.5 ± 15.1</td>
<td>32.7 ± 7.3</td>
<td>38.2 ± 13.4</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>43.7 ± 7.4</td>
<td>41.6 ± 10.6</td>
<td>44.8 ± 11.9</td>
<td>59.9 ± 15.5</td>
</tr>
<tr>
<td>NEFA (µM)</td>
<td>288.0 ± 63.3</td>
<td>341.7 ± 94.9</td>
<td>387.0 ± 86.6</td>
<td>294.2 ± 61.1</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>110.0 ± 10.9</td>
<td>104.0 ± 24.4</td>
<td>87.7 ± 20.0</td>
<td>106.4 ± 11.2</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>0.52 ± 0.13</td>
<td>0.48 ± 0.05</td>
<td>0.55 ± 0.17</td>
<td>0.51 ± 0.07</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>3.22 ± 0.51</td>
<td>2.98 ± 0.40</td>
<td>2.75 ± 0.49</td>
<td>3.27 ± 0.43</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SD (n=6-8). * versus CuMF; # versus CuMF+Saline; $ versus CuMF+Pre-GdCl3 (p<0.05). CuMF, marginal copper deficient diet +30% fructose drinking. GdCl₃, gadolinium chloride; Pre-GdCl₃, GdCl₃ start before the experimental diet; Post-GdCl₃, GdCl₃ start from 2 weeks of the experimental diet; BW, Body weight; EFW, Epididymal fat weight; NEFA, non-esterified fatty acid.
Figure 1

(A) AST (U/L) for CuA, CuM, CuAF, and CuMF.
(B) Triglyceride (mg/g liver tissue) for CuA, CuM, CuAF, and CuMF.
(C) H&E stained images at 200x magnification for CuA, CuM, CuAF, and CuMF.
Figure 3

A

Liver Emr1 mRNA (fold change)

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Pre-GdCl₃</th>
<th>Post-GdCl₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuMF</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Liver CD68 mRNA (fold change)

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Pre-GdCl₃</th>
<th>Post-GdCl₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuMF</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

Liver CD163 mRNA (fold change)

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Pre-GdCl₃</th>
<th>Post-GdCl₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuMF</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

B

CD68 X 100

Saline

Pre-GdCl₃

Post-GdCl₃

CuMF
Figure 3

C

![Bar charts showing gene expression levels](image)

- **Liver NFkB mRNA (fold change)**
- **Liver IL-1β mRNA (fold change)**
- **Liver TNF-α mRNA (fold change)**
- **Liver CCL2 mRNA (fold change)**

Comparing Saline, Pre-GdCl₃, and Post-GdCl₃ groups with CuMF treatment.
Figure 4

A

**WAT Emr1 mRNA (fold change)**

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Pre-GdCl₃</th>
<th>Post-GdCl₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuMF</td>
<td><img src="chart1.png" alt="Bar chart" /></td>
<td><img src="chart2.png" alt="Bar chart" /></td>
<td><img src="chart3.png" alt="Bar chart" /></td>
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</table>

**WAT CD68 mRNA (fold change)**

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Pre-GdCl₃</th>
<th>Post-GdCl₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuMF</td>
<td><img src="chart4.png" alt="Bar chart" /></td>
<td><img src="chart5.png" alt="Bar chart" /></td>
<td><img src="chart6.png" alt="Bar chart" /></td>
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</tbody>
</table>

**WAT CD163 mRNA (fold change)**

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Pre-GdCl₃</th>
<th>Post-GdCl₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuMF</td>
<td><img src="chart7.png" alt="Bar chart" /></td>
<td><img src="chart8.png" alt="Bar chart" /></td>
<td><img src="chart9.png" alt="Bar chart" /></td>
</tr>
</tbody>
</table>

B

**H&E X 100**

Saline | Pre-GdCl₃ | Post-GdCl₃

**Adipocyte size (µm²)**

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Pre-GdCl₃</th>
<th>Post-GdCl₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuMF</td>
<td><img src="chart10.png" alt="Bar chart" /></td>
<td><img src="chart11.png" alt="Bar chart" /></td>
<td><img src="chart12.png" alt="Bar chart" /></td>
</tr>
</tbody>
</table>

* p < 0.05 compared to Saline

* p < 0.05 compared to Pre-GdCl₃
Figure 4

C

- **WAT IL-1β mRNA (fold change)**
  - Saline
  - Pre-GdCl₃
  - Post-GdCl₃

- **WAT CCL2 mRNA (fold change)**
  - Saline
  - Pre-GdCl₃
  - Post-GdCl₃

- **WAT IL-6 mRNA (fold change)**
  - Saline
  - Pre-GdCl₃
  - Post-GdCl₃

*Note: CuMF indicates the presence of a specific condition or treatment.*
Figure 5

A

B

Plasma Ceruloplasmin (U/L)

Plasma Copper (μg/dL)

Liver Copper (μg/g dry weight)

Plasma Iron (μg/mL)

Liver Iron (μg/g dry weight)

CuA, CuMF, Saline Pre-GdCl₃, Post-GdCl₃, CuMF
Figure 6

A

Hepatocyte Iron (μg/mg protein)

CuA  CuMF

B

Kupffer Cell Iron (μg/mg protein)

CuA  CuMF

C

Spleen Iron (μg/g dry weight)

CuA  CuMF

D

Plasma Hepcidin (ng/mL)

CuA  CuMF
Figure 7

A

mRNA (fold change)

CuA
CuMF
CuMF+Saline
CuMF+Pre-GdCl3
CuMF+Post-GdCl3

Cpt1a
AcadL
Acadm
AcadVL
Hmgcs2
Acacb
CYP4a1

B

mRNA (fold change)

Srebf1
Fasn
Scd1

C

mRNA (fold change)

Mttp
ApoB

Legend:

* p < 0.05 vs. CuA
** p < 0.01 vs. CuA
*** p < 0.001 vs. CuA
### p < 0.001 vs. CuMF
#### p < 0.001 vs. CuMF

Note: The figure shows the mRNA expression levels of various genes under different conditions.
Figure 7

D

<table>
<thead>
<tr>
<th></th>
<th>CuA</th>
<th>CuMF</th>
<th>Saline</th>
<th>Pre-GdCl₃</th>
<th>Post-GdCl₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>mSrebp1/β-actin</td>
<td>1.00 ± 0.35</td>
<td>1.73 ± 0.46</td>
<td>2.03 ± 0.45*</td>
<td>0.82 ± 0.54$</td>
<td>0.51 ± 0.47#$</td>
</tr>
</tbody>
</table>
Figure 9

A

Plasma MCP-1 (pg/mL)

- CuA
- CuMF
- Saline
- Pre-GdCl₃
- Post-GdCl₃

B

MCP-1 (pg/mL)

- Control
- LPS
- LPS+L1
- LPS+DFO
Hepatocyte

Iron

Hepcidin

Liver Injury

Plasma Iron

CuMF

Cu

LPS

Spleen

Fat

SREBP1

Kupffer cell

MCP-1

TNF-α↑

IL-1β↑

Iron Chelator

Adipose tissue

IL-6↑